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JULS

Journal of Undergraduate Life Sciences

Celebrating 60 years of achievements in Transplantation

SPECIAL 10th EDITION

VOLUME 10 | ISSUE 1 | SPRING 2016


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ISSN 1911-8899 | University of Toronto Journal of Undergraduate Life Sciences (Print)
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VOLUME 10 • ISSUE 1 • SPRING 2016

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On the Cover

This year presents a unique triple anniversary for the field of transplantation. It was 60 years ago (in 1956) that Toronto scientists performed the first successful heart valve transplant, and introduced solid tissue transplantation as a major clinical practice to the world.

Thirty years later (in 1986) a Toronto Transplant Team again redefined this therapy by performing the world's first double lung transplant, establishing the gold standard therapy for end-stage pulmonary disease.

Finally, 30 years later in 2016, a Toronto Transplant team revolutionized Canadian transplantation once more by conducting the first hand transplant. JULS is proud to honor this long legacy and celebrate the many achievements of Canadian Transplantation.

Acknowledgements

JULS thanks the Toronto Transplant Institute at the University of Toronto for their support in the production of this issue's cover.

Staff List

Letter *from the* Editors

DEAR READER,

It is with great pleasure that we present you with the 10th edition of the Journal of Undergraduate Life Sciences (JULS). This issue caps off a transformative decade for JULS, and undergraduate research as a whole at the University of Toronto. Therefore, as our tenure as Editors-in-Chief concludes, we wish to take a moment to acknowledge the many staff, sponsors and volunteers whose tireless work allows us to produce this exemplary, peer-reviewed publication.

We also wish to extend a special note of gratitude to Her Honour, the Lieutenant Governor of Alberta, Lois Mitchell; the Premier of Ontario, Kathleen Wynne; and the President of the University of Toronto, Prof. Meric Gertler – who kindly took time to acknowledge the anniversary and achievements of JULS. We were touched by their messages of congratulations and their unequivocal support of the sciences and scientific training. As academic institutions around the world feel increasing fiscal strain and receive ever fewer resources, we understand that supporting research becomes increasingly difficult. However, should society demand the same, insatiable need for innovation and scientific advancement, then support of independent research, including at the undergraduate level must remain a priority.

The achievements in Transplantation, which we celebrate in this issue, are testament to the fruits of effective training. It was sixty years ago that Toronto doctors introduced heart valve transplants to the world, thereby providing an in-vivo mechanism to alleviate major cardiac disease for the first time. Thirty years later, the next generation of surgeons performed the world's first double lung transplant, establishing the gold-standard treatment for end-stage respiratory disease. Finally, early in 2016, in an oddly fortuitous thirty years after the first double-lung transplant, Toronto doctors performed Canada's first hand transplant, providing novel insights into advanced micro, muscular and neurological surgery. We congratulate the many surgeons, physicians and researchers who have given Toronto science this remarkable pedigree of innovation.

In this issue we offer an in-depth look into transplantation with no less than four independent literature reviews taking a closer look at the various intricacies surrounding this field. We also acknowledge the many other investigations examining broad questions across the life sciences. This year, we received a record number of submissions across all categories, and we are exceptionally grateful for all the authors who submitted their work to JULS. With increasing submissions, our editorial and peer-review teams must work ever harder to maintain the standards of publication, therefore we offer our sincere appreciation to the peer and faculty review boards for their diligent work and edits.

Finally, we wish to thank you for your readership. JULS exists to serve you, the undergraduate researcher, and we encourage you to pursue your own research ambitions, in whatever field they may be. You can find details of some research programs offered at the University of Toronto inside this issue.

We hope you will find the research presented herein insightful and illuminating.

Sincerely,

Imindu Liyanage & Charles Lee
Editors-in-Chief, 2015-2016

Message from the Lieutenant Governor of Alberta

As Her Majesty the Queen's representative, it's my pleasure to offer sincere congratulations to the Journal of Undergraduate Life Sciences (JULS) on its 10th Edition: *A Decade of Exemplary Research* and to recognize the many achievements of Canadian undergraduate researchers and the contributions made by them to the Life Sciences.

Over the past decade, JULS has played an instrumental role in advancing research and fulfilling its mission to provide an opportunity for undergraduate students to publish their research findings, to engage the undergraduate Life Sciences community to actively participate in the academic research process, as well as promoting an interest in the Life Sciences, encouraging participation in intellectual activity beyond the classroom environment and enhancing and broadening interdisciplinary knowledge and communication across the various Life Sciences.

This event signifies the importance of science to Alberta, to our country and to the world. JULS has provided a platform for evolving undergraduate research and has strengthened the scientific community in our province and nation. I commend the hard work and dedication of all the volunteers who have contributed to making this one of the most prestigious journals in North America.

Congratulations to all the outstanding contributors and volunteers of the Journal of Undergraduate Life Sciences on reaching this important milestone and best wishes for continued success in the future.

Lois Mitchell

Her Honour, the Honourable Lois Mitchell, CM, AOE, LLD
Lieutenant Governor of Alberta

Ontario

Premier of Ontario - Première ministre de l'Ontario

March 31, 2016

A Personal Message from the Premier

On behalf of the Government of Ontario, I am pleased to extend warm greetings to the editorial team, staff and readers of the Journal of Undergraduate Life Sciences (JULS), as you celebrate the publication's 10th edition.

Ontario boasts some of the world's foremost scientific expertise — expertise that is constantly bringing innovation to our life sciences sector and positioning our province as a major contender in the highly competitive knowledge-based economy. It is with this thought in mind that I wish to underscore the vital role undergraduates in the life sciences have had in pushing the frontiers of science forward in our province and beyond.

To write and publish a unique scientific publication of this scope requires hard work and determination. I commend the authors, reviewers, editors and layout personnel at JULS for volunteering their time and efforts to ensuring the ongoing success of this journal.

Again, congratulations on your 10th edition. Please accept my sincere best wishes for many more years of success.

Kathleen Wynne
Premier

UNIVERSITY OF TORONTO

OFFICE OF THE PRESIDENT

Meric S. Gertler, CM, FRSC, FBA, FAcSS
President

A Message from the President

On behalf of the University of Toronto, it is my pleasure to offer congratulations on the tenth edition of the *Journal of Undergraduate Life Sciences* (JULS).

Established in 2006, JULS has been an important forum for undergraduate students to showcase original research, and it has offered the rare opportunity for these young scholars to participate in the rigorous peer-review process as a first author. By engaging in the publication process, students gain essential experience, develop key skills, and refine their scientific writing.

In recent years, the University of Toronto has undertaken a fundamental transformation of teaching and learning—providing undergraduate students with ever increasing opportunities for research-based learning. In this context, the Journal's mandate has never been more relevant. JULS has encouraged the undergraduate life sciences community to participate actively in the academic research process and in academic activities beyond the classroom environment.

In closing, the remarkable growth and success of the *Journal of Undergraduate Life Sciences* can be attributed to the excellent leadership and dedication of the students and faculty members who have volunteered their services since its establishment. Thank you for your ongoing and extraordinary support across the years.

Again, I offer congratulations on this important milestone, and my very best wishes for continued success.

Sincerely,

Meric S. Gertler
President

Simcoe Hall, 27 King's College Circle, Room 206, Toronto, ON M5S 1A1 Canada
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Road ahead in teaching language to the needy

Sengottuvel Kuppuraj

Research shows that in child language learning, the acquisition of words and their associative meanings (e.g., armchair means furniture and comfort) are predominantly linked to the declarative memory system, which is an associative learning mechanism capable of learning through instructions. Grammar (e.g., inflecting words as per 'rules', i.e., I like vs She like-s), on the other hand, is predominantly acquired by the procedural memory system which possesses a complex statistical learning mechanism that is capable of tracking the regularities in the input over time (exposure). Children with language impairment (LI), who are otherwise known to be normal in every other aspect of development, have pronounced deficits in acquiring grammar, but not in word learning. Consequently, their procedural learning has been compromised in comparison to their declarative learning. Most language teaching methods for children with LI are instruction based which will enable grammar rules to be memorized in their declarative system rather than their procedural memory system. Although this may seem like a good solution for children with LI, this article will explore in-depth the downfalls of this method and alternative initiatives in light of these downfalls.

Past literature has shown that by using explicit instructions, grammar relations can be taught efficiently. However, a closer look at the method shows that these studies have mostly involved teaching of grammatical events relations that has high probability of co-occurrence (i.e., easily memorizable). For instance, Finestack and Fay (2009) examined the ability of LI children to learn the gender inflection through instructions. The LI children were instructed to mark '*pa*' or '*pu*' with the verb for '*male*' and *'female*' subjects respectively, which they learned efficiently. In our view, these LI kids simply memorized the rule that was given to them simply because memory functions through the declarative system. However, grammar relations in natural language are much more complex. For instance, the frame, 'I *want* to go running' could have many variations of 'want' such as 'I *want(1)/wanted(2)/ have been wanting(3)/ had been wanting(4)/ will be wanting(5)* to go running' (all legitimate) making the allowable transitional probabilities (TP) (TP is probability of 'B' given 'A') between variations of 'want' and other elements very low (*running* or *I* / variations of *'want'* is 1/5=0.2) in this context. In comparison to the probability relations examined by the studies by Finestack and Fay which have a high TP (*male* / *pa* is 1/1=1), the low TPs described in natural language could be extremely procedural and it is possible that even a highly flexible and intact declarative system would fail to learn such relations. It must be noted however, that the traditional instructional method is not completely inapplicable and it has worked under some circumstances. Alternative language intervention methods have yet to emerge as a viable replacement.

In light of the challenges posed above, research examining the possibility of recruiting the alternative default learning mechanism (i.e., procedural system) for grammar learning in children with LI is strongly recommended. Lum and his colleagues (2014) meta-analyzed studies that examined procedural memory in LI participants and reported that procedural learning is not all lost in LI children. That is, they still have the wiring to learn the probability relations (like grammar events) by exposure. However, what is not clear is, whether or not it is the capacity or the efficiency of the procedural system that is limited in LI. If the former is true, LI children may never possess the ability to acquire complex grammar (like the 'want' example) relations and if the latter is true they will be able to learn complex grammar structures with repeated exposure. Initiatives should be made in understanding the learning strengths of LI children so smooth transition to training methods can be made.

Putting Organ Donation into 'MOTION'

Linlei Ye

Transplantation, the preferred treatment for most end-stage organ diseases, offers patients better quality-of-life and a significantly greater lifespan than other traditional therapies. However, an alarming shortage of organ donation has crippled efforts to make it a broadly accessible treatment. Instead, despite its considerable benefits, it remains a remedy for only a select subset of the population: a so-called 'boutique treatment'. In fact, the average wait-time for a kidney in Ontario now exceeds 7 years. This has largely been a consequence of general public apathy and ignorance towards organ donation. Furthermore, unlike many other medical or social justice issues, very little public or academic attention is raised about this subject. In recognition of this, MOTIONS U of T (The Multi-Organ Transplant Insight Outreach Networking Society) was established as a tool to bring awareness to the fundamental issues in transplantation and organ donation and raise academic discourse about the issues surrounding organ donation.

This year, MOTIONS featured a dramatically new and innovative strategy to achieve these aims. We recognized that in order to effectuate major change, we had to take the message of Organ Donation beyond the comforts of U of T. In other words, we broadened our outreach beyond academia in hopes of engaging the general public, where advocacy in organ donation is scarce. In particular, we embraced the idea of visiting cultural centers and places of faith where the virtues of kindness, benevolence, and caring for humanity are fostered. Our team contacted several religious and cultural organizations in our local community with the proposition of bringing awareness to the issues surrounding organ donation through conversations, brochures, and seminars. The intent was to inform and educate without necessarily instructing. It was our hope that the dissemination of information and public discourse would influence a positive outlook and reduce stigma on the subject of organ donation. To our delight, the initiative was generally well received and we gained numerous invitations to visit events and services.

To date, MOTIONS has made its presence known at 10 community events this academic year alone. At each visit, we provided information pamphlets, pins, posters and ribbons that were generously provided by our partner, the Trillium Gift of Life Network as well as the willing expertise of our volunteer students. Moreover, by speaking with people from diverse backgrounds, we gained deeper insight into the various attitudes and opinions about organ donation. It was insightful to learn the reasons behind why an individual supported or opposed organ donation. However, it was clear that numerous stigma and misconceptions still exist and are prevalent among certain groups, which makes it all the more necessary to bring those issues to light and share the facts.

A particularly common misconception is that 'I am too old to donate', and therefore many potential donors do not consider registering. This is an entirely erroneous notion, espeically with novel organ repair strategies currently under extensive research. These may allow persons regardless of age or prior health status to donate, however if donors are not registered, then many potential life-saving transplants may be overlooked.

A final innovation MOTIONS introduced was to take the message of organ donation directly to broader institutions. Specifically, by invitation of office of the Archbishop of Toronto: His Grace Colin Johnson, we were afforded a rare opportunity to attend the Anglican Church's General Synod. Here, at a large gathering of all of the local clergy and parish representatives, we were able to persuade over 30 distinct churches in Ontario to raise the issue of organ donation in their home parishes. Such wider efforts are crucial to changing a deep-seated apathy and the general misconceptions about organ donation.

For more information about MOTIONS, or to connect with us please visit on beadonor.ca (www.beadonor.ca/organization-3053) or find us on Facebook.

MOTIONS at work: Outreach efforts to bring light to issues in organ donation, hosted by MOTION in partnership with various local and community partners.

Innovations in Immunotherapy: Conference on Curing Cancer and Autoimmunity

Tian Nie

Laboratory Medicine & Pathobiology Student Union Exec Team, Conference Organizers. Top row (L to R): Kevin Liu, Armin Farahvash, Stephanie Poon, Charles Lee, Jelena Tanic, Sudarshan Bala, Jerry Lin, Frank Hyun. Bottom row (L to R): Naijin Li, Anne Fu, Ashley Zhang, Ponta Pouramin, Lisa Qiu and Amber Cintosun

Guest speakers take questions from the audience during the panel discussion. Panel (L to R): Joey Silburt, Milica Tanic, Dr. Pamela S. Ohashi, Dr. Alan Lazarus, Dr. Donald R. Branch

On Saturday January 16th 2016, the Laboratory Medicine and Pathobiology Student Union (LMPSU) hosted its fourth annual research conference at the University of Toronto. The theme for this year was "Innovations in Immunotherapy: Conference on Curing Cancer and Autoimmunity", and its focus was on the research and development of various types of immunotherapy, and its multiple uses in different diseases. The event attracted over 250 participants from the community, featured seven speakers who are leaders of research in the field, and encouraged numerous engaging discussions among scholars of all levels.

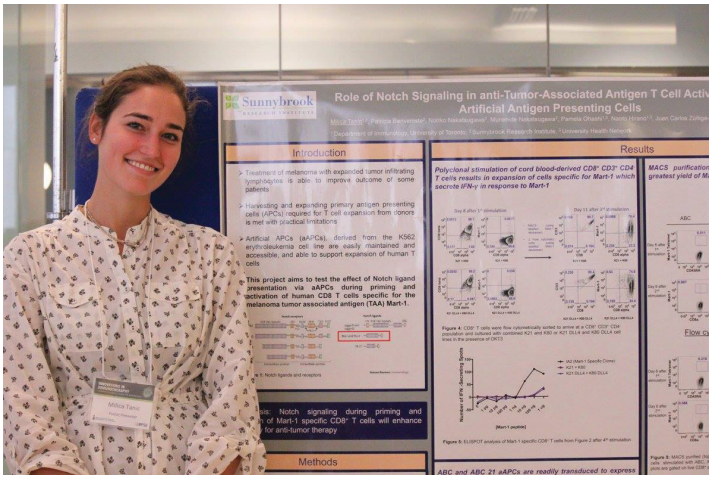
When asked why the topic of immunotherapy was chosen, the co-president of the LMPSU Charles Lee states: "Although it is in its infancy, immunotherapy can overcome numerous limitations of traditional treatments, and we wanted to highlight its promise. Some of the most amazing discoveries are being made right here at U of T, and we wanted other undergraduate students to be inspired and also pursue research in these exciting fields."

The conference began with a journey to the most fundamental part of our adaptive immune system – the generation of T cells from hematopoietic stem cells (HSC). Dr. Juan Carlos Zuniga-Pflucker, Chair of the Department of Immunology at the University of Toronto and Senior Scientist at Sunnybrook Research Institute, talked about how understanding HSC differentiation at the molecular level could lead to anti-tumor immunity and novel treatments of cancers such as leukemia.

In the following presentation, we travelled across species and evolutionary time to the doorsteps of the sea lamprey. Dr. Goetz Ehrhardt – Associate Director of the Toronto FOCIS Centre of Excellence and Assistant Professor in the Department of Immunology – aims to harness the adaptive immune system of the sea lamprey for biomarker discovery. Unlike their mammalian counterparts, the variable lymphocyte receptor (VLR) antibodies of the sea lamprey have radically different protein structures resulting in much higher stability and specificity. Their antibodies can circulate in the body for longer and attach more strongly to disease causing antigens. This has important implications in both research and clinical diagnoses. Sea lampreys also have the added benefit of tasting yummy. *

The first half of the conference closed off with a presentation from Dr. Alan Lazarus, Scientist at St. Michael's Hospital and Professor in the Department of Laboratory Medicine and Pathobiology. His work focuses on blood diseases, such as Immune Thrombocytopenia (ITP) and Haemolytic Disease of the Fetus and Newborn (HDFN), which arise when the immune system attack its own blood cells, In particular, he is looking at ways to modify or replace the antibodies involved, so they stop being self-reactive.

Dr. Juan Carlos Zuniga-Pflucker, Chair of the Department of Immunology, addresses the audience on the potential role of hematopoietic stem cells in conferring anti-tumour immunity.



Graduate student Milica Tanic presents her research on the "Role of Notch Signalling in anti-Tumour-Associated Antigen T cell Activity in Artificial Antigen Presenting Cells"

Lunchtime during the conference was filled with much conversation, with student research posters mounted for discussion for attendees and presenters alike. Afterwards, the conference split into two concurrent seminar sessions – Immunotherapy in Cancer, and Immunotherapy in Autoimmune Disease.

Both presenters in the cancer seminar session talked about their foray in linking T cells with cancer. Dr. Pamela Ohashi, Canada Research Chair in Autoimmunity and Tumor Immunity, is currently establishing clinical trials and turning her previous research in T cells into an immune therapy program. Dr. Naoto Hirano, Associate Director of the Research Immune Therapy Program at the Campbell Family Cancer Research Institute, also spoke about his research into artificial antigen presenting cells, with the goal of antitumor immunotherapeutic modalities.

Meanwhile at the autoimmune seminar, Dr. Edward C Keystone and Dr. Donald R. Branch spoke not only of the possible treatments for rheumatoid arthritis, Alzheimer's disease, and Multiple Sclerosis, but also about the current research environment for autoimmune disease research. There are multiple ways to target the same disease, and there are multiple diseases with similar pathogenesis. The potential in the field is enormous, and will only keep increasing as more research is conducted.

After the many informative talks, everyone gathered up for one final panel discussion. Guide questions were provided, but attendees were also invited to ask any questions they had during the course of the conference. These questions ranged from the molecular details of experiments, to the social impacts of immune therapy. In all, it was an excellent conference which introduced something new to all of the participants, and will inspired many more ideas to come.

In particular, Dr. Zuniga-Pflucker took some time for interview regarding his research motivations. (**TN** – Tian Nie; **JZ** – Dr. J. Zuniga-Pflucker).

TN – What made you chose to be a scientist? What got you into this field? What were your motivations?

JZ – My motivation to go into the field was my interest in developmental biology, and to try to understand how cells and tissues and organs come about from undifferentiated state. And that's sort of the big questions, and trying to understand the programming of cells. It's what I had originally found interesting as a third-year undergraduate student at the University of Maryland.

TN – I noticed you have a degree in zoology, so you developed from zoology into genetics now?

JZ – Well back then, as in the University of Toronto, zoology was what biology was called. The zoology department at U of T is now Cell Systems Biology. Biology is divided into botany, and zoology. Zoology deals with things that dealt with animal cells, or tissues, or organ, and things that dealt with plants were botany. So it was just an old name.

TN – Did you always know you wanted to go into the sciences?

JZ –As a high school student I knew I wanted to study biological systems.

TN – What type of advice would you give to people that are in high school?

JZ – Best thing to do is to do things you find interesting, things that you like. The rest will typically follow. So when I said I started in developmental biology, I ended up working in a lab that studied how the immune system develops. My view, that I wanted to do development, was really shaped by the environment I was working in. It was within the context of the immune system development that led me to be interested in the questions regarding immunology. So, it really was a naïve idea that I wanted to pursue an area that was very broad, developmental biology, that led to a focused way of understanding that led me to look at the thymus, that led me to look at T cells, and that's sort of how I developed. So in a way, just follow what you find interesting, and try to use that as your launching pad and then see where it takes you.

TN – What were some of the biggest challenges you've found, when getting to the point you are right now?

JZ – The biggest challenges are always trying to be the first to do something, trying to have the insight, or the cleverness to be able to ask the right questions to get the right answers and use the right tools and the right models. […]It's always been the ability to get fortunate enough to have confidence smile on you, so you can achieve those goals. It's a bit o luck, and a lot of hard work.

TN – Where do you see the future of the field?

JZ – When mean the field, do you mean immunology or immunotherapy?

TN – Immunotherapy.

JZ – Well, as Jelena pointed out earlier, it's an up and coming field in immunology. Earlier, when we heard Lazarus' talk, the idea of using antibodies was heralded in the eighties as the answer to everything, and now, it's becoming to be the case when we have a better way to make them with better ways to humanize them for reactivity. They're having an impact on multiple diseases, autoimmunity, cancer, and all the chronic diseases. Biomarkers, I think we're just at the beginning. One can take advantage of not only the immune system, but biology in general and use immunology, cells, antibodies, and compounds to affect the immune system and to affect health and disease. There's things we don't even know that are possible to do yet, in engineering these molecules to be more effective.

MR/IPH for diagnosing carotid artery stenosis: a potential cost-effective imaging tool

Hao Yue (Helena) Lan and Alana Man

With 50,000 cases reported each year, stroke stands as the third leading cause of death in Canada. Given that carotid artery stenosis (CAS) is responsible for approximately 30% of strokes, finding the right diagnostic approach for carotid disease is important to saving lives and healthcare resources. Drs. Tyrrell and Moody from the Department of Medical Imaging at the University of Toronto have recently looked to examine and improve the current model of care for CAS patients. We, Helena Lan and Alana Man, past Research Opportunity Program (ROP) students in the group, had the chance to revisit the topic and to discuss the current status of the project with Dr. Eli Lechtman, a third year medical student in the group.

(**AM** – Alana Man; **EL** – Eli Lechtman; **HL** – Helena Lan)

AM: What is the current practice of diagnosing CAS patients and why are we interested in assessing it?

EL: Ultrasound is the established first-line imaging modality. Although it can reveal the degree of stenosis, it cannot detect important biomarkers of atherosclerotic plaque vulnerability, such as intra-plaque hemorrhage (IPH), which has been shown to predict risk of future stroke. However MR/IPH, a new form of MRI technique developed by Dr. Moody's group at Sunnybrook, can offer this additional information [1]. With an optimized time per read, referring a patient suspected of CAS for an immediate MR/IPH may be justified.

AM: What steps have you taken to investigate this matter?

EL: After the review of the literature and general practices that Helena and you had completed, we, with the help of University of Toronto ROP students Kevin Chen, Sylvia Urbanik, Kiersten Thomas, and Indranil Balki, created a cost effectiveness model for carotid stenosis which compared the different patient outcomes and costs, depending on the patient's condition, imaging modality, and associated factors such as the sensitivity and specificity of the modalities.

HL: What did the model show?

EL: The results demonstrated MRI as an informative and cost-effective diagnostic imaging modality (see Table 1). With that knowledge in hand, we are currently collaborating with the Niagara (heartniagara.com) and Cambridge, Ontario regions to set up a knowledge translation project (with the help of ROP students Julia Robson and Muntaha Nadeem). The project's objective is to introduce a better way of assessing CAS at the primary care level by determining physicians' willingness to use MR/IPH as the primary diagnostic imaging modality for CAS and if IPH information is valuable for patients' risk-assessment of cerebrovascular disease.

HL: Upon gathering physicians' perspectives, what do you hope to achieve?

EL: We aim to propose the incorporation of the evidence-based diagnostic technology MR/IPH into current medical practice. With its ability to detect a strong predictor of ischemic events, we foresee that MR/IPH can aid physicians in making appropriate treatment decisions for patients with severe CAS. This new technology can lead to improvements in cost-effectiveness and patient care by potentially preventing 1,500 – 2,000 strokes per year in Canada [2].

Table 1. Lifetime Per-Person Clinical Outcomes and Cost Effectiveness Comparison of ultrasound and MR/IPH.

Strategy	First Stroke	Total stroke	Life years	NNS	Surgeries	QALY	Cost ($)	ICER
Initial age 70								
Ultrasound	0.107	0.121	15.63		0.044	12.14	5896.96	Reference
MRIPH	0.088	0.096	15.66	40.16	0.132	12.20	6075.60	$3300per/QALY
	Percent reduction	20.6				Cost difference	$178.64	

Notes: NNS=Number Needed to Screen, QALY=quality-adjusted life-year, ICER=Incremental Cost Effectiveness Ratio

JULS RESEARCH

Combinatorial Chemistry and Molecular Networking as the consequence of the Separation of Conformation and Reaction Subspaces for Multivariable Potential Energy Functions

Darius Hung[1], Anita Rágyanszki[2], Natalie J. Galant[1], Imre G. Csizmadia[1,2]

[1]Department of Chemistry, University of Toronto, Toronto, Canada M5S 3H6
[2]Department of Chemical Informatics, Faculty of Education, University of Szeged
Corresponding Author: Darius Hung (darius.hung@mail.utoronto.ca)

Abstract

Formaldehyde is a critically important species with implications in many processes such as isotope enrichment, air pollution, and synthesis of carbohydrates. Oxygen donor substituted carbenes have not been thoroughly studied, one prime example being hydroxymethylene. Hydroxymethylene is a key intermediate in the chemistry of formaldehyde tautomerization. This carbene intermediate is considered to be the parent of alkooxycarbenes, a family of compounds-which lie at the core of transition metal carbene chemistry. The ability to isolate and observe this highly unstable species is limited, therefore finding novel methods to study this species has always been an area of strong interest. The following study focuses on the conformational and reactive subspaces of this elusive molecule. The study successfully bridged the two subspaces, that are otherwise variedly distinct, by finding a commonality point. The subspaces were first analyzed and then compared. The commonality point was found to be the *trans* isomer of hydroxmethylene. The success of this study lays the groundwork for future work on combining reactive and conformational subspaces for larger, more complex molecules such as proteins of interest to better describe potential energy surfaces, hypersurfaces as well as conformation and reaction characteristics. More specifically, this study has particular implications on biological reactions that contain conformational modifications such as a *cis-trans* isomerization. Therefore the results of this investigation can be applied to a wide array of molecules within the life sciences.

Chemical background

Hydroxymethylene, HCOH, is the simplest alkoxycarbene and is a tautomer of formaldehyde. Formaldehyde is an important molecule that has implications in many processes. For example, the role it plays in laser isotope separation schemes to enrich hydrogen, carbon and oxygen isotopes [2]. Interstellar formaldehyde have been discovered, and is believed to be the most copious of all tetra-atomic interstellar molecules [3]. An important reaction of formaldehyde in interstellar clouds is the formation of protonated formaldehyde. Hydroxymethylene is suspected to be the product of a further reaction from protonated formaldehyde. The carbene intermediate is also found in abundance in interstellar clouds[4]. Moreover, formaldehyde has a substantial role in the photochemistry of air pollution [5-8]. The photolysis of formaldehyde gives two precursors to hydroperoxyl free radicals (HO_2). This source of H and HO_2 contributes to the rapid oxidation of NO to NO_2, which is an important initiator of photochemical smog formation. Furthermore the photolysis of nitrogen dioxide can lead to ozone production which is harmful to human health. Formaldehyde is also involved in a two step photocatalytic process to form carbohydates [9]. Formaldehyde acts as the intermediate in the direct synthesis of carbohydrate(s) from water and carbon dioxide.

In recent studies, Schreiner *et al.* described the generation of hydroxymethylene from glyoxylic acid, and the subsequent rapid rearrangement to formaldehyde had a half-life of 2 hours [11]-supporting the hypothesis made theoretically in 1977 that hydroxymethylene undergoes spontaneous isomerization to yield formaldehyde [12]. An important caveat to note is that Schreiner *et al.* only isolated the *trans*-isomer of hydroxmethylene. Although this can be attributed to the nature of the preparation of the carbene intermediate, rather than to say the *cis*-isomer cannot be experimentally isolated. The fast proton-tunneling in *trans*-hydroxymethylene possibly originated from the dipolar contribution in the ground state of hydroxycarbene [13] to facilitate the forma-

tion of formaldehyde via proton transfer from the -OH group to the carbon center (**Figure 1**).

Investigations were done into the topological properties of formaldehyde derivatives to predict the total potential energy hypersurface of HOCH represented by the conformational and reaction subspace. One important basis is that hydroxymethylene has stable S_0 *cis* and S_0 *trans* forms [11,14], this is paramount since *cis*-hydroxymethylene is the precursor for the formation of the products of dissociation, hydrogen gas and carbon monoxide, and to have the ability and thus observe conformational changes of the carbene realistically. By investigating the full potential energy surface of hydroxymethylene, this study hopes to assist better understanding of the torsional and reaction properties of important biological molecules by providing a novel analytical approach.

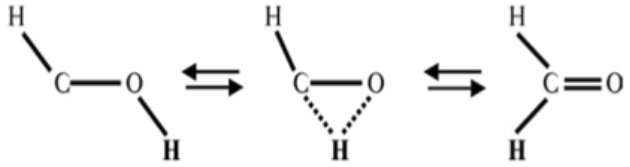


Figure 1. Reaction Progress for *trans*-hydroxymethylenecarbene via a 1,2 H-shift into formaldehyde. Dotted lines indicate partial bonds.

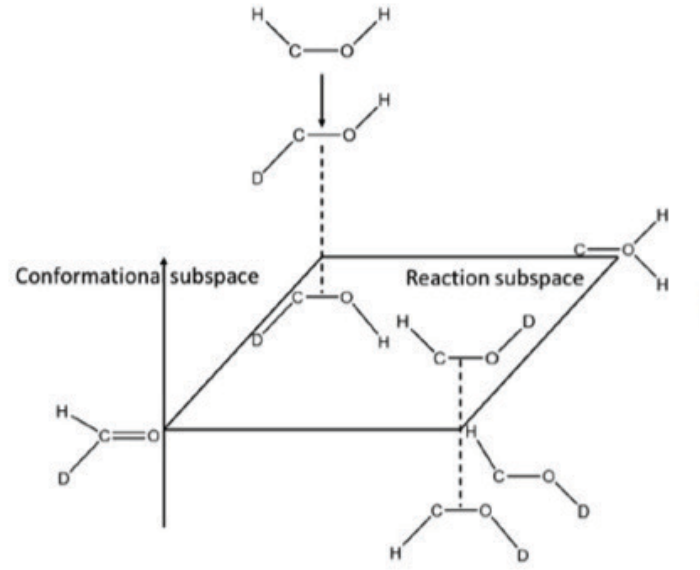


Figure 2. Schematic diagram showing the conformation and reaction subspace of the project.

Aims

A schematic diagram for my project is shown in **Figure 2**. This paper will analyze the conformational and reaction subspace of hydroxymethylene, with the aim of studying the link between the conformational properties and the intermolecular reaction characteristics. A special class of unimolecular reactions with relatively small activation energies that do not involve bond making or break can usually be considered conformational changes. **Figure 3** indicates the projected final result of the investigation. Min_2 represents the most stable conformer of hydroxymethylene and acts as the intermediate when the reaction proceeds along the edges of the plane. If the reaction proceeds along the edges of the plane, there can be two routes that can be taken (**Figure 2**,**3**). As a result, for each reaction pathway, the plot of energy versus reaction coordinate should in theory have 3 critical points (excluding the reactant and product endpoints), composed of 2 global maxima (transition states) and 1 local minimum (intermediate).

Further analysis of both subspaces are explored due to the potential of combining both subspaces. Both subspaces share a common minimum point or species, which is the most stable conformer of hydroxymethylene (**Figure 3**), potentially acting as a bridge between the subspaces. Also to note, deuterium has been used in place of hydrogen to better differentiate between the 2 hydrogens in the conformational subspace for clarity.

Computational Methods

Ab initio calculations were performed *in silico* using the Gaussian09 program package [15]. Electronic structure computations associated with internal rotation (**Figure 4**) and the optimum geometric structures were computed using the B3LYP/6-31G(d) level of theory [16]. The calculations were carried out for a sample of dihedral angle values, φ, for hydroxymethylene in the interval [-π,π] with grid points at 15° increments to generate the 1-dimensional PEC (**Figure 5**). Transition states and the reaction pathways were calculated by IRC calculation using Density Functional Theory with B3LYP/6-31G(d) basis set.

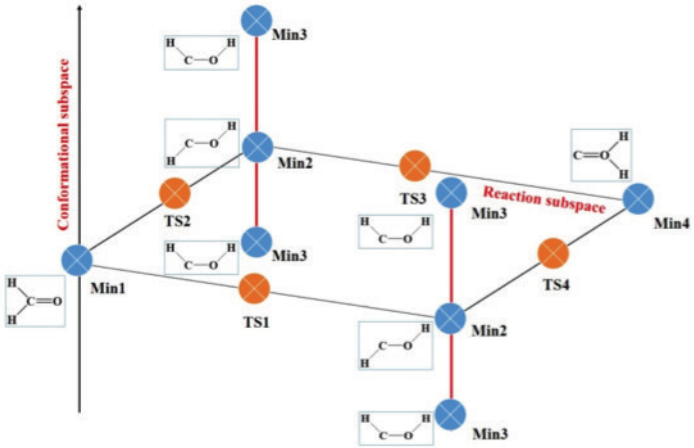


Figure 3. Schematic diagram showing the expected connection of the two surfaces. The red arrows denote conformational changes, making up the conformational subspace. The reaction subspace is represented by the plane, with the corners of the plane labeled as Min_1 and Min_4 being the reactant and the product in the reaction.

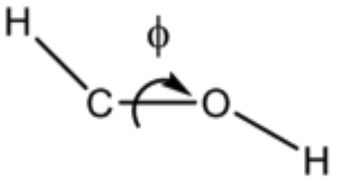


Figure 4. The internal rotation of hydroxymethylene

Results

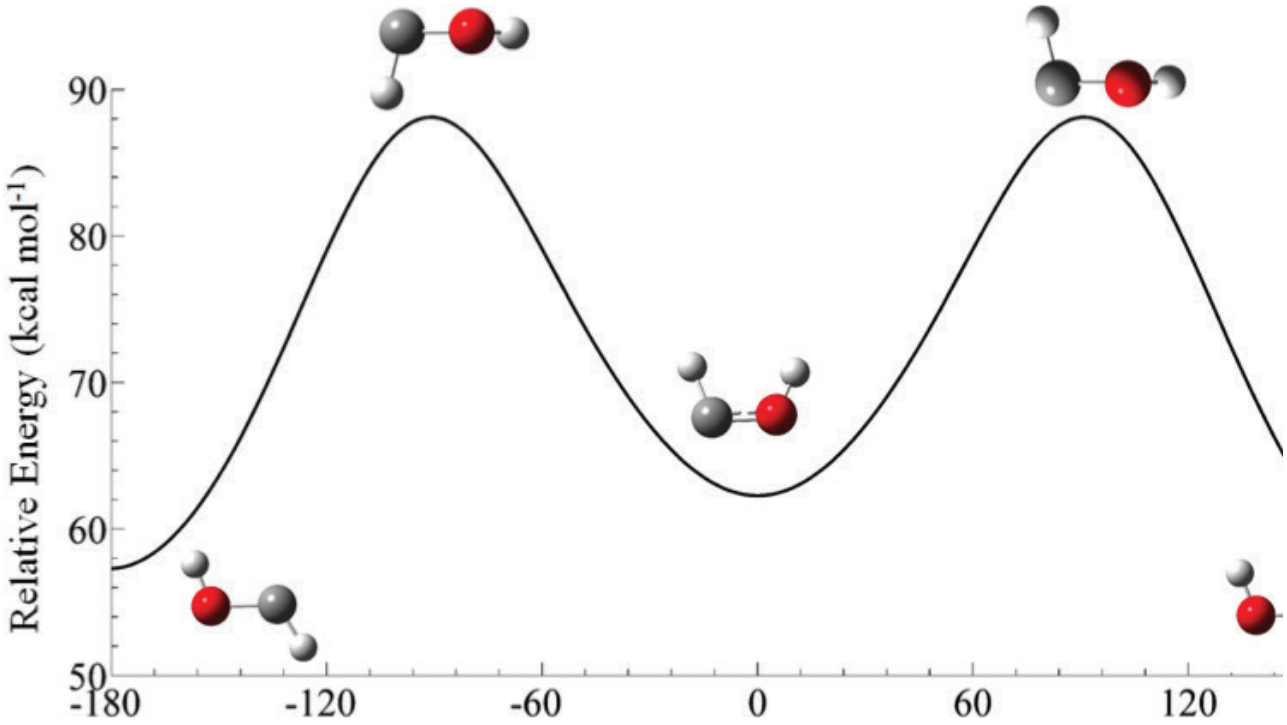


Figure 5. 1D scan of relaxed hydroxymethylene in [-π, π] interval.

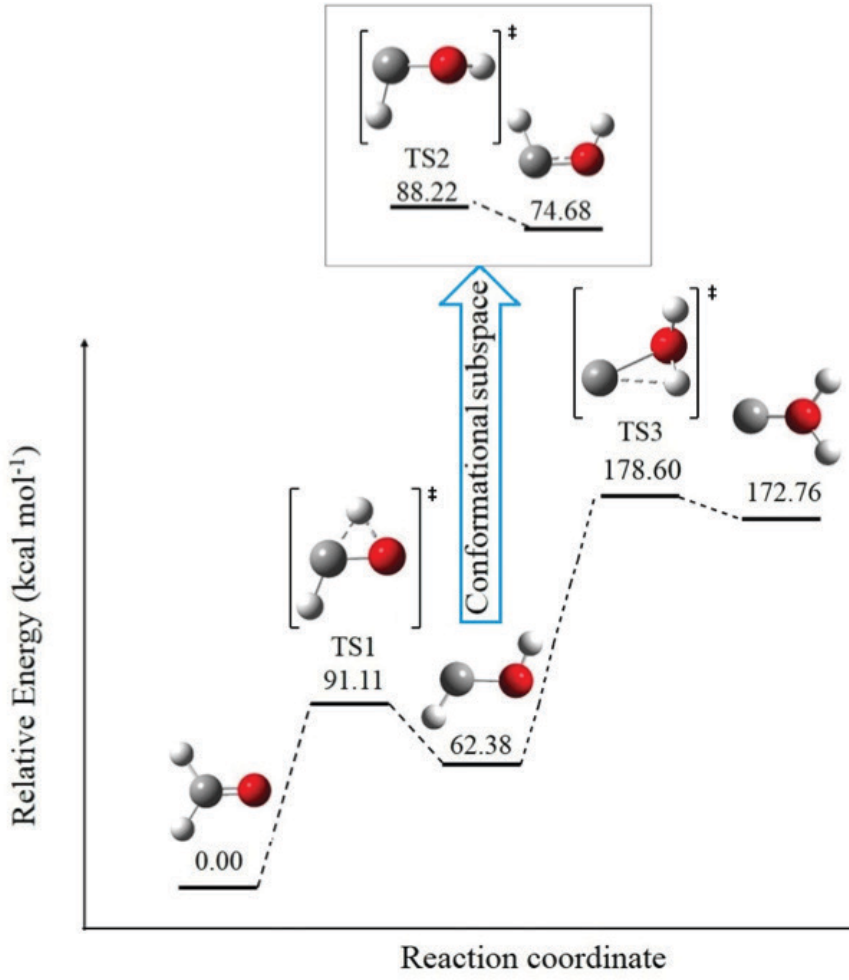


Figure 6. The reaction potential energy hypersurface of H–C–O–H.

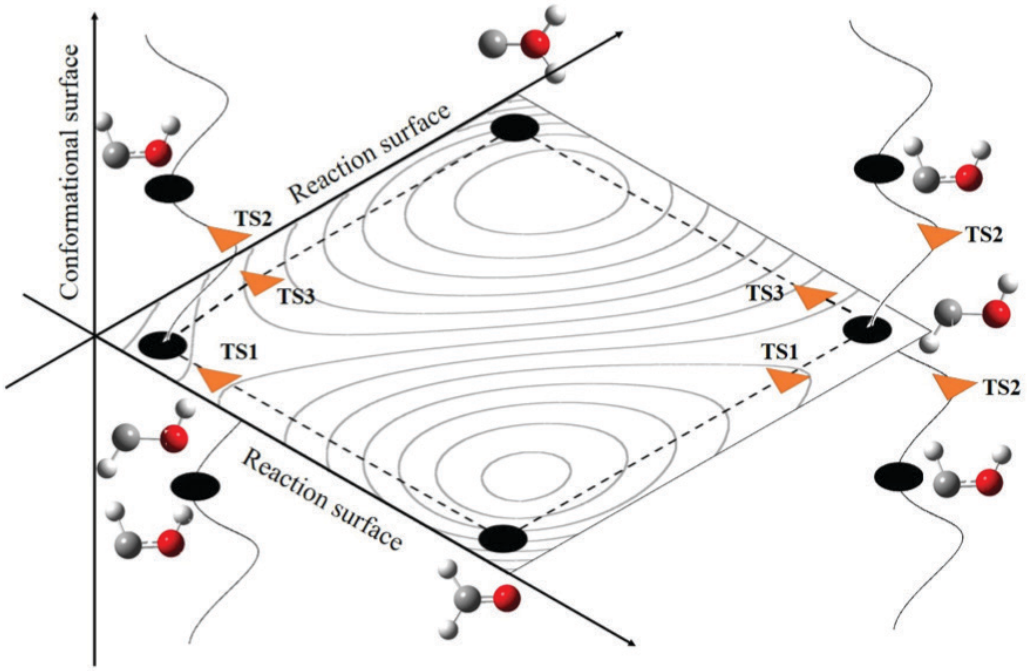


Figure 7. The full potential energy hypersurface of H–C–O–H

Discussion

Conformational Subspace

In comparing the two geometrical isomers of hydroxymethylene, the *cis* isomer exhibits some double bond character in the C-O bond. The C-O bond length is 1.31 Å, whereas the *trans* isomer has C-O bond length of 1.43 (**Table S2**). When you look at the two remaining bonds in hydroxymethylene, the C-H and O-H bonds in *cis* HOCH are noticeably longer, by around 0.06 Å and 0.03 Å respectively. This is intuitive because the *cis* isomer has greater electron density around the C-O bond leading to the elongation of the remaining two bonds due to electron repulsion. In addition, the close proximity of the hydrogens lead to repulsion between the 1s electron in each atom, destabilizing the *cis* isomer by 12.3 Kcal/mol versus the *trans* isomer. This energy difference is slightly higher than reported in literature [9], however energy calculations can vary by the computational method. Nonetheless, the calculations performed show that the trans isomer is still the more energetically favorable isomer, in accord with literature.

The torsional potential energy curve of relaxed hydroxymethylene is shown in **Figure 5**. The conformational scan was conducted at 15° increments in the range [-π,π] to give 24 data points. At first glance, it is most apparent that the curve contains 2 absolute minimas, 2 absolute maximas and 1 local minimum. For both scans, the absolute minima occurs at φ=180, representing the *trans* isomer. The local minimum occurs when φ=0.044 which is the *cis* isomer. The absolute maxima is when φ=91.5, and this is a transition state (abbreviated as TS2). A *trans-cis* isomerization represents an energy barrier of ΔE=25.8 kcal/mol, whilst the backward reaction has an energy barrier of ΔE=13.5 kcal/mol.

Reaction Subspace

Transition states were obtained by IRC calculations and the reaction coordinate schematic diagram is shown in **Figure 6**. The Hammond postulate states that the transition state of a reaction will resemble either the reactants or the products, to whichever it is closer in energy. Thus it can be observed that for the studied endothermic reactions, the transition states are closer in energy to the products than the reactants and so will closely resemble the products. As previously predicted, the reaction coordinate diagram contains 3 critical points excluding the endpoints. These points are transition state 1 (TS1) and 3 (TS3) and *trans*-hydroxymethylene intermediate.

The overall reaction is endothermic, however it can also be divided into two separate endothermic reactions. The trigonal planar formaldehyde requires 91.11 kcal/mol of energy to reach the intermediate whilst the back reaction requires only 28.73 kcal/mol. However as reported by Schreiner *et al*, *trans*-hydroxymethylene bypasses this energy barrier through proton tunneling to form formaldehyde. In TS1, one of the hydrogens in H-C-O-H forms partial bonds with carbon and oxygen to form a 3 membered ring with HCO and HOC bond angles of 54.3° and 60.8° respectively (**Table S2**). The HCO bond angle is expected to be less than the HOC bond angle because oxygen has greater electronegativity than carbon. Meanwhile the other hydrogen not in the ring has OCH angle of 113.8°. TS1 subsequently breaks of the C-H partial bond and rearranges to form *trans*-hydroxymethlyene. A noteworthy point is that the C-O bond length in TS1 is 1.31Å which implies it has greater double bond character than *cis*-hydroxymethylene and suggests the transition state is somewhat stabilized by π electron delocalization.

The final product resembles that of a solved carbon atom, where a carbon atom is bonded to a water molecule via the oxygen atom. This planar tautomer of formaldehyde is high in energy and is unstable with respect to rearrangement back to hydroxymethylene, and formaldehyde as well. Moreover, the aqua moiety in the solvated carbon atom product has slightly longer OH bonds at 1.05Å compared to waters 0.96Å, however it has an almost identical HOH bond angle to water, 104.3° versus water's 104.5°. TS3 is the most unstable structure with an energy of 178.6 kcal/mol (**Table S1**). Notice that there is only a small energy difference of 5.8 kcal/mol between TS3 and the final product, therefore the structure of TS3 closely resembles the product. In TS3, the carbon, oxygen and one hydrogen atom are roughly in the same plane, with a weak partial C-H bond (bond length of 1.75Å). The remaining hydrogen is going away from the plane. To form the 3rd transition state, the hydrogen atom in the C-H bond of the intermediate is attracted to the electronegative oxygen atom to form a bond with it. Further analysis into this molecule reveals the reason for the high instability. Formation of TS3 from the intermediate is unfavorable because it yields a very long C-O bond length of 1.83Å that is caused by the repulsion between electronegative oxygen and the 3

lone pairs on carbon. This structure should be short-lived because once the C-H partial bond breaks, it will rapidly rearrange itself so that the two hydrogen become symmetrically oriented away from the carbon atom to form the aqua moiety of the relatively more stable final product.

Full Potential Energy Surface

In order to show and visualize the possibility of separating the two subspaces, a simple molecule had to be chosen, yet must contain at least 4 molecules. The simple molecule H-C-O-H was picked to convey both the bridging and differentiating aspects of two different subspaces. The complete, full potential energy surface that incorporates the conformational and reactive subspaces of hydroxymethylene is shown in **Figure 7**. The reaction subspace that contains 2n-3 reaction coordinates is represented by the horizontal plane, and the conformation subspace containing n-3 conformational coordinates is the vertical axis/line perpendicular to the horizontal plane. The dotted lines on the horizontal plane indicate the reaction pathways. Comparing **Figure 7** to previous reaction coordinate diagrams, you notice the bond length labels are omitted. To obtain Figure 7, the bond lengths C-O and C-H in formaldehyde were varied, however labels have been omitted in the reaction plane because it is evident that the 2 mechanistic routes are identical, and so changing the C-O bond length has the same effect as changing bond length C-H in terms of attaining the final product. However, it is true that if the hydrogen atoms are replaced by deuterium, then it would become easier to observe which bond length is changing. Since the 2 mechanistic routes are the same, the reaction coordinate curves acquired from each route will be identical, with identical energies. This curve is exactly **Figure 6**.

In **Figure 7** you can clearly see a connection point between the two subspaces, and that is the intermediate *trans*-hydroxymethylene. As well, TS2 is within the conformational surface and thereby is a part of the reaction surface. Ultimately meaning that it is also part of the full PES. It is well-known that a molecule's movements can be categorized into bends, stretches and torsional changes, although the important point is the visual representation of the subspaces in 3-dimension. As said before, *trans*-hydroxymethylene acts as the crucial bridge between subspaces, which makes separation of reaction and conformation surfaces possible using X, Y, Z axes (3-Dimension). Moreover, the remaining isomers of hydroxymethylene are conformational changes via a unimolecular reaction. Such conformational changes have not been reported by previous studies.

Conclusion

Despite the interest in hydroxymethylene, the molecule's inherent instability led to problems isolating the molecule for observation. This has been true even in the most recent study by Schreiner *et al*. With the conformational and reactive subspaces characterized and investigated, this *ab intio* study presented here show hydroxymethylene's stability in relation to other potential isomers of formaldehyde and helps cement the foundation for further studies into this molecule. Moreover, this study was able to successfully link the two subspaces together originating from a mutual structure *trans*-hydroxymethylene. The bridging of conformational and reaction subspaces have yet to be reported and so this paper leaves a framework to be built upon for future studies dealing with potential energy surfaces and hypersurfaces of a molecule. Investigations into full potential energy surfaces and hypersurfaces are critical to understanding a molecule's behavior in isolation and with nearby reactants. The novel methodology of this study can be extended to marcomolecules such as proteins to obtain extensive knowledge about a molecule's conformational and reactive properties. In particular, this paper can act as a precursor for analysis of biological reactions, one example being molecular redox switches that have conformational modifications that affect enzyme activity and binding. As a result, this novel study of potential energy hypersurfaces can alter the method in which molecules and reactions are studied in the life sciences.

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Acknowledgements

Above everyone else, I would like to thank my supervisor, Professor Imre. Csizmadia for his passionate, enthusiastic and continuing support he has provided me with. I would like to also thank Anita Rágyanszki for her invaluable assistance, Natalie Galant for her guidance and feedback, and my mentors Nidaa Rasheed, Tommy Sung, Craig Yu, Jay Lee for general support through technical assistance. Additionally, I want to extend my gratitude to the Department of Chemistry and the Research Opportunity Program (ROP299) at the University of Toronto for allowing me to partake in this research project. Furthermore, I would like to thank the University of Szeged for offering the software enabling my molecular calculations.

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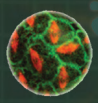

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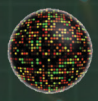

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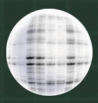

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JULS RESEARCH

Imaging Research and the Potential for Collaboration across Jurisdictions

Aya Mahder Bashi[1], John R. Harvey[1], Alan R. Moody[1] and Pascal N. Tyrrell[1]

[1]Department of Medical Imaging, University of Toronto, Toronto, Ontario, Canada.
Corresponding author: Pascal N. Tyrrell

Abstract

This study investigates the requirements for sharing medical imaging data with a third party for research purposes. It aims to do so by identifying the main ethical, privacy and confidentiality, security, and administrative guidelines that need to be adhered to in order to successfully create links between Medical image Network Enterprise (MiNE) data centers, both locally—within Toronto—and globally. MiNE is an e-infrastructure that aims at housing an electronic image based inventory to support and encourage the research community to use existing clinical image data efficiently. The jurisdiction investigated in this study was the Kingdom of Jordan. Radiology consultants in different hospitals, institutional review board (IRB) coordinators, as well as picture archiving and communication system (PACS) product line managers were interviewed. This qualitative project used both literature reviews as well as informal, semi-structured interviews of potential stakeholders in Jordan. A total of six stakeholders from Jordan were interviewed. Four hospitals expressed interest in establishing a link with MiNE for collaborative work. Interoperable PACS is in place in several hospitals in Jordan, which could be utilized in a research network. Ethical and security approval procedures in Jordan exist but are not as tightly regulated. Restricted resources at Jordan's public sector medical centres would likely preclude active participation in research from radiologists at those centres and constitute the biggest hurdle. Collaborative research could be conducted, provided that the differences in regulations are understood, and compensated for where possible to ensure best possible outcomes from such links.

Introduction

Medical imaging encompasses diverse modalities that are used to image the human body, and plays an integral role in the understanding, diagnosis, and treatment of a wide variety of diseases. But while the clinical (or diagnostic) use of X-rays, CT scans, MRIs etc. is prevalent and needs little justification, the research (or analytical) use of the data they contain is hindered in comparison. The result is suboptimal use of medical imaging resources and lost opportunities to explore image data for patterns that might reveal new correlations and biomarkers for disease [1]. While the collection of medical images for secondary analysis has great potential to improve our understanding of disease and its impact, such collection is challenged by technological hurdles as well as by rules surrounding the retention, disclosure and use of image data. These challenges can be daunting and the result is an extensive gap in knowledge gain and translation, such that the research community cannot say what may impede collaboration or what potential solutions are available [2].

In response to this gap, the Medical image Network Enterprise (MiNE) was created [3]. The purpose of MiNE is to provide an engaging environment that supports every aspect of medical image research: including idea conception, image storage and analysis, and the publication of results. To this end, MiNE has defined and implemented a scalable and secure web-based portal to an image inventory, with related software tools. This implementation has taken place in three phases: (i) design consultation with interested parties; ii) recruitment and training of highly qualified personnel to populate the portal development chain: including the disciplines of computer science and engineering, data science, and design; (iii) installation and testing of e-infrastructure (hardware, middleware, software); (iv) completion of stable guidelines and their implementation for the efficient and privacy-sensitive management of and access to research data, resources, documentation, and educational material.

The next step in the development of MiNE is the establishment of ethically sound data links amongst MiNE's University of Toronto teaching hospital sites, as well as between these sites and future participating sites. The Kingdom of Jordan was chosen as a possible site for future participation, and this study was undertaken to explore the feasibility of medical image data-sharing with that jurisdiction from both technical and ethical perspectives. Jordan was selected by way of the relationship established there by MiNE's industry partner, Merge Healthcare (Merge)(4). MiNE enjoys an active, research-driven partnership with Merge, an IBM company that provides picture archiving and communication system (PACS) and medical data management systems internationally. Merge had been retained by the Jordanian Ministry of Health as a PACS provider under the Jordan Health Initiative (JHI). The JHI was launched in 2010 to promote accessible quality care and

cancer screenings to Jordanians through off-site diagnosis and telemedicine consultations between specialists at urban and rural centres. Accordingly, Merge, in partnership with Cisco Systems [5], implemented a proof-of-concept pilot project to feature and promote its latest PACS technology product line in what was then a new market. Merge installed a PACS for viewing access and storage of images from different modalities [6] as well as a Vendor Neutral Archive (VNA) to ensure interoperability in image sharing. [7]

The same Merge products that were installed in Jordan also provide the foundation for MiNE's Toronto-based e-infrastructure. MiNE's interconnected components include: i) a Research PACS with data de-identification and role-based viewing capabilities; ii) web-based gateway software that provides connection to the PACS from remote desktops, cross-enterprise Document Sharing and data de-identification on upload; iii) Electronic Data Capture through Structured Reporting and Clinical Trial Management applications; iv) a Data Centre; v) a Members' Community Forum for Q & A on research-related topics; vi) a Research App Store with license management for established, new and experimental research applications; vii) a comprehensive data backup and recovery protocol. A diagram of the server setup and data input/output streams that underlie MiNE, as well as its expected data transfer pathway, is shown in Figure 1.

Compatibility amongst Merge products implies that data sharing between researchers in Toronto and Jordan through MiNE is technically achievable. Nonetheless, MiNE recognizes that an assessment of non-technical challenges is required before collaboration with any given site can be achieved. In the case of Jordan, such challenges include possible jurisdictional differences with regard to ethical guidelines for medical research [8], interest in collaboration on the part of radiologists, and the perception of research held by radiology staff in-general.

The objectives of this study were to: i) assess the current level of medical imaging research in Jordan and the level of stakeholder interest in such research; ii) determine Jordanian research ethics and privacy requirements; iii) compare Jordanian requirements with local requirements (as would apply to researchers from the University of Toronto and participating Toronto-based sites), and; iv) propose solutions for ethics, security, privacy, and data transfer agreements.

Methods

An Undergraduate Ethics Review Protocol for student-initiated projects was approved by the University of Toronto's REB (Protocol Reference # 31578). Registration was made with Safety Abroad training from the Center for International Experience (CIE) at the University of Toronto. Permission was obtained from Merge Healthcare to meet with and interview representative personnel in Jordan.

This study followed case study methodology. Data collection was through face-to-face interviews conducted using the Sawatsky Method. This method was perceived as most suitable to our target sources: it encourages an informal, semi-structured and direct style, without preamble or commentary and based on prior knowledge gained through background research. This method also stresses the avoidance of trigger words and hyperbole [9]. Information was collected through note-taking during interviews and de-briefings.

The information sought during interviews fell into two categories: i) medical research and ii) ethics, privacy and confidentiality. **Table 1** presents the interview questions.

For a case study methodology, subjects are chosen intentionally rather than randomly. Merge facilitated a meeting with the first three participants in the study: managers from CISCO. Merge had collaborated with CISCO in Jordan to install the e-infrastructure currently in place. The remaining participants interviewed were recommended by these managers. The IRB coordinator chosen was located in the only office of scientific affairs and research that was accessible for interviews.

Information was collected through note-taking during and following interview sessions. Analysis of the data included the interpretation of the answers provided. Validation of the data regarding research ethics was conducted through literature review.

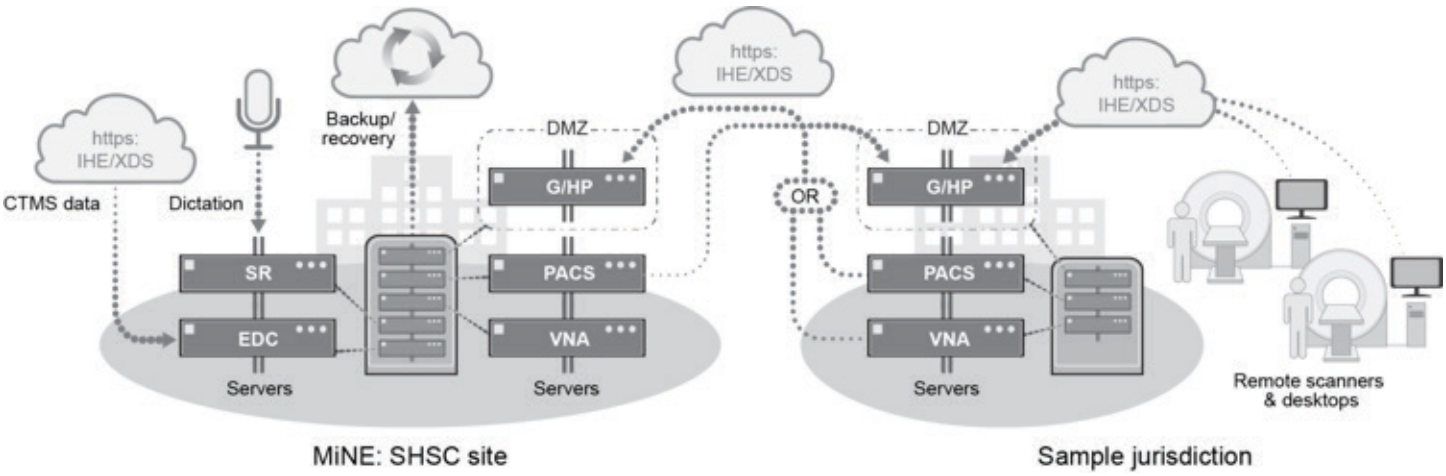

Figure 1. MiNE's basic server configuration, showing data input and output. CTMS: Clinical Trial Management System; DMZ: Demilitarized Zone; EDC: Electronic Data Capture; G/HP: Gateway/Holding Pen; https: Hypertext Transfer Protocol Secure; IHE: Integrating the Health Enterprise profile; PACS: Picture Archiving and Communications System; SR: Structured Reporting; VNA: Vendor Neutral Archive; XDS: Cross-enterprise Document Sharing.

Table 1.

Medical research	
1	What is your involvement in medical imaging research?
2	How do you feel about being (or getting) involved in medical imaging research?
3	Which medical and/or academic centres in Jordan are involved with medical imaging research?
4	What do you think about sharing medical images for research within Jordan?
5	And outside Jordan, with the University of Toronto?
Ethics, privacy and confidentiality	
6	What governs research ethics at your centre or institute?
7	And across Jordan?
8	How is research ethics approval obtained?
9	What are the regulations and guidelines at your institution for maintaining patient privacy and confidentiality in medical research?
10	What are the laws/regulations put in place to protect patients if there is a need for sharing their privacy-sensitive information with a third party?
11	What if this information was de-identified?
12	centre/institution and the University of Toronto?

Results

Timeline and interviewees

Interviewer arrival to Jordan was in April 2015 and interviews began shortly thereafter. Interviews were conducted from April 29th through July 1st, 2015, using both English and Arabic. Nineteen subjects were contacted via wwemail, telephone and office appointments during business hours. Interviewees included radiologists, PACS product line managers and a Jordanian medical ethics authority. The subjects who agreed to be interviewed, along with their roles and interview dates, are listed in **Table 2.**

Technical overview

Merge facilitated a meeting with PACS/RIS product line managers associated with the JHI endeavour. Interviews with these managers provided an overview of the e-infrastructure that was installed under the JHI. A central PACS server had been installed at the Al-Bashir Hospital in Amman, Jordan's main public sector hospital. Distributed PACS gateways—also referred to as thin clients—had been installed on workstations at the Al-Mafraq Government Hospital in the northeast of Jordan, and at the Queen Rania Hospital in the south. Upon gateway installation, these centres could connect to the Al-Bashir Hospital. This connection was internet-based, using standard browsers and HTTPS authentication for secure upload and viewing of patients' images. To provide a companion Radiology Information System (RIS), Cisco had installed a Service Exchange Platform (SXP). SXP also supports video conferencing through WebEX, a web-based application that enables peer-to-peer communication. Eligible users included technicians and radiologists from any of the three hospitals. A demonstration of the gateway described the requirement for the assignment of a username, password and access level by a central PACS administrator at Al-Bashir. Access to a patient's medical images is dependent on the user's role assignment: not every role is entitled to view a particular patient's image or information.

Interviews

April 29th, 2015;
Aiman Alrawbdeh; CISCO/Senior Program Manager
Amer Madaeen; CISCO/Project Manager
Malek Karadsheh; Optimiza/PACS & RIS Product Line Manager

Operational information about PACS was provided during the first meeting. According to, Aiman Alrawabdeh, Senior Program Manager at CISCO, PACS serves to collect medical images, store them, and provide doctors with off-site access to them. The main purpose of installing this system through CISCO under the Ministry of Health, Jordan Health Initiative is to enable hospitals that are located in remote areas and have limited human resources to provide patients' with the best healthcare possible; by enabling more radiologists to view medical images for diagnostic purposes. Malek Karadsheh, PAC Product Line Manager at Optimiza, further added that PACS has a community of users who are eligible to access it using an assigned username and password, including technicians and radiologists. It provides different privileges to different users; including different levels of access, such that not all users are able to view patients' private information. Amer Madaeen, Project Manager at CISCO, explained that SXP service exchange platform has been provided by CISCO, in addition, peer to peer communication is facilitated by WebEX. It was further acknowledged by all three participants that the presence of such a system enables images to be used in research as they can be easily accessed offsite.

May 7th, 2015; Dr. William Maaiah; Ministry of Health/ consultant radiologist

Dr. William Maaiah, the consultant at Al-Bahir hospital, was interviewed. The following are the responses he gave to the interview questions. When asked about his involvement of research he stated that he is not involved in large-scale studies, but participates in individual case studies that the hospital sometimes conducts for special cases. His answer to the second interview question expressed his interest in conducting research then communicated his concern about the inability to do so saying: "Funding for research is a problem. Jordan does not allocate many funds for research; therefore research in public hospitals is almost nonexistent. Conducting future studies would be hard as well". Answers to questions three

Table 2.

	Name	Position/Institution	Date of interview
1	Mr. Aiman Alrawbdeh	Senior program manager for Cisco Systems	April 29, 2015
2	Mr. Amer Madaeen	Project manager for Cisco Systems	April 29, 2015
3	Mr. Malek Karadsheh	PACS/RIS product line manager for Optimiza	April 29, 2015
4	Dr. William Maaiah	Consultant radiologist for the Jordan Ministry of Health	May 7, 2015
5	Ms. Linda Kateb	IRB coordinator at King Hussein Cancer Center, Amman	June 15, 2015
6	Dr. Mohammad Hiaari	Consultant radiologist for Jordan Hospital, Amman	July 1, 2015

and four were "not sure". He answered question five saying that he is very interested in the potential of sharing images with the University of Toronto for research purposes. He added that due to the hospitals' lack of resources and the consequent inability to conduct research, the only part the three hospitals can play in such an initiative is to provide data. He concluded his answer to question five by adding "Since there isn't much research going on in Jordan, research findings using our data would greatly benefit doctors here." Answers to questions six, seven, and eight were "not sure". He answered question nine saying "For Medical Imaging clinical purposes, access to PACS is restricted by username and password, protecting patients' privacy." No answer was provided for question ten. He answered question eleven stating that to share images with the University of Toronto, permission from Ministry of Health (MOH), then permission of the radiology department – which is his jurisdiction – are needed. For question twelve, he stated that he provided permission for him to be contacted later if needed.

July 1st, 2015; Dr. Mohammad Hiaari; Jordan Hospital / Consultant Radiologist

Dr. Mohammad Hiaari, a consultant radiologist at Jordan Hospital was interviewed.

The following were his answers to the interview questions. When asked about his involvement in medical imaging research, he stated that he has done various research studies in the past in this area. He did not provide further specifics to the type/number of studies that he has taken part in. He answered the second interview question stating he has a very strong interest in conducting medical imaging research, he further added that the teaching hospital has ten residents and five fellows who will be assigned to participate in research studies. He answered the third question saying that teaching hospitals, like Jordan Hospital and King Hussein Cancer Center, as well as universities should be the ones conducting studies. He further added that universities do not conduct a lot of research. Answers to questions four was "not sure". He answered question five saying that he thinks sharing images with the University of Toronto for research purposes is a great idea. Answers to questions six, seven, eight and nine were all the same, stating that the department of continuing studies at the hospital is in charge of all the research ethics issues. He had no further comment. As for question ten, he stated that there are no clear guidelines for maintaining patient privacy and confidentiality in medical research, and the de-identification of data is sufficient to share patient images with a third party. He answered question eleven saying that to share medical images from Jordan Hospital, his approval would have to be given. Images have to be de-identified. Moreover, he stated that compatible e-infrastructures allow easy sharing of images. He answered question twelve saying that he gives his consent to be contacted later if needed. The interview was concluded by a final comment from him stating that while being interested in all areas of Medical Imaging research, he has particular interest in Neuroradiology as well as Vascular radiology research.

June 15th, 2015; Linda Kateb; King Hussein Cancer Center / IRB coordinator at the Office of Scientific Affairs and Research

As an institutional review board (IRB) officer, Linda Kateb could not provide answers to the first five questions. She answered the sixth and seventh question saying: "research ethics are governed IRB which functions in accordance to Good Clinical Practice (GCP), Helsinki Declaration and the International Conference on Harmonization (ICH) guidelines on clinical trials". She further added that the IRB at KHCC is approved by the Jordan Food and Drug Administration (JFDA). It is also registered with Federalwide Assurance (FWA) allowing KHCC institutional IRB to act as a central IRB for any institution/third party that wishes to conduct research but lacks an IRB. She answered the eighth question stating that a research proposal must be sent to the IRB office, which will then undergo preliminary review, after which an appropriate form is determined and must be filled out to gain final approval by IRB, upon approval, a primary investigator (PI), from the institution, is assigned. The entire process of getting an approval for research studies usually takes less than a month. Her answer to question nine was "not sure". She answered question ten saying that to her knowledge there are no specific requirements in place to guide how patients data is to be protected if there is a need for sharing their information with a third party, and de-identification of data should be sufficient. She didn't answer question eleven, as it was not within her area of expertise. The interview concluded with her answering question twelve by giving consent to be contacted later if needed.

Discussion

Our investigation revealed the completion of a pilot project by Merge and Cisco that could technically support image data sharing with MiNE. Our study unveiled the ethics and security related guidelines and steps that shall apply in the event of collaborative research with appropriate institutions in Jordan. Although, there was interest for establishing a link between Jordan and MiNE among the radiologists interviewed it remains difficult to gauge the level of imaging research taking place in Jordan, as well as the overall interest of the Jordanian radiology community to participate in collaborative research.

A PACS/VNA with capability for remote access, image upload and viewing was successfully installed in 2015 as a proof-of-concept under the JHI. This installation implies that future collaboration between Jordanian hospitals and MiNE's Toronto-based PACS is technically achievable. It also implies that links to medical centres outside MiNE's Toronto base, whether in Jordan or Northern Ontario for example, will require similar e-infrastructure, namely remote access to a PACS on a secure internet-based network that resembles tele-health in its security, quality assurance and capacity for real-time online communication. The JHI e-infrastructure was installed for clinical purposes: exchanged patient data is identifiable and therefore not available for research according to privacy regulations. It is nonetheless theoretically available thanks to Merge's scan de-identification technology and MiNE's data scrubbing protocol, which includes removal of any patient identifiers that may remain following upload.

Restricted resources at Jordan's public sector medical centres would likely hinder active participation in research from radiologists at those centres. Nonetheless there is willingness to act as data collection and contribution hubs for MiNE. In comparison to Jordan's public healthcare sector, its private sector is well funded, and the response of private sector personnel to our inquiry was enthusiastic. It has been shown that the quality of patient care and patient satisfaction are higher for the private than for the public sector, [10] and Jordan's health tourism has grown to net one billion dollars annually for its private centres. [11] Interestingly, according to our findings, very little medical imaging research is conducted in this sector. Radiology training in privately held centres is in the practice of clinical skills and does not include research outside of case studies. In light of these findings, MiNE has described possible challenges from this sector as: i) commitment to research outcomes in face of clinical demands, and; ii) the observance of unfamiliar, research-driven acquisition protocols. A final challenge addressed later in this study was to determine whether image data transfer between Jordan's private and public centres is ethically permissible.

Jordan's Clinical Trial Law entitles both private and public institutions to conduct medical research through their respective IRBs. The law delegates ultimate responsibility for harm that may result from a research study to that study's PI and her team, provided that this PI is a member of a designated institution. The law also states that the insurance given to a study participant must be equivalent to that given in other countries (conducting the same trial). In addition, the law: i) seeks to ensure that subjects complete all necessary tests and give consent prior to participation; ii) identifies criteria for institutions conducting clinical trials, and; iii) requires translation into Arabic of all information provided to participants (2011 amendment). With regard to privacy and confidentiality, the law reflects the Declaration of Helsinki by placing responsibility for any confidentiality-related procedures on the participant's physician. With regard to the reuse and sharing of identifiable medical data, consent is required unless impossible, impractical or threatening to research validity. With regard to the reuse and sharing of de-identified data, such as images acquired either during a trial or clinically (prior to the study), the law is silent: there is no requirement for consent from either the participant or her physician. [10-12] We can assume therefore that Jordanian law permits the sharing of de-identified subject data between private and public hospitals for research led by a private sector PI.

The protocol for ethics approval in Jordan differs from Canadian protocol not in principle but in the level of detail for implementation: while the laws protecting patient privacy in both Jordan and Canada derive from the Declaration of Helsinki, the Canadian legal landscape surrounding the conduct of research and the protection of patient data is more granular and requires greater diligence. Canadian rules are informed by the Tri Council Policy Statement (Statement) on the Ethical Conduct for Research Involving Humans (2010), such that Canada's federal research agencies require all institutions seeking funding to adhere to its principles. These principles refer to respect for human subjects, concern for their well-being and their equitable treatment. In keeping with the Declaration of Helsinki, the Statement describes the need for free and informed consent, protection of vulnerable persons, maintenance of privacy and confidentiality, assessment of risks and benefits, and minimization of harm. The statement also details the role that Canadian REBs play in the execution of the policy. With regard to privacy and confidentiality in Canada, these protections are outlined by Canada's Personal Health Information Protection Act (PHIPA), which is implemented by the Privacy Commissioner of each province and which regulates the gathering, use and disclosure of PHI in a privacy-sensitive and confidential manner within provincial and territorial jurisdictions. The act also describes best practices regarding access to PHI without consent. [13,14] Both PHIPA and Clinical Trial Law permit such access provided that the local IRB/REB approves a research plan that describes why consent is not being sought as well as who will have access to this information. It should be noted that, like Jordan's Clinical Trial Law, PHIPA applies to identifiable patient data only: de-identified data is exempt from regulations under the act. The difference between the laws that is of greatest concern to MiNE is the description (in PHIPA) of the liability of information technology service providers with regard to the collection, use, modification, disclosure, retention and disposition of PHI. In face of PHIPA, it becomes all the more important for MiNE to ensure compliance through a tested de-identification protocol.

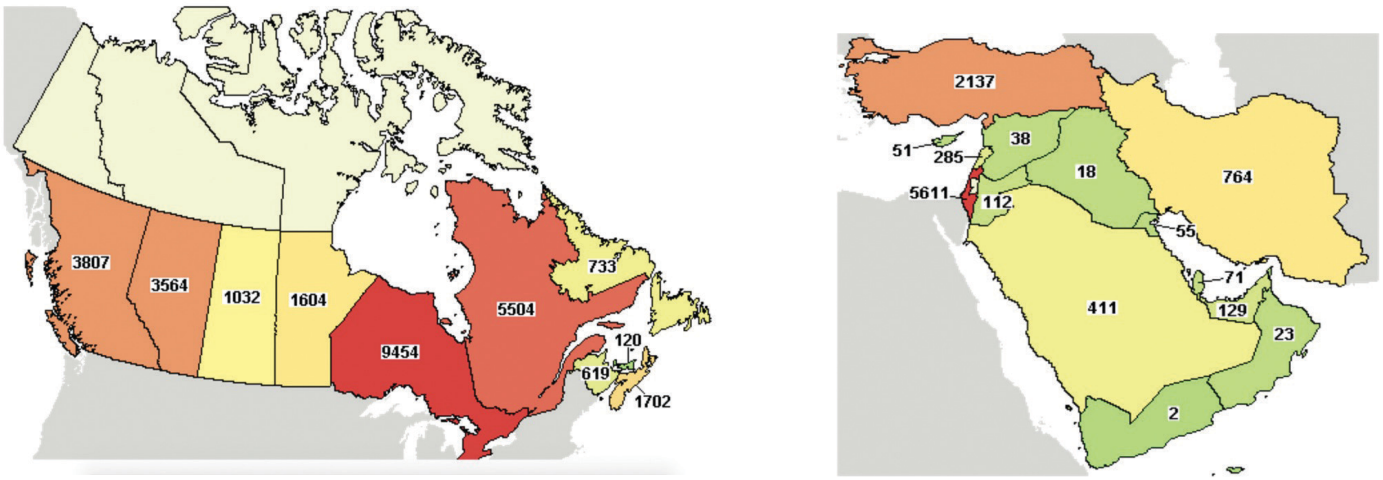

Figure 2. A map comparison of clinical trial density between Ontario (left) and Jordan (right).

From MiNE's perspective, understanding the research potential of an expanding economy like Jordan is extremely important, in no small part because of how efforts there can inform our approach to potential regional partners in Canada. Clearly, there are economic differences between the two countries that affect the likelihood of participation. If we infer healthcare-related spending from the Gross Domestic Product (GDP) of each country, Jordan's healthcare budget—per citizen—is one tenth that of Canada's budget. [12] Similarly, private medical research spending can be inferred by comparing the number of clinical trials being conducted: 14,000 in Canada but only ninety-four in Jordan (data from 2015). However, Ontario and Jordan are similar in that they both have the highest number of clinical studies being conducted relative to their surrounding provinces/ countries. [13] **Figure 2** shows a data visualization map of clinical trial density in these jurisdictions. It is undeniable that the electronic collection of personal data has grown exponentially and will inevitably reach every community, regardless of its locale. Any e-infrastructure put in place for such purpose—such as MiNE—must be scalable, interoperable, auditable and secure.

Limitations

This study was limited by the small number of interviews conducted. This limit was largely due to a lack of understanding and permission at the departmental and office administration level. Had all those contacted for an interview been made accessible, we would likely have gained a more comprehensive understanding of radiology in Jordan, including the current degree of research participation and the willingness to participate in future research.

Conclusion

The technology underlying MiNE's e-infrastructure has been proven at MiNE's Toronto site and was demonstrated successfully in Jordan, indicating that image data sharing between these jurisdictions is technically achievable. Interviews conducted in Jordan provided insight into the readiness for image sharing and collaborative medical image research.

Acknowledgments

We would like to thank Merge Healthcare for its support of this study. We would also like to acknowledge Neda Ghiam and Jennifer Liu for their assistance in this project.

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JULS RESEARCH

Investigation on the Ring Flipping of Vicinal Disulfide Rings Found within Nicotinic Acetylcholine Receptors Using Eight-Membered Ring Model Systems

Filip Dinic[1], Anita Rágyanszki[2], Natalie J. Galant[3], Imre G. Csizmadia[1,2]

[1]Department of Chemistry, University of Toronto
[2]Department of Chemical Informatics, Faculty of Education, University of Szeged
[3]Department of Medical Biophysics, University of Toronto

Corresponding Author: Filip Dinic (filip.dinic@mail.utoronto.ca)

Abstract

Previous studies have demonstrated that the ring flips undergone by vicinal disulfide rings (VDR) play a significant role in the bonding function of nicotinic acetylcholine receptor's (nAChR), whereas due to the change in confirmations, the nAChR will also undergo a change in shape. These nAChR's are mainly found in axon terminals, and have a role in nicotine addiction and diseases such as Parkinson's and Alzheimer's. These receptors are potential drug targets, and understanding the change in properties as the ring flips between its conformers can lead to potential drugs which could minimize or prevent these illness from occurring. This ab initio study investigates the nature of various eight-membered rings and their associated ring flips, quantifying and analyzing its effects of thermodynamics and structure. It was determined that while this process is spontaneous the associated activation energy with this transition is high. In addition, the S-S linkage was found to impose a great deal of strain, leading to deformation of the ring. This study elucidates the process of various eight membered ring flips, allowing for a better understanding of its influence on nAChR's.

Introduction

Cycloctane is an eight membered carbon ring, which readily switches between its conformers via ring flipping [1]. The most stable form being the chair boat conformer [2]. Ring flipping is a process whereby a molecule adopts a new spatial conformation, where each of the eight members of the ring undergoes a change, from either *cis* to *trans* or vice versa. The process of changing the spatial orientation of atoms in the ring can lead to a more energetically stable species due to the removal of steric strain, **Figure 1**. One example of an eight membered rings appearing in nature is the vicinal disulfide ring (VDR, **Figure 2**). VDR's are formed when a disulphide bond is formed between two adjacent cysteine residues [3]. These rings contain 4 heteroatoms and are extremely rare in nature, approximately 1.1% of known proteins contain this structure [4]. The rarity of this ring in nature is indicative of its unique and specialized function, arising from its potential to ring flip. Like regular cycloctane rings, these rings can undergo conformational changes associated with an eight membered ring flip (**Figure 1**), where the change from *cis* to *trans* and vice versa, can drastically change the structure of proteins. The ability of a VDR to change the local or overall structure of a protein is a proposed model for the nicotinic acetylcholine receptor's (nAChR) binding site [5]. Upon ligand and receptor interaction, the ring undergoes a conformational change, making it resistant to reduction of two orders of magnitude [5]. These nAChR's are mainly found in axon terminals, and have a role in nicotine addiction and diseases such as Parkinson's and Alzheimer's [6]. These receptors are a potential drug target, and since VDR's play a key role in nAChR's binding site, it is important to understand the change in properties as the ring flips between its conformers. Part of this, is understanding the purpose of which heteroatoms play in the ring flipping process.

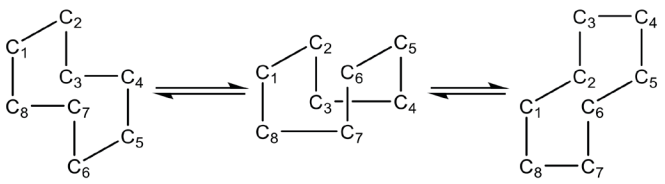

Figure 1. Model of which cycloctane rings undergo ring flipping. The carbons have been labelled in order to demonstrate the *cis* to *trans* and vice versa process.

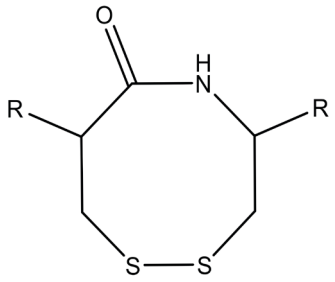

Figure 2. Structure of vicinal disulfide rings, where the disulfide bridge is opposite to the peptide bond in the ring.

With VDR ring flips being a crucial part of nAChR binding, it is important to assess the influence that heteroatoms have on this conformational change. Through the introduction of heteroatoms, either via the replacement or addition of carbons, or alternative functional groups, the respective activation energy associated with the ring flip may change. In this study, through the use of various isodesmic reactions, the energy associated with the cycloctane ring flip will be analyzed in order to investigate the effects of heteroatoms on the ring flipping of cycloctane.

Three different isodesmic reactions have been studied to observe the effect of the addition of heteroatoms on the energy associated with ring flipping.

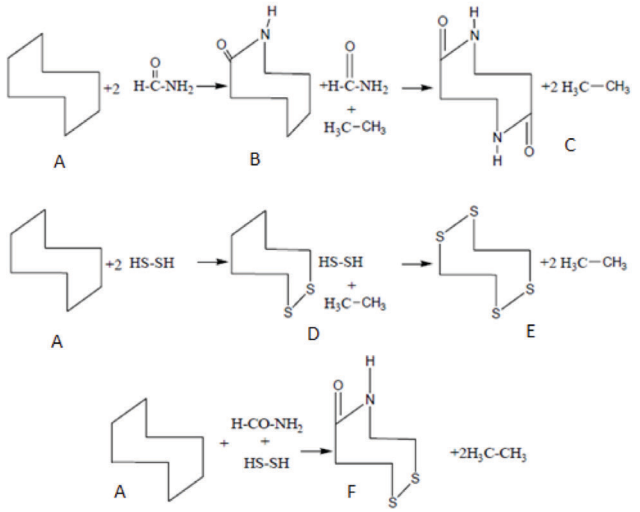

Figure 3. The six studied molecules along and their associated isodesmic reactions.

Methodology

The thermodynamics for Gibbs free energy, entropy and enthalpy and geometric properties such as bond length, bond angles and dihedrals were calculated using the B3LYP/6-31G(d) level of theory (DFT) using the Gaussian 09W [23,24] software package. The total energies and the thermodynamics obtained for the molecules were found in Hartrees, as well as geometric data was tabulated in tables 1-5 of the supplementary section. However, their relative thermodynamic properties were calculated in kilojoules per mole through the use of a conversion factor where, 1 Hartree = 2625.49963 kJ/mol.

Results

The results obtained from the Gaussian program can be found in the supplementary section.

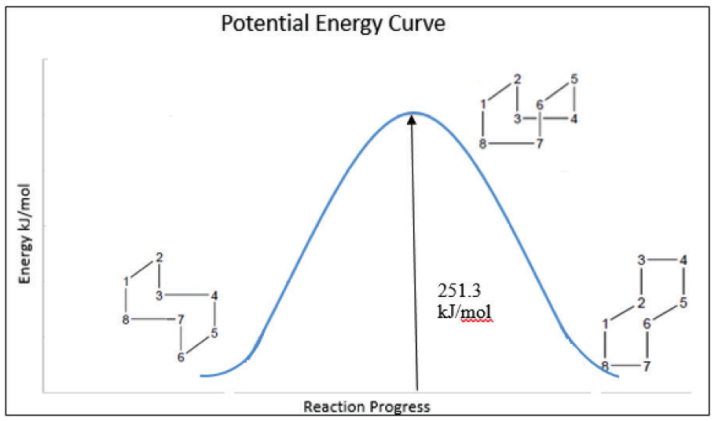

Figure 4. The potential energy curve of the structure A's ring flip. The associated thermodynamics with this process is 251.3 kJ/mol, where both the final and initial forms are identical in energy.

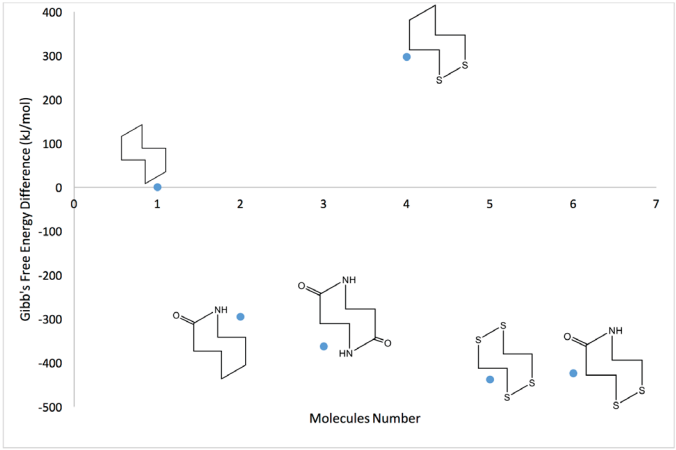

Figure 5. Change in Gibbs free energy (ΔG) during the transition from the initial to final chair, depicting the spontaneity of the ring flip. Where molecule A is represented by 1, molecule B by 2, molecule C by 3 and so forth.

Discussion

The energy comparison between the initial and final energy of the six chair conformers, are quite similar within a range of 285.1 – 420.2 kJ/mol. In general there is a slight decrease in the overall thermodynamics of the final chairs in comparison to the initial chair compounds. The most probable explanation behind this phenomenon is potentially due to shifting from *cis* to *trans*, resulting in a decrease in steric strain. However the notable exception to this pattern will be the compound D. The large sulfur atoms caused a great deal of strain within the ring, mainly due to its large electronegativity and its large atomic bond of 2.2 Å. As a result, a large amount of deformation will occur as an attempt to accommodate this addition strain. As a result, this will lead to a higher ring strain, ultimately causing the final form to be more strained.

Similarities between the six compounds

Since chair F, has similar substitutions as the structures B and D, one would expect an energetic and geometric relationship between those molecules. Within the F chair, one can observe similar characteristics in the rings structure within the other rings. For instance, the S-S linkage has a dihedral angle which is very similar compared to chair D, varying from 0.407 to 1.89 degrees. However, the bond angles for the peptide bond group, in compound B and F, will also be similar but will vary more, anywhere from 16.2-0.96 degrees.

When one adds the energies of structures B and D, it is equal to the energies of structure F. In addition a similar trend is noticed for the compounds which contain heteroatoms and functional groups opposite to each other in the ring. If one adds the energies from structures C and E, and divided it by two, one would, once again receive the energy of the sixth chair conformer. This phenomenon can be applied to both the initial and final chair compounds.

Structural Analysis of the compounds

While in the initial *cis* form, this ability to deform is easily achievable since the heteroatoms are more mobile. However for the *trans* form, the ability for the ring to deform will be significantly reduced. This is especially significant when comparing the dihedral angle between the final and initial structure of molecules D and F. In both cases, the dihedral angle decreases, indicating that while in the *trans* form, the ring has a lower degree of mobility and therefore, it will be more unstable compared to the initial form.

When analyzing the dihedral of both compound D and F one observes an average dihedral angle of 89.6°. Upon further investigation, the dihedral of a hydrogen disulfide, the simplest sulfur-sulfur bond, will have an ideal dihedral angle at 91°. As a result, one can suggest that the most energetically stable dihedral angle will occur at around 90 degrees, shifting based on the presence of other heteroatoms in the ring and the groups orientation. When the S-S group is in the *trans* form, the dihedral is reduced to 86.22°. This result effectively explains why there is such a high degree of distortion within the compounds D and F.

Biological implication of the Energetics

Using the relative energies of cyclooctane, the activation energy (Ea, kJ/mol) was predicted. The predicted activation energy is relatively high (251.3 kJ/mol) and as a result one can envision that some catalytic or enzymatic process might occur which would regulate and activate the ring flipping of eight membered rings such as in VDR's.

In order to anticipate the spontaneity of the ring flip, one can analyze the change in the Gibbs free energy (ΔG). For spontaneous systems, the change in G will be negative, while for non-spontaneous systems, it will be positive. Most molecules, to the exception of ring number four, the ring flip process is spontaneous, including the VDR ring **Figure 5**.

However in order for the VDR to ring flip between both *cis* and *trans*, it must be able to alternate from one chair to the other. As a result, some sort of enzyme, protein, is required to act like a catalyst, in order to assist the ring in transition from the final chair to the initial chair.

Conclusions

While previous studies have shown that VDR ring flips play a significant role within the nAChR receptor's bonding site (Kao, 1986), the ring flipping process is not very well understood. Within this *ab initio* study it was determined that while this process is spontaneous and the associated activation energy with this transition is high. As a result, in order for this process to be regulated and occur in a controlled and desired manner, there must be an external influence which catalyzes this conformation change. Future research is required to investigate possible enzymes and signals which catalyzes such a change.

Acknowledgements

I would like to thank Professor Imre Csizmada for the opportunity to have an independent research project and for all the advice he has given me. In addition, I would like to thank the Research Opportunity Program and the chemistry department of the University of Toronto for creating this opportunity. In addition I would like to thank the University of Szeged for supplying the computational power needed to run the software needed for energy calculations. Furthermore I would like to thank Anita Rágyanszki and Natalie J. Galant for their irreplaceable assistance and guidance. Finally I would like to thank Nidaa Rasheed, Craig Yu, Jay Lee and Tommy Sung, for their help with technical issues and for their support.

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JULS RESEARCH

Generating *Saccharomyces cerevisiae* models of Lenz-Majewski Syndrome for cellular and genetic studies

Wan Ting Huang[1]

[1]Faculty of Arts and Science, University of Toronto

Corresponding Author: Wan Ting Huang (tinaw.huang@mail.utoronto.ca)

Abstract

As the only known human disease caused by disrupted phosphatidylserine (PS) metabolism, Lenz-Majewski syndrome can offer insight into the physiologically important, yet not well understood PS pathway. *Saccharomyces cerevisiae* (budding yeast) was used to produce working models of the disease for genetic and cellular studies, which to the author's knowledge are the only models available for Lenz-Majewski Syndrome. PS expression levels in yeast were manipulated using a combination of wild-type, PS decarboxylase 1 knockout (*psd1Δ*), or *psd1/psd2Δ* cells and overexpression of plasmid borne CHO1 that encodes PS synthase. Experiments investigated cell growth, cellular PS distribution, and global PS levels to confirm and further define phenotypes. Results show extensively impaired cell growth in all *psd1/psd2Δ* cells, especially for *psd1Δ* cells that constitutively expressed CHO1. In parallel experiments using galactose inducible plasmids, wild-type and *psd1Δ* cells that overexpressed CHO1 on galactose plates regained non-glucose associated phospholipid depletion and grew better compared to counterparts transformed with vector. Analysis of PS distribution revealed increasingly aberrant PS accumulation in early endosomes and vesicles for wild-type cells that overexpressed CHO1, *psd1Δ* cells with vector, and *psd1Δ* cells that overexpressed CHO1. Similarly, cells experienced loss of polarity and were larger and rounder. *Psd1Δ* cells that overexpressed CHO1 were on average 99.7% greater in area compared to wild-type cells. Attempts to investigate global PS levels were unsuccessful. The study reveals that high PS levels result in impaired cell growth and PS accumulation in intracellular structures.

Introduction

In eukaryotic cells, lipid membrane composition varies significantly among organelles and tissues and is a functionally essential and highly regulated process [1]. Phosphatidylserine (PS) is a membrane phospholipid that constitutes only 2 – 10% of all phospholipids [1]; however, it plays a key role in several important processes including second messenger transduction, blood coagulation, and apoptosis [2,3]. The physiological importance of PS is largely attributed to its unique biochemical properties, cellular distribution, and tissue variations [2]. Unlike phosphatidylcholine (PC) and phosphatidylethanolamine (PE) that are zwitterionic and together make up over 60% of phospholipids, PS is anionic [3]. PS is also preferentially localized in the inner leaflets of lipid bilayers, most notably in the plasma membrane and endocytic membranes [1]. Hence, large endowment of PS causes an electrostatic surface potential that enhances recruitment of cationic proteins and those with cationic motifs, a process essential for survival [3]. In multicellular organisms, PS variations in different tissues is also functionally important [3].

In mammalian cells, two PS synthases (PSS1/PSS2) localized in the ER have been identified, encoded by the *PTDSS1* and *PTDSS2* genes respectively [4]. PSS1 synthesizes PS via an exchange of L-serine with PC while PSS2 converts PE into PS through a parallel base - exchange reaction [2]. PS is degraded by phosphatidylserine decarboxylase (PSD) found in outer areas of the inner mitochondrial membrane [3]. PS is also hydrolyzed by phospholipases located in the plasma membrane [3]. In *Saccharomyces cerevisiae*, PS is synthesized by PS synthase in the ER encoded by a single gene (*CHO1*) and converted into PE extensively by PSD1 localized in the mitochondria and minimally by PSD2 localized in the Golgi complex/vacuole membranes [3].

PS is formed in the ER yet found in highest concentrations in the plasma membrane and early endosomes [3]. However, PS intracellular transportation is not well understood [3]. PS is synthesized in the ER and integrates into mitochondrial-associated membranes that communicate between mitochondria and the ER [3]. However, neither the ER nor mitochondria is PS rich [3]. In the ER, newly synthesised PS travels to the Golgi intermediate compartment and eventually the trans-Gogi network via the secretory pathway [3]. PS transport then continues to the plasma membrane or traverses to prelysosomal endocytic compartments [3]. There is rich PS endowment in the plasma membrane and early endosomes; however, the steps that concentrate PS in the organelle membranes are unknown [3]. One theory is that PS concentration

occurs in the trans-Golgi network secretory vesicles that deliver lipids to the plasma membrane [3]. Another theory explains that PS enrichment may be due to preferential internalization of plasma membrane subdomains that exclude PS rich regions [3].

Sousa et al. (2013) described gain-of-function mutations in *PTDSS1* that cause the rare genetic condition known as Lenz – Majewski Syndrome (LMS) [4]. Cases are isolated in ancestry, which in addition to a distinct and consistent phenotype, indicates *de novo* heterozygous dominant mutations believed to be in the single *PTDSS1* gene [4]. The disorder is characterized by skeletal abnormalities, intellectual impairment, and several craniofacial, dental, cutaneous, and distal – limb abberations [4]. Progressive generalized hyperostosia associated with LMS also affects the cranium, vertebrae, and diaphysis of tubular bones, resulting in severe growth restrictions [4].

In vitro experiments comparing PS synthesis in control and fibroblasts harvested from LMS individuals found enhanced PSS1 mediated PS synthesis as a result of reduced end-product inhibition of PS in cells from LMS individuals [4]. Interestingly, despite increased PS synthesis, the amount of cellular PS, PC, and PE determined by cell lysate mass remained unchanged [4]. Global phospholipid homeostasis appeared to be tightly regulated; however, cellular PS distribution was not investigated [4]. Furthermore, PS is recognized for its calcium – binding properties that are important in bone metabolism, a process severely deviant in individuals with LMS [4,5]. Although no direct link had been found between PS metabolism and increased bone density (observed in LMS), Sousa et al. (2013) proposed that bone mineralization, calcification, and dentine formation may be linked to PS synthesis dysfunction due to the key role of PS in apoptosis and enrichment of released vesicles [4]. These processes are tightly regulated in osteoclasts and related lineages [5]; however, the author is not aware of studies investigating PS dysfunction in these cells.

The objective of this project is to create *S. cerevisiae* models of LMS for genetic and cellular studies. Phenotypes will be observed for both constitutive and inducible overexpression of *CHO1* in wild-type, PS decarboxylase 1 knockout (*psd1Δ*), and *psd1/psd2Δ* mutant strains. Experiments will focus on characterizing cell growth, PS distribution, and global PS levels.

Methods

Three *S. cerevisiae* strains were used: BY4741 (*WT*), *psd1Δ* and *psd1/psd2Δ*. All strains carry the genotype of ura3, leu2, and his3, rendering them unable to synthesize uracil, leucine, and histidine. Each strain was transformed following the protocol developed by Gietz and Schiest (2008) [6] to constitutively express plasmid borne *CHO1* (p416-GDP CHO1) or plasmid vector (p416-GDP) as controls. The plasmids were of the pRS series derived from Sikorski & Hieter (1989) [7] and contain the URA3 gene. Transformed cells were plated on Synthetic Complete medium that lacks uracil (SC-Ura) to select for successful transformants. *Psd1Δ/psd2Δ* cells were grown on ethanolamine enriched plates to compensate for ethanolamine auxotrophy.

Parallel experiments for galactose inducible *CHO1* cells were performed to enhance cell growth. Another set of *WT*, *psd1Δ*, and *psd1/psd2Δ* cells were transformed with galactose inducible *CHO1* plasmids containing the GAL1 promotor (pESC-LEU CHO1) or plasmid vector (pESC-LEU) as controls [6]. pESC-LEU plasmids contain the LEU2 gene and thus standard gene complementation was used to determine transformation success on SC-Leu plates. Similarly, *psd1Δ/psd2Δ* cells were grown on ethanolamine enriched plates. Cell proliferation assays were used to analyze *S. cerevisiae* growth by spotting serial dilutions on SC-Leu, SGal-Leu (glucose replaced with galactose), and SRaf-Leu (glucose replaced with raffinose) plates [8]. *Psd1Δ/psd2Δ* cells did not grow and due to time constraints p416-GDP/CHO1 cells were not used for experiments after spotting serial dilutions. Hence, only *WT* and *psd1Δ* strains transformed with either pESC-LEU or pESC-LEU CHO1 plasmids were used from this point forward.

Cho1p (PS synthase) overexpression was investigated using western blots. Cell samples were grown overnight in 3mL SC-Leu medium shaken overnight at 30°C. In the morning, half of each sample was saved and the other half back inoculated into SGal-Leu to induce pESC-Leu/CHO1 plasmids. After four hours, samples were prepared in 200µL 4x SDS-PAGE sample loading buffer [9]. We had obtained two custom rabbit monoclonal antibodies described by Choi et al. (2010), one against the N-terminus and one against the C-terminus of Cho1p [10]. Hence, two SDS-PAGE gels were probed to assess each antibody. Following the ladder, eight samples were loaded per gel: *WT* pESC-Leu, *WT* pESC-Leu CHO1, *psd1Δ* pESC-Leu, and *psd1Δ* pESC-LEu CHO1 cells all grown in glucose, and *WT* pESC-Leu, *WT* pESC-Leu CHO1, *psd1Δ* pESC-Leu, and *psd1Δ* pESC-LEu CHO1 cells all grown in galactose. SDS-PAGE was performed on 14% polyacralimide gels [11]. Proteins were transferred onto PVDF membranes and incubated with either the N-terminus or C-terminus primary antibody at 1:2000 dilution and HRP-conjugated anti-rabbit IgG secondary antibody at 1:5000 dilution [12], then imaged with *ChemiDoc™ MP*.

The distribution of PS in cells was investigated by transforming the PS biosensor fused to GFP (GFP-LactC2) that has a C2 domain that binds PS highly selectively [13]. GFP-LactC2 contains the complementary URA3 gene, therefore cells were plated on SC-Leu-Ura plates to select for successful transformants. Samples were prepared in 3mL SC-Leu-Ura media grown overnight. In the morning, half the cells of each sample were back-inoculated into SGAL-Leu-Ura media and allowed to grow for three hours. A total of eight cell samples were then imaged with confocal microscopy. Using the *Fiji program*, images were further analyzed by obtaining diameters of twenty different cells measured along the longest side for each sample condition.

Global levels of PS in cell samples were also investigated and compared. Cells were grown overnight in SC-Leu media and switched to SGAL-Leu media in the morning and allowed to grow for four hours. After concentrating cells through centrifugation, lipids were extracted and separated by Thin Layer Chromatography using a solvent of 6.25mL 1-propanol, 6.25mL chloroform, 6.25mL methyl acetate, 2.5mL methanol, and 2.25mL 0.25% KCl. Plates were dried in an oven and sprayed with primulin to visualize under UV light [14].

Results

WT and psd1Δ p416-GDP/CHO1 spotted serial dilutions on SC-Ura plates showed similar growth, except *psd1Δ* p416-GDP CHO1 cells that grew poorly (Figure 1a(i)). Upon closer inspection, there were large colonies growing amongst smaller ones (Figure 1a(ii)).There were no visible differences on SC-Ura and SRaf-Ura plates, although the latter grew more poorly overall (Figure 1b, c). On the SGal-Ura plate, *WT* and *psd1Δ* pESC-Leu CHO1 cells grew better than their pESC-Leu counterparts (Figure 1d).

Only the N-terminus antibody produced a western blot with distinct bands (Figure 2a). Bands at 27kD and 30kD represent unphosphorylated and phosphorylated forms of Cho1p and are faint

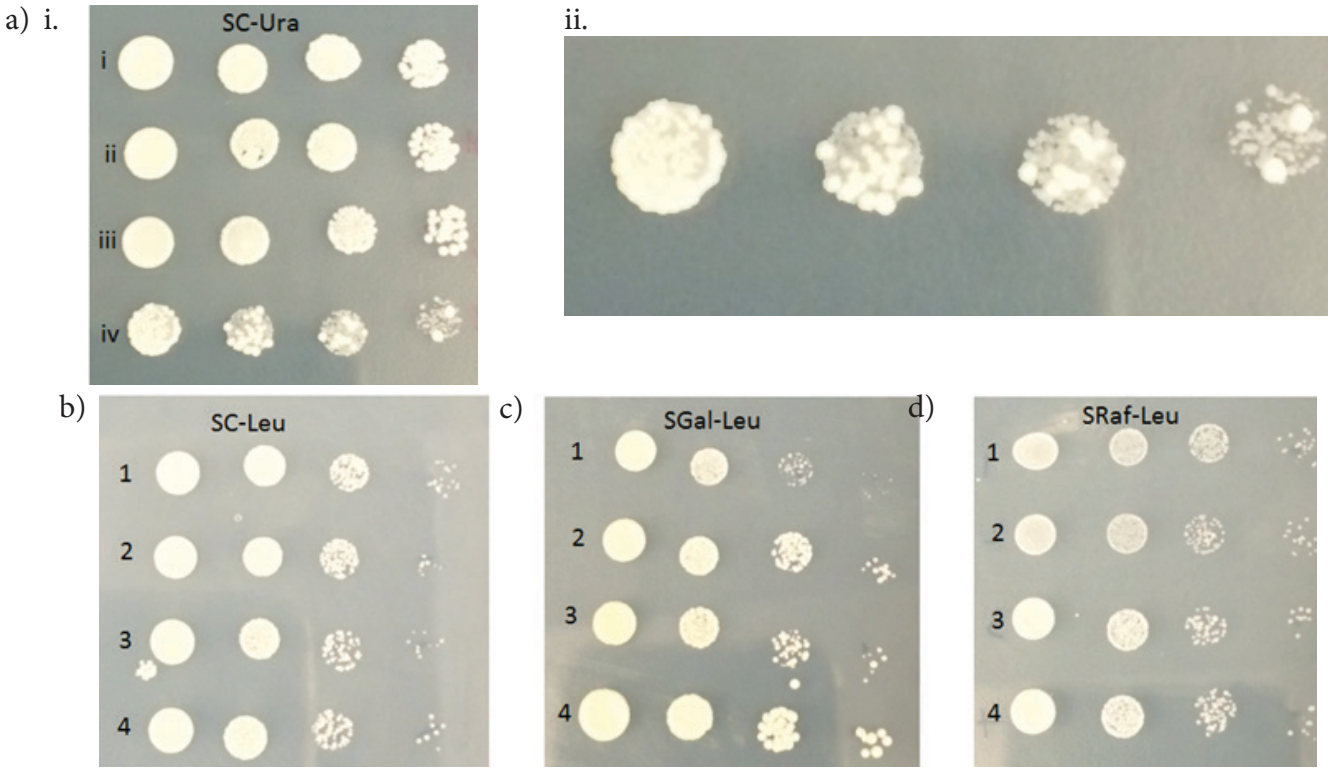

Figure 1. *S. Cerevisiae* spotting serial dilution growth assays. a) i. *WT* and *psd1Δ* cells that constitutively express p416-GDP/CHO grown on SC-Ura plates. Serial dilution samples are as follows: (i) *WT* p416-GDP, (ii) *WT* p416-GDP CHO1, (iii) *psd1Δ* p416-GDP, and (iv) *psd1Δ* p416-GDP CHO1 cells. *ed* p416-GDP CHO1 cells show poorer growth. ii. Magnification of *psd1Δ* p416-GDP CHO1 cells revealed several large colonies mixed with smaller ones. b-d) Samples are as follows: (1) *WT* pESC-Leu, (2) *WT* pESC-Leu CHO1, (3) *psd1Δ* pESC-Leu, (4) and *psd1Δ* pESC – Leu CHO1 cells. b) *WT* and *psd1Δ* cells grown on the SC-Leu plate that contained glucose inhibited expression of the pESC-Leu/CHO1 plasmid. There were no visible differences between samples. c) Cells grown on SGal-Leu plates with galactose induction of the pESC-Leu/CHO1 plasmid. Better growth displayed by *WT* pESC-Leu CHO1 and *psd1Δ* pESC-Leu CHO1 cells. d) Cells grown on SRaf-Leu plates that contained raffinose. No visible differences between cell types but poorer growth compared to cells grown on SC-Leu plates.

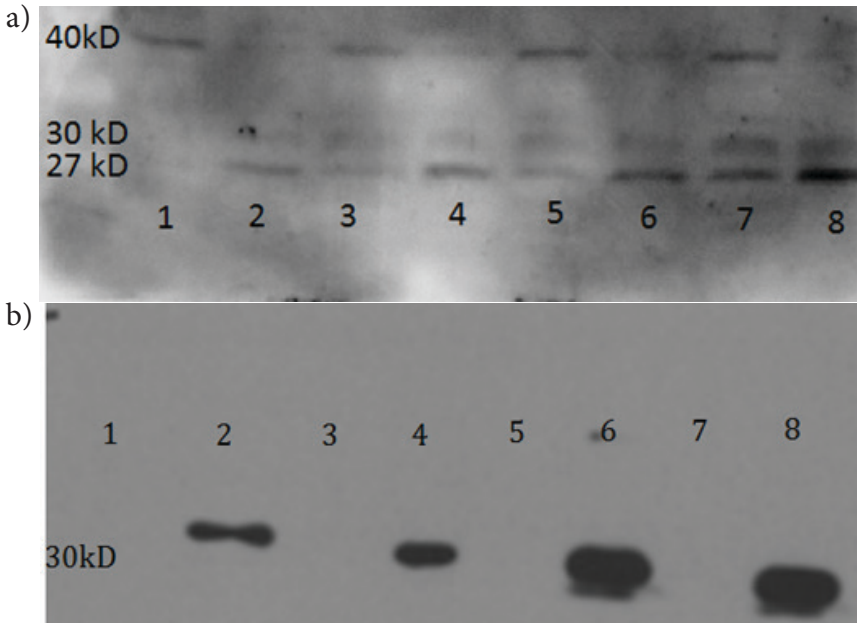

Figure 2. Western blots using rabbit primary antibody against the N-terminus of Cho1p. Samples are as follows for both blots: (1) *WT* pESC-Leu cells grown in glucose, (2) *WT* pESC-Leu cells grown in galactose, (3) *WT* pESC-Leu CHO1 cells grown in glucose, (4) *WT* pESC-Leu CHO1 cells grown in galactose, (5) *psd1Δ* pESC-Leu cells grown in glucose, (6) *psd1Δ* pESC-Leu cells grown in galactose, (7) *psd1Δ* pESC-Leu cells grown in glucose, and (8) *psd1Δ* pESC-Leu CHO1 cells grown in galactose. a) Phosphorylated and unphosphorylated forms of Cho1p form a doublet at 30kD and 27kD respectively [10]. There was increased Cho1p concentration in galactose conditions (2, 4, 6, and 8) compared to their glucose counterparts (1, 3, 5, and 7). Upregulation of protein(s) in glucose condition at 40kD was also observed. b) Addition of anti-dolichol phosphate mannose 1 as load control yielded massive upregulation of protein(s) in galactose conditions (2, 4, 6, 8), especially in *psd1Δ* pESC-Leu and *psd1Δ* pESC-Leu CHO1 cells (6,8).

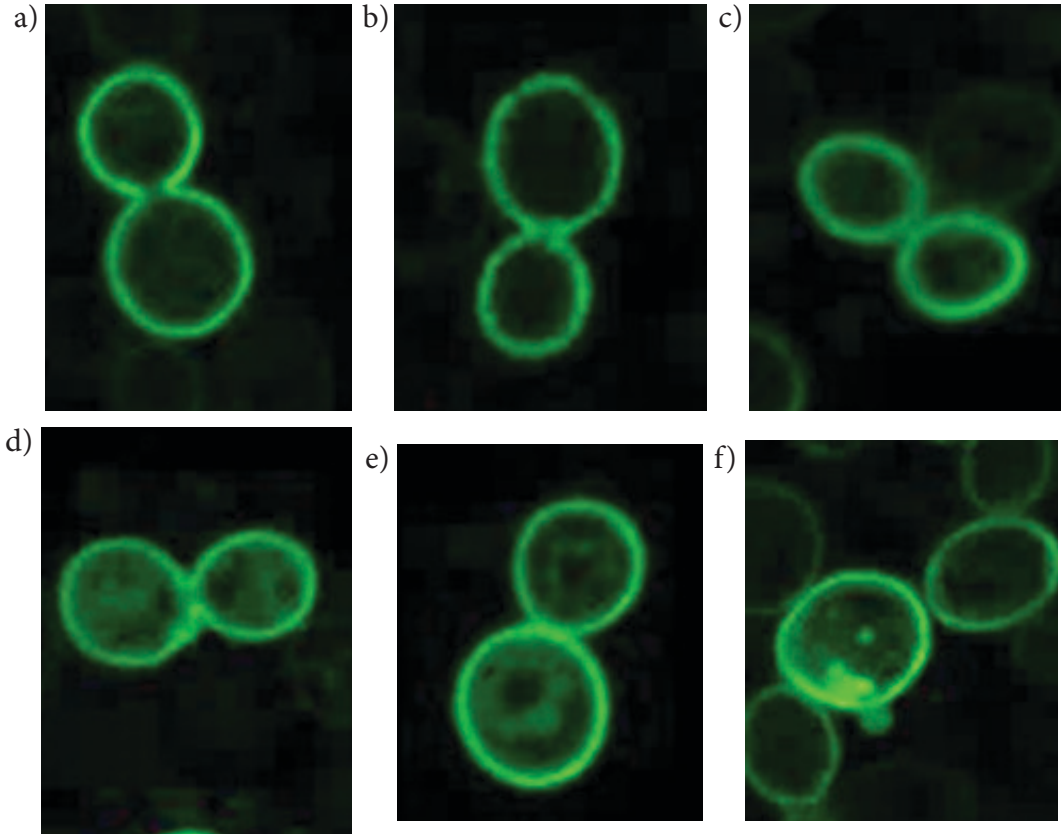

Figure 3. PS localization in *WT* and *psd1Δ* pESC-Leu/CHO1 S. Cerevisiae visualized through confocal microscopy. LactC2-GFP probe that binds to PS with high affinity was used. a) *WT* pESC-Leu cells grown in glucose, b) *WT* pESC-Leu CHO1 cells grown in glucose, and c) *WT* pESC-Leu cells grown in galactose show PS localization almost exclusively to the plasma membrane. d) *WT* pESC-Leu CHO1 cells grown in galactose show diffuse staining in cytosol. e) *Psd1Δ* pESC-Leu cells grown in galactose show a ring-like circular staining pattern surrounding a large unstained structure, likely to be the vacuole. f) *Psd1Δ* pESC-Leu CHO1 cells grown in galactose show several bright dot-like stains on the plasma membrane and non-diffuse circular staining in the cytosol, likely to be trans-Golgi network vesicles delivering lipids to the cell surface. Scale is consistent across all images. *Psd1Δ* pESC-Leu/CHO1 cells grown in glucose not imaged.

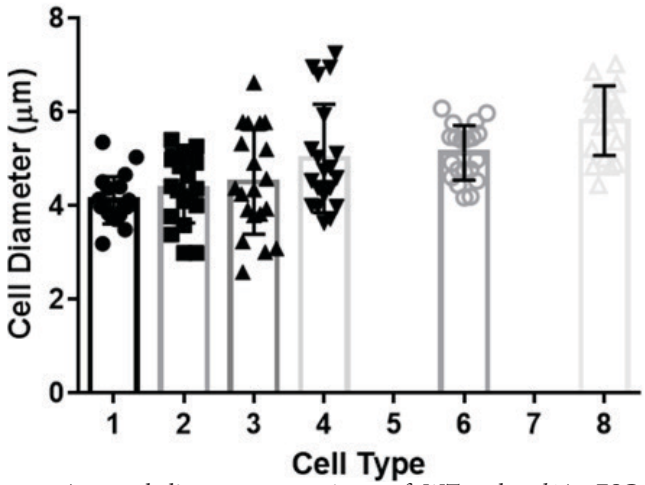

Figure 4. Averaged diameter comparisons of *WT* and *psd1Δ* pESC-Leu/CHO1 *S. cerevisiae* cells imaged by confocal microscopy. Diameter length measured from longest side was obtained for 20 individual cells per condition using the *Fiji program*, then averaged and graphed using *Graphpad Prism 6*. Cell types are as follows: (1) *WT* pESC-Leu cells grown in glucose, (2) *WT* pESC-Leu cells grown in galactose, (3) *WT* pESC-Leu CHO1 cells grown in glucose, (4) *WT* pESC-Leu CHO1 cells grown in galactose, (6) *psd1Δ* pESC-Leu cells grown in galactose, and (8) *psd1Δ* pESC-Leu CHO1 cells grown in galactose. *Psd1Δ* pESC-Leu CHO1 cells were on average 99.7% larger than *WT* pESC-Leu cells. No data collected for *psd1Δ* pESC-Leu/CHO1 cells grown in glucose (5, 7).

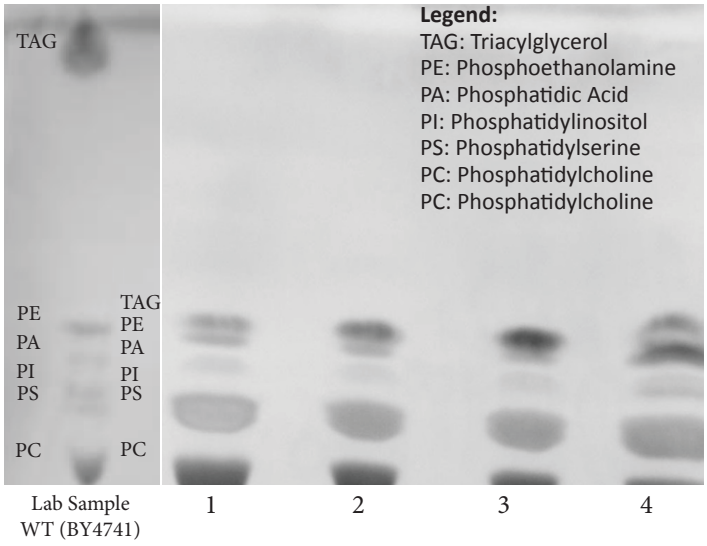

Figure 5. Thin Layer Chromatography of *WT* and *psd1Δ* pESC-Leu/CHO1 *S. Cerevisiae* cells to visualize global cellular PS levels. Lab sample *WT* (BY4741) shows reference separation of lipids. Cell types are as follows: (1) *WT* pESC-Leu, (2) *WT* pESC-Leu CHO1, (3) *psd1Δ* pESC-Leu, and (4) *psd1Δ* pESC-Leu CHO1 cells all grown in galactose. Results were inconclusive due to incomplete separation of lipids. In particular, phosphatidylinositol (PI) and PS bands were merged.

for *WT* pESC-Leu cells in glucose and similar for *WT* pESC-Leu cells in galactose, *WT* pESC-Leu CHO1 in glucose, *WT* pESC-Leu CHO1 in galactose, and *psd1Δ* pESC-Leu cells in glucose. In comparison, *psd1Δ* pESC-Leu in galactose, *psd1Δ* pESC-Leu CHO1 in glucose, and *psd1Δ* pESC-Leu CHO1 cells in galactose have darker bands and thus higher concentrations of CHO1p. Overall, there was increased CHO1p concentration from *WT* pESC-Leu in glucose to *psd1Δ* pESC-Leu CHO1 (left to right of the blot). The western blots incubated with anti-dolichol phosphate mannose 1 as load control yielded massive upregulation of protein(s) in galactose conditions, especially for psd1Δ pESC-Leu and psd1Δ-pESC-Leu CHO1 cells (Figure 2b).

The LactC2-GFP probe (and therefore PS) localized to the plasma membrane with little staining of intracellular structures in all glucose conditions and *WT* pESC-Leu cells in galactose (Figure 3a, b, c). Due to technical difficulties, *psd1Δ* pESC-Leu/CHO1 cells in glucose were not imaged. *WT* pESC-Leu CHO1 cells in galactose showed diffuse cytosolic staining (Figure 3d). *Psd1Δ* pESC-Leu cells had small round staining surrounding a large circular unstained structure. (Figure 3e). *Psd1Δ* pESC-Leu CHO1 cells had bright florescent dots on the plasma membrane and diffuse circular stains in the cytosol (Figure 3f). Cell diameters were similar for glucose conditions, greater for *WT* pESC-Leu CHO1 and *psd1Δ* pESC-Leu cells in galactose, and greatest in *psd1Δ* pESC-Leu CHO1 cells in galactose (Figure 4). Given that cells were roughly spherical, *psd1Δ* pESC-Leu CHO1 cells (average diameter = 5.80μm) were on average 99.7% larger in area than *WT* pESC-Leu CHO1 cells (average diameter = 4.11μm). Thin Layer Chromatography to visualize global PS levels for cells in galactose yielded merged bands (Figure 5).

Discussion

The extremely poor growth of *psd1/psd2Δ* cells even on ethanolamine enriched plates indicated high toxicity when there was accumulation of PS due to complete knockout of both PS decarboxylases. In contrast, previous studies demonstrate that *cho1Δ* cells were able grow on ethanolamine enriched plates [3], suggesting that increased PS levels impairs growth more than absence of PS. The *psd1Δ* p416-GDP CHO1 cells appeared to exhibit poorer growth because spot dilutions were less concentrated (Figure 1a(ii)). However, the mixture of large and smaller colonies suggests that some colonies may have acquired suppressor mutations to overcome the growth deficiency from reduction in PS decarboxylation and increase in PS synthesis (Figure 1a(ii)). Knocking out PSD1 or upregulating Cho1p on their own did not result in visible differences, which was not surprising due to the strict PS pathway regulation [3]. Results were consistent with Sousa et al. (2013), where overexpression of PSS1 or PSS2 did not increase amounts of PS in HEK293 and McArdle hepatoma cells [4]. As expected, there were no visible differences in the serial dilution spotting of *WT* and *psd1Δ* pESC-Leu/CHO1 cells grown in glucose because the plasmids were inhibited (Figure 1b). There was overall poorer cell growth for cells grown on raffinose plates: attributed to a decrease of PS relative to the entire phospholipid pool from 8% in glucose to 2% in raffinose as noted by Klose et al. (2012) [15] (Figure 1d). It is possible that there was similar decrease in PS representation in galactose grown cells. Hence, the galactose induced pESC-Leu CHO1 plasmid may have partially restored PS levels in *WT*, and *psd1Δ* pESC-Leu CHO1 cells resulted in better growth compared to *WT* and *psd1Δ* pESC-Leu cells (Figure 1c).

Increased expression of Cho1p in *WT* and *psd1Δ* pESC-Leu CHO1 cells in galactose compared to their glucose counterparts was expected because galactose promotes - while glucose inhibits - expression of the plasmid (Figure 2a). Interestingly, cells grown in glucose had protein(s) visualized by 40kD bands (Figure 2a). It is possible that there are metabolic differences for cells grown in galactose that caused down regulation of specific glucose metabolic protein(s) and upregulation of other proteins that convert galactose into glucose for glycolysis [16]. Despite several attempts, the western blot had large amounts of non-specific binding (Figure 2a). Antibodies developed by Choi et al. (2010) may have degraded over time and currently there are no alternatives available. Increased Cho1p concentrations from *wt* pESC-Leu to *psd1Δ* pESC-Leu CHO1 cells (left to right on blot) could be explained by non-uniform amounts of total protein. The massive upregulation of protein(s) in galactose grown cells when dolichol phosphate mannose 1 was used as load control was especially prominent for *psd1Δ* pESC-Leu and *psd1Δ* pESC-Leu CHO1 cells, suggesting greater total protein amounts in those conditions (Figure 2b). Actin as load control can be used to repeat the experiment. However, these results do suggest that galactose grown cells have different metabolisms compared to their glucose counterparts.

Localization of LactC2-GFP probe (and therefore PS) almost exclusively to the plasma membrane for cells grown in glucose and *WT* pESC-Leu cells in galactose is consistent with previous studies [3] (Figure 3a, b). *Psd1Δ* pESC-Leu/CHO1 cells in glucose can be imaged in the future. Diffuse cytosolic staining in *WT* pESC-Leu CHO1 cells in galactose suggests that excessive PS was incorporated into cytosolic structures (Figure 3d). Stained structures were likely early endosomes, which are known to be PS rich and/or trans Golgi network vesicles delivering lipids to the cell surface [3]. The strongest phenotype in *psd1Δ* pESC-Leu cells that showed distinct

circular staining (Figure 3e) were likely early endosomes surrounding the unstained nucleus or vacuole [3]. *Psd1Δ* pESC-Leu CHO1 cells did not display the ring-like staining pattern; however, these cells had brightly stained spots on the plasma membrane and circular non-diffuse cytosolic staining that may be PS in vesicles (Figure 3f). These results that show abnormal cellular PS distribution supplements earlier experiments that found no global PS differences [4]. *Psd1Δ* pESC-Leu cells and especially *psd1Δ* pESC-Leu CHO1 cells in galactose (Figure 3f) were larger and rounder than their glucose counterparts and *WT* cells. Cells experienced loss of polarity that indicates severe growth abnormality since polarity is strictly regulated in *S. Cerevisiae*. It should be noted that the large colonies in *psd1Δ* pESC-Leu CHO1 cells discussed earlier (Figure a(ii)) may simply be larger cells. Thin Layer Chromatography yielded incomplete separation of lipids, especially PS and phosphatidylinositol (Figure 5). Hence, repetition of the experiment would be beneficial.

In the future, yeast models could be used for high-throughput synthetic lethality/sickness screens to identify potential modifiers of enhanced PS synthesis and any abnormal phenotypes determined by growth assays will be defined and pathway interactions characterized. An alternative mammalian cell model of LMS using monocytes, the precursor to osteoclasts, could be generated and compared to the *S. cerevisiae* models to investigate the link between PS and abnormal bone metabolism described in LMS.

Acknowledgements

The author thanks Dr. Greg Fairn for his contributions and guidance throughout the project.

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JULS RESEARCH

Comparing post-exercise recovery of blood pressure and heart rate following arm cranking and leg cycling aerobic exercise in young healthy men

Cindy H. Nguyen[1], Danielle Bentley[1], Scott Thomas[1]

[1]Faculty of Kinesiology and Physical Education, University of Toronto

Corresponding Author: Cindy H. Nguyen (cindyhp.nguyen@mail.utoronto.ca)

Abstract

Aerobic exercise is commonly used as a non-pharmacological intervention to manage high blood pressure. Post-exercise cardiovascular responses (blood pressure and heart rate) may be important to long term adaptations. Little is known about cardiovascular recovery following different modes of aerobic exercise with distinct muscle mass involvement. The primary research objective was to characterize and directly compare the post-exercise recovery of cardiovascular variables (blood pressure and heart rate) following arm cranking and leg cycling aerobic exercise. Eight young healthy, normotensive men, (mean±SD; age 21.9 ± 2.3 years) were recruited. Following a familiarization session, participants randomly completed three separate 15-minute intervention sessions: control (seated rest), arm cranking (at 40% heart rate reserve (HRR)), and leg cycling (at 40% HRR). The 30-minute post-exercise time was divided into two recovery periods (R1: 0-15min & R2: 15-30min). Length and circumference measurements confirmed greater thigh compared to arm mass. Recovery following exercise cessation was similar between exercise modalities. Specifically, there was an insignificant interaction effect of time x modality for the systolic blood pressure ($p = 0.66$), the diastolic blood pressure ($p=0.33$), and the mean arterial pressure responses ($p=0.59$). The response of HR showed a main effect of time (R1: 12.1 ± 7.8bpm, R2: 9.5 ± 8.9bpm, $p < 0.05$) and a main effect of modality (Arm: 8.3 ± 8.4bpm, Leg: 13.2 ± 9.1 bpm, $p < 0.05$) with an insignificant interaction ($p = 0.90$). These findings suggest that among young, healthy men, muscle mass may not influence cardiovascular recovery following aerobic exercise.

Introduction

High blood pressure, also referred to as hypertension, is a major risk factor for cardiovascular diseases (CVDs), such as stroke and heart attack. It is estimated that one Canadian dies from CVD every seven minutes [1]. In order to lower the risk of developing CVD, research has focused on determining evidence-based practices to reduce one's resting blood pressure (BP) [2, 3]. Although there are numerous interventions for the prevention and treatment of hypertension, patients have recently expressed an interest in non-pharmaceutical interventions to manage their health [4]. Aligned with these interests, exercise has been identified as an effective strategy for both the prevention and treatment of hypertension [5].

In developing a full understanding of the physiological mechanisms responsible for the BP lowering effects of exercise, the acute response both during exercise and following exercise cessation has been explored. Post-exercise hypotension (PEH) is the temporary reduction in BP below resting levels [6] which has been reported to last up to 13 hours [7], with systolic blood pressure (SBP) reductions greater than diastolic blood pressure (DBP) reductions [6]. This acute cardiovascular phenomenon has been correlated with long term reductions in resting BP as a result of exercise training [8].

Studies of acute BP responses following exercise have demonstrated PEH in cohorts of males [9-11], females [10, 11], young [12, 13], and elderly [9, 13]. Previous research has shown a strong correlation between acute BP reductions and long-term BP reductions [8]. However, there is little research that compares the acute PEH response between different modalities of aerobic exercise. Leg cycling and arm cranking are forms of aerobic exercise with distinct muscle mass involvement. Previous research has demonstrated PEH following both leg cycle ergometry (LCE) [15, 16] and arm cranking ergometry (ACE) [17]. Muscle mass may be a potential mediator between exercise and magnitude of PEH as it has been thought that exercising muscles release compounds that may elicit PEH [14]. It is unclear though how the magnitude of PEH response of an individual compares across exercise modalities.

Therefore, the primary objective of this research was to characterize and compare the post-exercise cardiovascular recovery patterns of young healthy men following both LCE and ACE. With

intra-individual comparisons, this research reveals if cardiovascular recovery patterns are exercising-limb dependent. In accordance with previous research that elicited PEH when comparing these two forms of exercise among young, borderline hypertensive men and women [17], it was hypothesized that both exercise modalities will elicit PEH among young, normotensive men. In addition, it was hypothesized that there will be a greater magnitude of PEH following LCE compared to ACE. Characterizing and understanding the impact of exercising muscles on PEH can contribute to the growing body of knowledge on the use of exercise to prevent hypertension thereby helping to combat the global burden of CVD.

Methodology

Participants

Participants were recruited from the University of Toronto using word-of-mouth communication strategies and were invited to the Human Health and Performance Laboratory at the University of Toronto. Based on the inclusion criteria, all participants were young, male, recreationally active, non-smoking, free from chronic disease, and had no history of cardiovascular disease or concussions. Written informed consent was obtained from all participants prior to participation in the study. All study procedures were approved by the University of Toronto Faculty of Kinesiology and Physical Education Delegated Ethics Review Committee and are in accordance with the guidelines set forth by the Declaration of Helsinki of the World Medical Association [18].

Experimental Design

A randomized crossover design was employed where each participant completed three experimental conditions (LCE, ACE, and Control) on separate testing days and an initial familiarization visit (Figure 1). In total, testing was completed over four separate visits, separated by at least 24 hours. Prior to each session, participants completed a 4 hour fast, a 4 hour abstinence from caffeine, and a 24 hour abstinence from alcohol and strenuous exercise. These abstinences controlled for external factors that could temporarily affect BP. All sessions were completed between 2 and 8 pm to control for diurnal rhythms and BP variation [19]. During the initial visit, candidate participants were screened for inclusion criteria and familiarized to the study by completing mock procedures, with data recorded but not analyzed. Participants were characterized during this initial session using the Physical Activity Readiness Questionnaire Plus (PARQ+) [20], and the Rapid Assessment of Physical Activity (RAPA) [21]. Anthropometric measurements including height, weight, and limb dimensions were also obtained. Limb dimensions were averaged between right and left. Arm length was measured from the antecubital fossa to the anterior border of the acromion process of the scapula and circumference was measured at the midway point of arm length to the nearest 0.1 cm. Thigh length was measured from the anterior superior iliac crest to the base of the patella and circumference was measured at the midway point of thigh length to the nearest 0.1 cm.

Each of the three randomized experimental sessions followed a similar sequence of events, as seen in Figure 1. Upon arrival at the laboratory, participants were positioned in an upright, seated position with the left arm supported and fitted with an automated oscillometric brachial cuff (BpTRU Vital Signs made by BpTRU Medical Devices, model #BPM-100, British Columbia, Canada). Following 10 minutes of quiet rest with no data collection, discontinuous BP and heart rate (HR) recordings were acquired at 2 minute intervals, for a total of 10 minutes. A similar procedure, lasting a total of 30 minutes, was followed for post-exercise BP and HR measurements with recordings at 3 minute intervals. The LCE and ACE interventions involved 15 minutes of exercise at 40% of their heart rate reserve (HRR), calculated using the Karvonen method of [HRR = [((220-age) – resting HR) x 0.4 + resting HR]). HR was monitored continuously (Ambit2 made by SuuntoHR, Vantaa, Finland). The control session was time-matched to the exercise sessions and consisted of 15 minutes of quiet, seated rest.

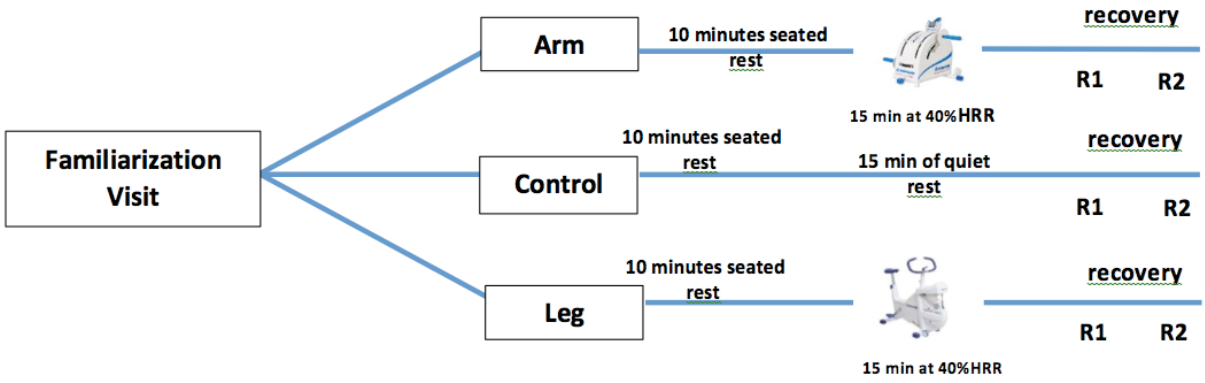

Figure 1. Randomized cross-over study design. R1: 0-15 minutes post-exercise. R2: 15-30 minutes post-exercise.

Statistical Analysis

Data have been presented as mean ± SD, unless otherwise stated. Day-of pre-exercise resting values of SBP, DBP, and HR were calculated using the BpTRU measurements. Six values were recorded in two minute intervals, with the highest and lowest values for each variable removed and the remaining four values averaged. The day-of resting values from each of the three experimental visits were used to determine internal consistency between visit dates (Cronbach's alpha).

Post-exercise recovery was characterized as the change between day-of rest and thirty minutes following exercise cessation measures. PEH is defined as a reduction in post-exercise SBP from resting greater than 3 mmHg. To directly compare recovery between the two exercise modalities the recovery time was segmented into R1 (0-15mins) and R2 (15-30mins). Post-exercise cardiovascular recoveries were analyzed using two-way analyses of variances (ANOVAs) with repeated measures (time (R1,R2), modality (LCE, ACE)), as compared to control. Exercise adherence was measured based on the prescribed exercise intensity and continuous during-exercise HR data. Statistical analyses were completed using IBM SPSS

Statistics 23 (SPSS, Chicago, IL) and significance was set at a P value of 0.05.

Results

A total of 8 healthy, young (mean ± SD, range) (21.9 ± 2.3, 19-25 years) men completed this study. Participants were moderately active (RAPA: 7.0 ± 2.1, 3.0 to 10.0), slightly overweight (BMI: 25.3 ± 6.1 kg/m2, 18.4 to 37.1), and normotensive (SBP: 107.2 ± 8.7 mmHg; DBP: 71.7 ± 5.7 mmHg). Participants had greater leg lengths and circumferences (length: 26.7 ± 2.9cm, circumference: 28.4 ± 4.9cm) compared to arm (length: 47.4 ± 4.3cm, circumference: 51.4 ± 7.4cm), suggesting greater muscle mass in the legs. All participants self-reported to be non-smoking, without any current medical conditions, and not currently taking any prescription medications. Due to connection inconsistency with the HR monitor chest strap, during-exercise HR data was excluded for one participant. All other data is complete.

Between experimental visits there was a high level of internal consistency in resting BP (SBP: α = 0.94, DBP: α = 0.81). Resting HR displayed lower consistency (α = 0.66). Due to the variability of HR between session visits, day of resting HR values were used to calculated exercise HR intensity. As such prescribed exercise HR at 40% of HRR was 122.4 bpm for ACE and 120.8bpm for LCE, with corresponding exercise adherence of 98.5% and 107.6% respectively which was statistically similar ($t(6)=-2.07$, $p = 0.084$).

Post-exercise recovery for each exercise modality is visualized in Figure 2 as change in mean arterial pressure (MAP) from rest over the entire 30 minutes post-exercise. Statistical comparisons of BP reveal that recovery following exercise cessation was similar between exercise conditions (Table 1). Specifically, there was an insignificant interaction effect of time x modality for the SBP response ($p = 0.66$), the DBP response ($p = 0.33$), and the MAP responses ($p = 0.59$). The HR response showed a main effect of time (R1: 12.1 ± 7.8bpm, R2: 9.5 ± 8.9bpm, $p < 0.05$, $r = 0.80$) and a main effect of modality (ACE: 8.3 ± 8.4bpm, LCE: 13.2 ± 9.1 bpm, $p < 0.05$, $r = 0.74$) with an insignificant interaction ($p = 0.90$), signifying similar HR recovery patterns over time. PEH was not observed following ACE and LCE.

Table 1. Post-exercise recovery variables represented as change scores from day-of resting values segmented into R1 (0-15 minutes) and R2 (15-30 minutes). **significant change from rest, $p < 0.01$.

Mean ± SEM	Arm Cranking Ergometry (ACE)		Leg Cycle Ergometry (LCE)	
	R1(0-15mins)	R2 (15-30mins)	R1(0-15mins)	R2 (15-30mins)
Systolic Blood Pressure (SBP)	3.0 ± 2.7	0.31 ± 3.4	0.12 ± 3.3	-1.2 ± 2.9
Diastolic Blood Pressure (DBP)	-7.6 ± 4.3	-5.3 ± 3.3	-2.6 ± 2.1	-2.7 ± 1.8
Mean Arterial Pressure (MAP)	-4.1 ± 3.6	-3.5 ± 3.1	-1.7 ± 2.4	-2.2 ± 2.0
Heart Rate (HR)	9.6 ± 2.7**	7.0 ± 3.3	14.6 ± 3.3**	11.9 ± 3.2**

Discussion

This cohort of young, healthy men did not show PEH following either ACE or LCE. PEH has been clearly observed in a wide range of pre-hypertensive and hypertensive cohorts [22]. Health benefits as a result of PEH, such as reduced CVD risk, may be especially beneficial for these hypertensive individuals. However, observations of PEH following acute bouts of exercise have been inconsistent among normotensive males [23-25]. One explanation for this inconsistency may be the various methods being used to calculate PEH, including averaging post-exercise recovery [26] and comparing it to control sessions [27]. Alternatively, the lack of PEH in this study may be due to the short exercise durations as other researchers have used exercise durations of 30 minutes [16, 28] to 50 minutes [15]. However, previous researchers have determined that PEH magnitude does not depend on exercise duration when comparing submaximal (70% VO2peak) exercise at durations of 15, 30 and 45 minutes of leg cycling among young normotensive males [29]. This cohort of young, healthy men likely did not show PEH due to the lower exercise intensity levels and not necessarily due to the exercise duration. This suggests that perhaps this cohort may simply require a more robust stimulus in order to elicit PEH.

Muscle mass in the thigh segments primarily used in LCE tends to be greater than the muscle mass in the arm segments used

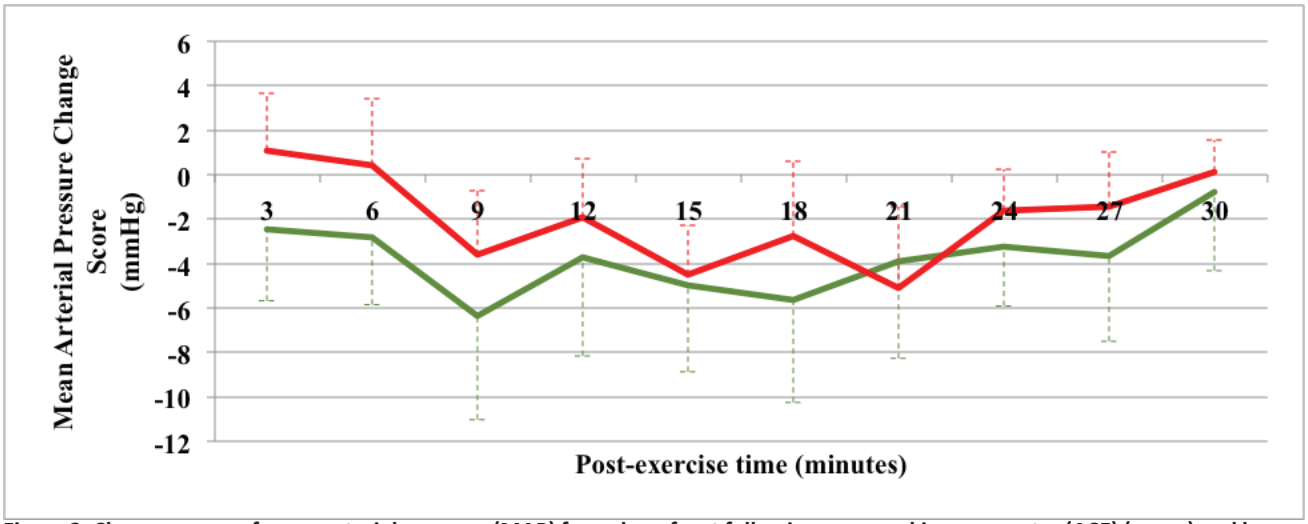

Figure 2: Change scores of mean arterial pressure (MAP) from day-of rest following arm cranking ergometry (ACE) (green) and leg cycling ergometry LCE (red). Error bars represent SEM. There was no significant difference in post-exercise recovery between ACE and LCE.

in ACE [30, 31]. ACE can be a good alternative aerobic exercise to LCE for those who prefer upper body exercises. Using length and circumference measurements, it was confirmed that arm segments were smaller than thigh segments in this cohort. This suggests that the muscle mass in the thighs is greater than the muscle mass in the arms, which is consistent with the assumptions made in previous research [17]. Results from this study suggest that the amount of muscle involved with exercise does not influence cardiovascular recovery following acute moderate aerobic exercise. This is consistent with previous literature comparing moderate intensity arm cranking and leg cycling among borderline hypertensive individuals, which also found that the muscle mass involved with exercise did not impact BP post-exercise [17]. When comparing different exercise modalities (cycling, running and walking) in maximal cardiopulmonary exercise testing, PEH magnitude is partially dependent on exercising muscle mass and only occurs following an acute bout of running [27]. Therefore, this current study supports previous research that the influence of exercising muscle mass on PEH magnitude with aerobic exercise may vary across different exercise intensities. Future research investigating the influence of differing exercise intensities and exercise modalities on the magnitude of PEH is required to corroborate these findings.

This research is not without limitations. First and foremost, this study had a small sample size, limited by the realistic constraints of a short term research project. Pooled recovery comparisons yield low effect sizes (Cohen's d) of current BP comparisons (SBP: 0.26, DBP: -0.45) predominantly driven by the large variance in responses. Increasing the sample size would increase the power of this analysis and improve confidence in the insignificant results. In addition, the real-time BP responses during the exercise bouts were not measured, limiting our ability to characterize and compare exercising BPs during different exercise modalities. The original research design included the collection and assessment of continuous HR for the during-exercise assessment of HR variability. However due to technical issues with the HR collection tools, it was not possible to analyze HR variability, which would have given insight into the activation of the autonomic nervous system. It is possible that selection bias may have influenced the results of this study as all participants were recruited through word of mouth and poster advertisements at the University of Toronto. Future research with larger, comprehensive designs are required to confirm these preliminary findings. Given that this research was dedicated towards the assessment of healthy young men, these results have inherently limited generalizability and should not be extrapolated to other populations, such as older individuals or women.

Moving forward, future research which characterizes and describes the during-exercise cardiovascular responses for ACE and LCE would provide insight into the physiological mechanisms involved. In addition, the investigation of HR variability during different exercise modalities would help elucidate the autonomic responses to better understand the mechanisms behind PEH. The influence of exercise modality and exercising muscle mass should also be investigated among other cohorts, including female and hypertensive participants, to determine if these findings remain consistent. Muscle mass in the exercising limbs should also be accurately measured in future investigations to strengthen these study findings. Finally, studies of exercise interventions with more robust stimuli to elicit PEH would help determine if the post-exercise recovery of the cardiovascular system continues to be independent of muscle mass among young, healthy male participants.

Conclusion

In conclusion, this research provides evidence that acute bouts of LCE and ACE at moderate intensities of 40 %HRR do not elicit PEH among young, healthy, normotensive men. Intra-individual comparisons reveal that exercising muscle mass may not impact the post-exercise cardiovascular recovery. Arm cranking can be a good alternative aerobic exercise to leg cycling for those who prefer upper body exercises. Taken together, this research suggests that exercise modality may not influence PEH and that young males may require higher intensity aerobic exercises in order to consistently elicit PEH.

Acknowledgements

Thank you to Dr. Scott Thomas, Dr. Danielle Bentley, and Dr. Tri Nguyen for their continual guidance and support. This work was supported by the Faculty of Kinesiology and Physical Education at the University of Toronto.

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JULS RESEARCH

Anti-proliferative effect of Brusatol and Brucein D on human breast cancer cells

Natalia Marta Blicharska[1], Lin Zhixiu[2]

[1]Department of Pharmacology, University of Toronto
[2]School of Chinese Medicine, Chinese University of Hong Kong

Corresponding Author: Natalia Marta Blicharska (n.blicharska@mail.utoronto.ca)

Abstract

Breast cancer is the leading cause of cancer-related deaths in women. Metastatic breast cancer (MBC) is a difficult disease to treat and patients diagnosed with MBC often succumb to the disease. The difficulty in treating MBC is due to the heterogeneity of breast cancers which limits the number of effective treatment options available to patients and due to the development of chemoresistance in breast cancer cells. As a result, it is imperative to identify and develop new anti-cancer agents that can provide additional treatment options to patients diagnosed with breast cancers. Recent studies have demonstrated the anti-proliferative effects of Brusatol and Brucein D, two quassinoids isolated from Brucea javanica, in multiple cancer cell lines. The present study tests the anti-proliferative effects of Brusatol and Brucein D in two human breast cancer cell lines: MDA MB-231 and MCF-7. Results of MTT assays determine that both Brusatol and Brucein D exhibit concentration-dependant anti-proliferative effects in MDA MB-231 and MCF-7 breast cancer cells lines.

Introduction

Breast cancer is the leading cause of cancer-related deaths in women worldwide, claiming the lives of over 520 000 women in 2012 [13]. Despite recent advancements in cancer research and therapy, breast cancer continues to be a burden and is projected to worsen with a growing and aging world population [13].

Over the past few decades, advancements in breast cancer treatment and prevention have seen improvements in overall breast cancer survival rates [4,6,12]. Screening and early detection, reduction in use of menopausal hormone replacement therapy and better and more appropriate use of treatment options such as surgery (breast conservation surgery or mastectomy), radiation, chemotherapy, hormone therapy and targeted therapy [1,4] have reduced the incidence of deaths from breast cancer in developed countries [1,4,6,13]. However, despite the wide range of treatment options available, metastatic breast cancer (MBC) continues to have poor prognosis, and the majority of patients diagnosed with MBC die of the disease [12].

This is because the selection of appropriate course of treatment is complex and determined by many factors, predominantly the biology of the cancer, such as the presence of estrogen and progesterone receptors and HER2 status [12]. Breast cancer heterogeneity limits the number of available treatment options for patients as well as overall impact of chemotherapy [6].

The development of drug resistance by breast cancer cells is an additional challenge to treatment and the major reason for the failure of currently available chemotherapeutics [6,9]. Eventual development of chemoresistance to anthracyclines and taxanes is particularly problematic in treating MBC as these 1st-line therapeutics are two of the most active anti-cancer drug classes currently available [6]. Therefore, it is of paramount importance to identify and develop new pharmaceutical agents for use in treatment of breast cancer, especially for the treatment of MBC for which current treatment options are "deemed a merely palliative treatment [given] to relieve cancer-related symptoms" [6, pg. S38].

Recent decades have seen a surge of interest in the development of novel anti-cancer agents from medicinal plants [5]. Many anti-cancer drugs of clinical importance, such as the taxane diterpene taxol, have been isolated from medicinal plants [5]. The herb Brucea javanica is one plant undergoing significant investigation.

Recent studies investigating the potential of B. javanica as a source of potential anti-cancer agents provide evidence of the value of using B. javanica in cancer research. Lau et al demonstrated in their 2008 study that B. javanica fruit extract had anti-proliferative and cytotoxic effects on three pancreatic cancer cell lines [7]. Further investigation demonstrated that Brucein D isolated from the extract possessed anti-proliferative and apoptogenic activity in the pancreatic cancer cells [8]. These findings were further supported by Serasanambati et al who demonstrated in their 2014 study the concentration-dependant inhibition effect of Brucein on MDA MB-231 breast cancer cells [11]. The importance of Brusatol in cancer treatment was illustrated by Ren et al who determined that Brusatol enhances the efficacy of chemotherapy in cancer cells by inhibiting the Nrf2 mediated chemoresistance pathway [9].

These studies have successfully demonstrated that the quassinoids Brusatol and Brucein D, found in abundance in B. javanica, exhibit anti-cancer effects (Lau et al, 2008; Lau et al, 2009; Ren et al, 2011; Serasanambati et al, 2014). Due to the successful demonstration of the anti-cancer effects of Brusatol and Brucein D in previous studies, the present study aims to expand on the Serasanambati et al study to determine whether Brusatol and Brucein D have anti-proliferative effects on two human breast cancer cell lines: MDA MB-231 and MCF-7.

Materials and Methods

Materials

The two breast cancer cell lines (MDA MB-231 and MCF-7) examined in this study were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). MDA MB-231 and MCF-7 cell lines are adenocarcinoma breast cancer cell lines derived from metastatic sites (pleural effusion) in 51 year old and 69 year old, Caucasian females respectively (2,3). MDA MB-231 cells are aneuploid cells that express epidermal growth factor receptor (EGF), transforming growth factor alpha receptor (TGFα) and WNT7B oncogene [2]. MCF-7 cells express estrogen receptor and WNT7B oncogene in addition to expressing Rh+ antigen [3]. The cell lines form adherent cell cultures and are grown in complete DMEM culture medium supplemented with 10% FBS and 1% penicillin/streptomycin (P/S).

Cell culture reagents used in this study, namely DMEM medium, Fetal Bovine Serum (FBS), penicillin and streptomycin (P/S), DPBS, Trypsin, DMSO and MTT dye were purchased from Invitrogen (Grand Island, NY, USA).

Brusatol and Brucein D used in this study were isolated from Brucea javanica L. fruit in lab as described previously by Lau et al [7,8]. For quality control purposes, the identities of the two compounds were confirmed by subjecting the compounds to nuclear magnetic resonance (NMR) and mass spectroscopy and comparing resultant profiles with available published data [10]. HPLC analysis determined that purity of the two compounds was greater than 95%.

Brusatol and Brucein D stock solutions were prepared by dissolving BR and BD in DMSO. Serum-free 1640 cell culture medium was added to each solution to obtain a final concentration of 20 μg/mL Brusatol stock solution and 500 μg/mL Brucein D stock solution. Final concentration of DMSO in stock solutions was <0.1%. Stock solutions were then diluted using serum-free DMEM culture medium to create appropriate treatment concentrations (Brusatol treatments = 0, 0.01, 0.1, 1, 10 μM; Brucein D treatments = 0, 0.01, 0.1, 1, 10, 100 μM).

General cell culture methods

MDA MB-231 and MCF-7 human breast cancer cell lines were cultured and maintained in DMEM cell culture medium, supplemented with 10% FBS and 1% P/S. Cell cultures were incubated at 37 °C, 5% CO_2 and 80% humidified air.

Drug Treatment Preparation

MDA MB-231 and MCF-7 cells were seeded into 96-well cultures plates at a density of 5000 cells per well. After incubating seeded cell plates for 24 hours, cells were treated with increasing concentrations of either Brusatol (control= 0, 0.01, 0.1, 1, 10 μM) or Brucein D (control= 0, 0.01, 0.1, 1, 10, 100 μM). Cells were then incubated for 72 hours prior to performing MTT assays.

MTT Assay

The anti-proliferative effect of Brusatol and Brucein D on the MDA MB-231 and MCF-7 breast cancer cells was determined using the MTT assay. MTT assay is a cell-based assay used to determine anti-proliferative or cytotoxic effects of test molecules on cells [10]. The assay uses the reduction of the tetrazolium compound MTT to formazan as a measure of metabolic activity to determine cell viability and consequently, to infer extent of inhibition on cell proliferation [10].

Anti-proliferative effects of Brusatol and Brucein D was determined by adding 20μL of MTT to each well followed by a 4 hour incubation period, after which all medium was removed from 96-well plates and 150μL of DMSO was added. Optical density was measured at 570nm using BioTek ELx808 absorbance microplate reader. Figure 1 illustrates a schematic of the experimental design.

Statistical Analysis

Results obtained were analysed using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test for multiple comparisons in which the untreated group (i.e. 0 μL of either Brusatol or Brucein D) served as the control. Statistical analysis of data was performed using Microsoft Office Excel 2007 and Minitab 14 Student. Differences were considered significant at $p < 0.05$. Results are presented as mean of each treatment group + SEM.

Results

Observations

Prior to performing MTT assays, 96-well plates (treated with either Brusatol or Brucein D and subjected to MTT for 4 hours) were observed under the inverted microscope. Cells appeared to have long, thin, needle-like filaments emanating from the cell. The presence of these cells decreased in a concentration-dependant manner as the concentration of treatment increased.

Brusatol exhibits anti-proliferative effects on MDA MB-231 and MCF-7 cancer cell lines

Brusatol exhibited anti-proliferative effects on MDA MB-231 and MCF-7 human breast cancer cells (**Figure 2**). Treatment with Brusatol had statistically significant inhibitory effects on MDA MB-231 cells at each treatment concentration. In MCF-7 cells, Brusatol had a statistically significant inhibitory effect at 0.1, 1, 10 and 100 μM concentrations. The anti-proliferative effect of Brusatol appeared to be concentration-dependant in both cell cultures. The IC50 of Brusatol was determined to be 3.90 μM in MDA MB-231 cells and 2.08 μM in MCF-7 cells.

Brucein D exhibits anti-proliferative effects on MDA MB-231 and MCF-7 cancer cell lines

Brucein D exhibited anti-proliferative effects on MDA MB-231 and MCF-7 human breast cancer cells (Figure 3). As was seen with Brusatol treatment, MDA MB-231 cells treated with Brucein D had statistically significant inhibition at each treatment concentration level. MCF-7 cells only exhibited statistically significant inhibition at 10 and 100 μM concentrations. The anti-proliferative effect of Brucein D appeared to be concentration-dependant in both cell cultures. The IC50 of Brucein D was determined to be 26.09 μM in MDA MB-231 and 37.18 μM in MCF-7 cells.

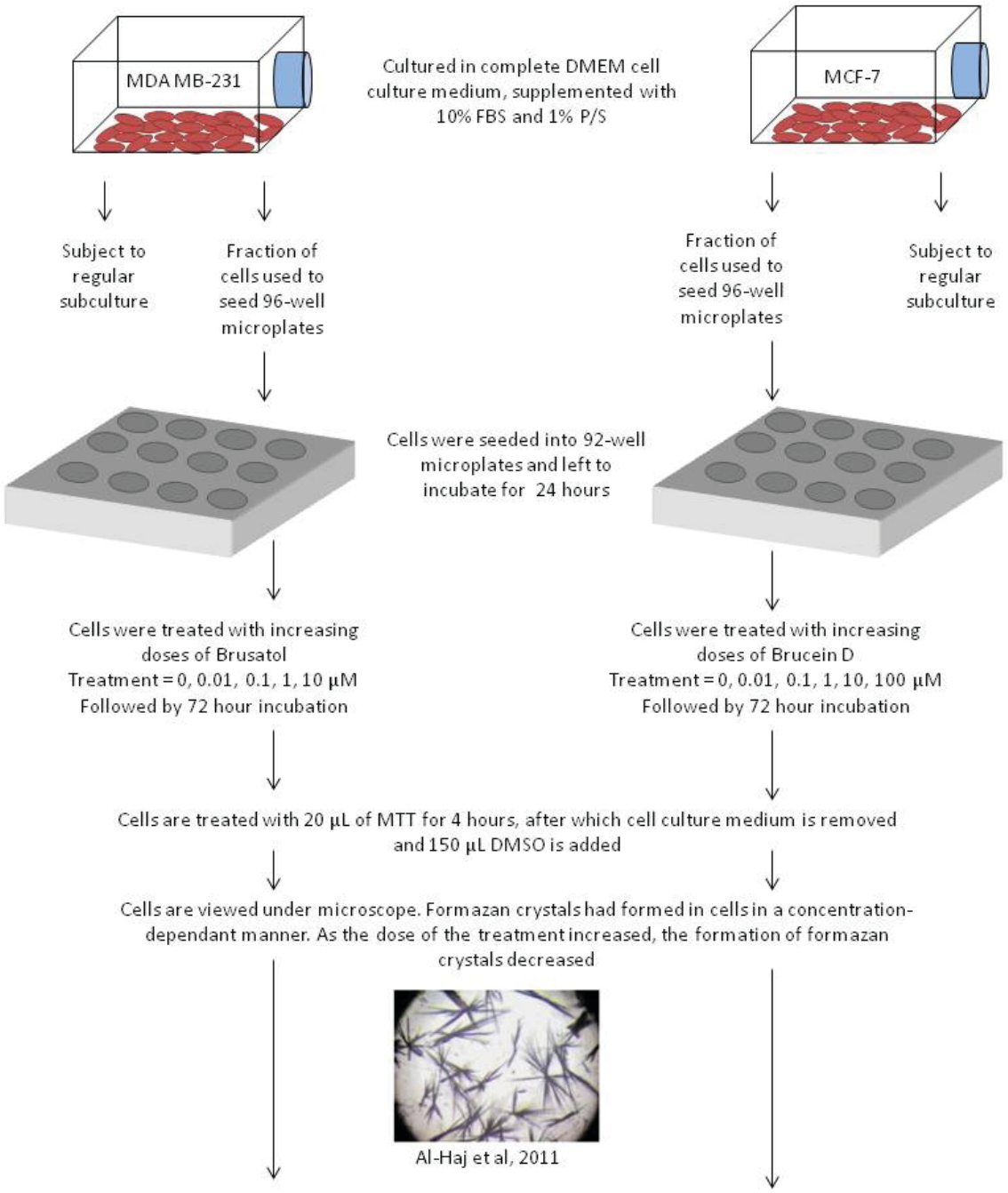

Figure 1: Schematic representation of experimental design

Discussion

Brusatol and Brucein D have demonstrated in multiple studies to have anti-proliferative effects on different cancer cell lines [7,8,11]. Thus as was initially expected, the results obtained from the present study provide further evidence to support this fact, as both Brusatol and Brucein D have demonstrated to have anti-proliferative effects on two human breast cancer cells lines: MDA MB-231 and MCF-7.

Microscopic observations of 96-well plates treated with either Brusatol or Brucein D indicated the presence of breast cancer cells with needle-like protrusions. Needle-like protrusions are the result of cellular reactions in which the tetrazolium compound MTT is converted by viable cells through a reduction reaction into insoluble formazan crystals [10]. The insoluble formazan crystals are deposited near the cell surface, often protruding from the cells into the cell culture medium [10].

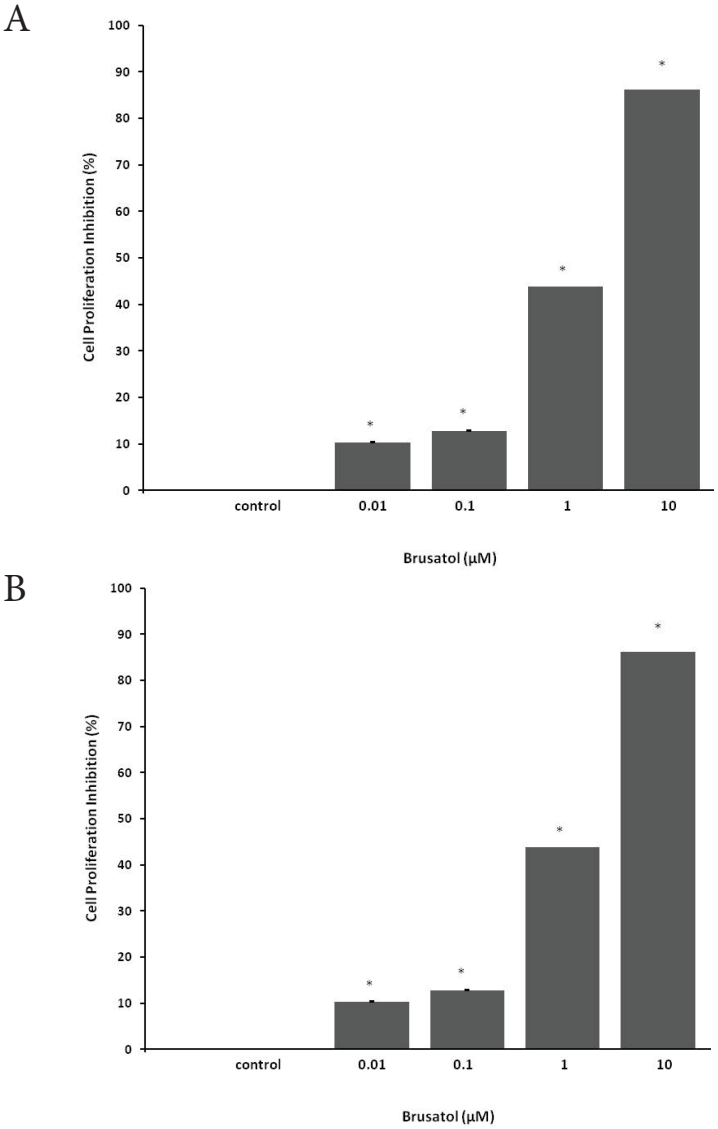

Figure 2. Effect of Brusatol on MDA MB-231 (A) and MCF-7 (B) breast cancer cells. Drug treatment was added to 96-well plates 24 hours after seeding and left to incubate for 72 hours. MTT assay was used to assess cell viability following treatment. * represents statistically significant results, $p<0.05$

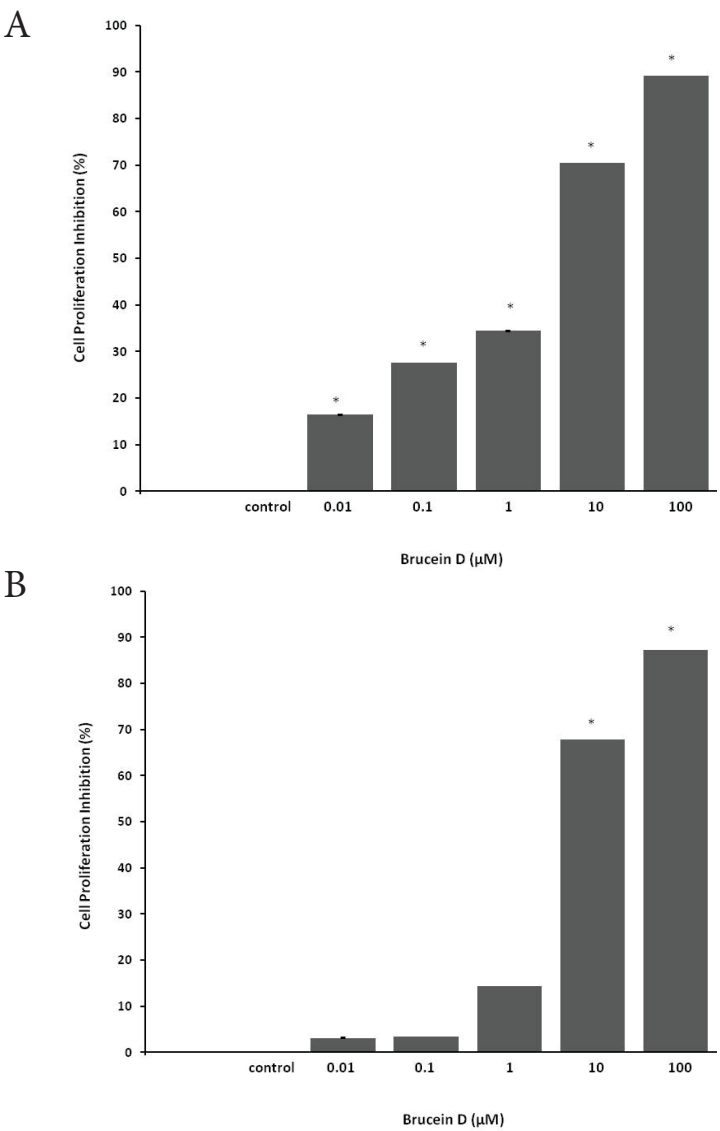

Figure 3. Effect of Brucein D on MDA MB-231 (A) and MCF-7 (B) breast cancer cells. Drug treatment was added to 96-well plates 24 hours after seeding and left to incubate for 72 hours. MTT assay was used to assess cell viability following treatment. * represents statistically significant results, $p<0.05$

The formation of formazan crystals indicates the presence of viable cells in the 96-well plates following drug treatment. Since the presence of these cells decreased in concentration-dependant manner, it is indicative of a reduction in cell viability [10] with an increasing treatment dose of Brusatol or Brucein D. Consequently, the reduction in cell viability of treatments compared to their respective controls can be used to infer a reduction in cell proliferation. These findings are further corroborated with the results of the MTT assays which quantify the extent of cellular inhibition.

The MTT assays used to assess the extent of cellular proliferation recorded a decrease in optical density absorbance with increasing treatment concentrations (**Figures 2 and 3**). This demonstrates an increase in cellular inhibition with an increase in treatment concentration. Analysis of the findings reveals that Brusatol and Brucein D have statistically significant inhibitory effects on cell viability in MDA MB-231 and MCF-7 cancer cells lines, demonstrating that Brusatol and Brucein D have anti-proliferative effects in these two cancer cell lines. Moreover, the anti-proliferative effects of Brusatol and Brucein D inhibit cancer cells in a concentration-dependant manner.

This finding has tremendous implications for future development of Brusatol and Brucein D as potential chemotherapeutic agents. The fact that the two test compounds produced inhibitory effects in a concentration-dependant manner allows for predictability of results. As the treatment concentration increases, one can anticipate an accompanying increase in the response to the treatment. This is important in drug discovery and development because concentration-dependant relationships allow one to determine important pharmacological values such as the concentration at which 50% of cell proliferation is inhibited (IC50) as well as therapeutic and toxic doses. Had the inhibitory effect of either Brusatol or Brucein D occurred in a sporadic nature, where statistically significant cellular inhibition occurred at any treatment concentration in a non-concentration-dependant manner, the drug under examination would not be clinically useful as predicting the appropriate treatment concentration that would exert anti-

proliferative effects on cancer cells would not be possible.

Further investigation of the anti-proliferative effects of the two test compounds reveled that Brusatol exerts a stronger inhibitory effect on MDA MB-231 and MCF-7 cells. This is evident from the IC50 values determined for Brusatol (IC50 = 3.90 μM and IC50 = 2.08 μM) and Brucein D (IC50 = 26.09 μM and IC50 = 37.18μM) in the MDA MB-231 and MCF-7 cell line respectively. Despite its weaker performance, Brucein D exhibited significant inhibitory effects, particularly on MDA MB-231 cells. These results support the findings obtained by Serasanambti et al in their 2014 study.

The differential abilities of the two quassinoids to inhibit cancer cell proliferation should not be interpreted as evidence to suggest that Brusatol is a better breast cancer drug candidate than Brucein D. The different extents of inhibitory action on cell proliferation are the result of different underlying mechanisms of action, both of which may prove to be useful in cancer treatment.

According to recent studies investigating the underlying mechanism of Brusatol's inhibitory action, Ren et al provide evidence for their hypothesis that Brusatol inhibits Nrf2-mediated chemoresistance and thereby enhances the efficacy of chemotherapy treatment; according to their findings, Brusatol does not directly induce apoptosis in cancer cells [9]. Nrf2 is a transcription factor that contributes to chemoresistance in cancer cells [9]. Activation of the Nrf2 pathway due to high constitutive expression of Nrf2 (as is the case in many cancer cells) results in the formation of a protective environment that allows for cancer cell survival during unfavourable environmental conditions, such as during chemotherapy [9]. Inhibiting this pathway results in a reduction in Nrf2 and consequently sensitizes cancer cells to chemotherapy, thereby reducing chemoresistance [9]. Since Brusatol selectively reduces cellular Nrf2 levels, Brusatol selectively targets the pathway responsible for chemoresistance in cancer cells [9]. As a result, Ren et al are able to provide evidence for the potential therapeutic use of Brusatol in chemotherapy treatment to reduce chemoresistance.

Brucein D, on the other hand, exerts its anti-proliferative effects via another mechanism. Studies investigating the mechanism by which Brucein D is capable of inhibiting cellular proliferation have provided evidence for the hypothesis that Brucein D inhibits cellular proliferation by inducing apoptosis [8]. This is believed to be accomplished by activating the p38-MAP kinase pathway and the subsequent activation of the caspase cascade pathway responsible for cellular changes that lead to apoptosis [8].

The activation of the p38-MAPK pathway to promote apoptosis is a pathway currently exploited by multiple chemotherapeutic drugs including Cisplatin, the anthracycline Doxorubicin and taxane Taxol [8]. This makes Brucein D a useful drug candidate for treating cancer cells not only because it has clear efficacy (due to its mechanistic similarity with currently available drugs that utilize the same pathways) but also because it is capable of inducing apoptosis in cancer cells while having lower cytotoxicity in non-cancer cells [8].

Despite the fact that monotherapy is the preferred method of breast cancer treatment [6,12], the different underlying mechanisms of action of Brusatol and Brucein D can potentially be useful in multi-therapy treatment. By implementing a treatment regime that targets two different pathways responsible for the proliferation of breast cancer, one has a better chance of ameliorating breast cancer. This is because targeting the cell proliferation pathway as well as the drug resistance pathway increases the likelihood that at least one targeted pathway will be affected by treatment. Moreover, the use of Brusatol in combination therapy can enhance the efficacy of 1st line chemotherapeutic drugs [9], and thereby increase the efficacy of treatment.

Collectively, the microscopic observations and results of the MTT assays provide clinically important findings. These findings are observed during a time when current chemotherapeutic agents have limited efficacy in treating breast cancer, (especially metabolic breast cancer) due to the heterogeneity of the disease and development of chemoresistance. The ability of Brusatol and Brucein D to significantly inhibit cellular proliferation in multiple cancer cell lines [7,8,11] and to increase the efficacy of currently available chemotherapeutics in the case of Brucein D [9] provides evidence to warrant the further investigation and potential development of these compounds into novel chemotherapeutic agents. Moreover, areas of future research should include, determining the potential anti-proliferative effect of these compounds *in vivo* using rodent models in addition to determining the usefulness of Brusatol and Brucein D in monotherapy and adjuvant chemotherapy. Engaging in these successive research projects will bring science closer to the development of new chemotherapeutic drugs and to administering these drugs to those patients in clinical trials.

Acknowledgement

I would like to thank Lin Zhixiu, Yang-Fang Xian and Jianhui Xie for their help and guidance both in the lab and during the preparation of this manuscript.

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JULS RESEARCH

Is Genomic Imprinting related to the presence of miRNAs within the Sfmbt2 Locus?

Cathy Su[1], Susannah Varmuza[2]

[1] Department of Biological Physics, University of Toronto
[2] Cells and Systems Biology, University of Toronto

Corresponding Author: Cathy Su (c.su@live.ca)

Abstract

Sfmbt2 is a gene maternally imprinted in mouse trophoblast tissues. Whereas the majority of imprinted genes are clustered beside each other, exist near an imprinting control region, and are regulated mainly by DNA methylation and long non-coding RNAs, none of these statements appear to hold true for Sfmbt2. Its mechanism of imprinting is currently unknown. However, one unique attribute of this gene is the block of miRNAs present in its tenth intron, whose function has yet to be characterized. To determine if this miRNA block is involved in the imprinting of Sfmbt2, a CRISPR-Cas9 system with two gRNAs was used to delete this region within a mouse trophoblast cell culture. This deletion had no effect on imprinted expression as determined by cDNA sequencing. However, methylation analysis of bisulfite sequencing data for this cell culture revealed that the cultured trophoblast stem cells used in this study had a different epigenetic profile than cells in vivo because of methylation around the promoter region of Sfmbt2. As such, additional studies are needed in order to determine if this miRNA block is driving genomic imprinting at Sfmbt2 in vivo.

INTRODUCTION

About 100 genes in mammals are imprinted, which refers to the fact that they are expressed from only one parental allele [1]. Genomic imprinting was discovered separately by two different labs in the 1980's, both of whom failed to grow diploid uniparental embryos. McGrath and Solter (1984) as well as Barton et al. (1984) found that replacing the pronucleus from one-cell embryos with a pronucleus from the opposite parent generated diploid embryos that failed to properly develop [2, 3]. Further investigation revealed that epigenetic marks were responsible for differentiating the parental genomes, and thus genetic contributions from both parents were necessary for development [4]. This epigenetic phenomenon is categorized as genomic imprinting. Since imprinted genes are silenced according to their parent of origin, maternally imprinted genes are paternally expressed and paternally imprinted genes are maternally expressed. Consequently, both gynogenetic and androgenetic embryos are unable to survive to adulthood because they lack the necessary complementary gene expression facilitated by epigenetic marks acquired through germ cell production [1].

Imrpinting is maintained through epigenetic marks

The mechanism of imprinting, by definition, allows the imprint to be erased in the germline and re-established in a parent-of origin fashion. Most imprinted loci are maintained through the regulation of DNA regions defined as 'imprinting control regions' (ICRs), because their deletion causes a loss of imprinting. These regions are regulated epigenetically by cis-acting mechanisms including differential methylation, histone modifications, enhancers, and/or non-coding RNAs [5]. However, differential methylation by methyltransferases DNMT1 and DNMT3A/B/L at CpG islands within ICRs seem to not only control expression at that locus, but are also the only epigenetic mark known to be erased and re-established in the germline [6]. Classical ICRs are characterized by differential methylation between the parental alleles which is preserved after fertilization [7]. Imprinting can take place due to cis or trans acting effects. Typically, the cis-acting element is the ICR while trans-acting factors usually include regulatory proteins [1].

Non-coding RNAs are also an important regulator of imprinted regions. Well known long non-coding RNAs which regulate imprinting include Airn, H19, and Nespas, all of which are host transcripts for small RNAs [5]. Many classes of small non-coding RNAs (21-23 nucleotide) function in gene regulatory pathways such as the RNA interference pathway, including miRNAs, siRNAs, and small RNAs which interact with the slicer protein PIWI (piRNAs) [8]. As yet, the role of small non-coding RNAs in co-transcriptional silencing has not been clearly deciphered in mammalian cells [9].

Irpinting in embryonic development

The placenta is an extra-embryonic organ important for embryonic development which supports nutrient exchange, releases

hormones to the mother, and provides immune and mechanical support to the offspring [10]. The early embryo is made up of the trophectoderm and inner cell mass [10]. The trophectoderm is a precursor for trophoblast stem cells (TSC) which differentiate into the various cell lineages that form the placenta [10]. Interestingly, gynogenetic embryos have a deficit of trophoblast while androgenetic embryos have hypertrophic, or enlarged, trophoblast [11]. Neither type is viable past mid-gestation, as shown by McGrath and Solter (1984) as well as Barton et al. (1984), but the difference in trophoblast quantity suggests that maternally imprinted genes are essential towards placental development [11].

In 2008, the Varmuza lab screened imprinted genes which might explain this effect using a microarray of cDNA from androgenetic, gynogenetic and biparental blastocysts [11]. Sfmbt2 was discovered as a previously uninvestigated gene with a large difference in murine expression between androgenetic and gynogenetic embryos [11]. Sfmbt2 is a Polycomb-group gene, a group of genes which encode parts of the Polycomb Repressive Complexes that associate with heterochromatin to negatively regulate gene expression [12]. Restriction fragment length polymorphism (RFLP) analysis confirmed that murine Sfmbt2 is maternally imprinted, but only in extraembryonic tissues [11]. Sfmbt2 and its antisense ncRNA appear to be the only imprinted genes in a large 4.3 Mb region domain [7]. It has two transcriptional start sites (TSS) of which the 5' TSS is specific to extraembryonic tissues while the 3' TSS is ubiquitous, although weak. A sparsely methylated CpG island exists at its two transcriptional start sites, but there is no nearby region which fits the classical criteria for an ICR [7]. Bisulfite sequencing has revealed that Sfmbt2 imprinting is unaffected by the loss of methyltransferases essential for establishing methylation in the genome [13]. Overall it seems that Sfmbt2 does not occur in an imprinted gene cluster, does not have an ICR, and is not regulated by DNA methylation. These characteristics together distinguish Sfmbt2 from other imprinted genes.

Sfmbt2 comatins a miRNA block

A large miRNA cluster is annotated in intron 10 of Sfmbt2 in the NCBI murine genome database [7]. This miRNA block is found in all mammals in which Sfmbt2 is imprinted including mice and rats, but is not present within organisms for which Sfmbt2 is not imprinted such as humans, cows, and deer mice [7]. In previous experiments, a male mouse with a gene-trap (gt) null allele of Sfmbt2 was used to study the role of this gene. The gt construct contained a splice acceptor site, β-geo cassette and poly-A signal. The splice acceptor site allowed the construct to be expressed, the β-geo cassette encoded β-galactosidase and neomycin resistance, and the poly-A signal was bound by poly-A polymerase to generate the adenine tail which characterizes the termination of mRNA transcripts [14]. The β-geo cassette confers G418 resistance to cells which express the gt construct and allows the expression pattern to be visualized using lacZ activity, while the poly-A signal terminates the transcript when expressed [15]. As a consequence, only one exon of Sfmbt2 is expressed, and the miRNAs predicted to be processed from the primary transcript are assumed to be lost (Figure 1). Offspring with genotype +/gt were found to express Sfmbt2 from the maternal allele, suggesting that the miRNA block on the paternal allele might confer silencing effects that are absent when it is deleted [16]. Since the gt allele contains a strong polyadenylation signal before the miRNA block, the re-activation of maternal Sfmbt2 expression in +/gt offspring could reflect loss of paternal allele miRNAs with loss of imprinting of the maternal allele. Together with the pattern of its imprinting across species, this suggests that the presence of the miRNA cluster in Sfmbt2 may drive imprinted expression of this gene.

If the miRNA block is necessary for imprinted expression of Sfmbt2, deletion of this block should result in reactivation of the maternal allele. CRISPR-Cas9 genome editing technology allows this hypothesis to be tested by editing the miRNA block inside of murine trophoblast stem cells (TSC). These cells are derived from trophoblast cells that normally differentiate into the cell lineages of the placenta, and were chosen as a model system because Sfmbt2 is imprinted in the trophoblast in vivo and in TSC [11].

The CRISPR (clustered regularly interspaced short palindromic repeats) system originated in prokaryotes and involves three steps which have been reviewed previously [17]. First, short repetitive DNA elements are inserted into foreign DNA. Secondly, the integrated genetic material, termed a CRISPR array, is transcribed into a small RNA (crRNA). Lastly, the foreign DNA is then targeted with the help of the crRNA by a complex containing Cas (CRISPR associated protein). Although the CRISPR system is intended to protect against foreign DNA in prokaryotes, it has been successfully repurposed for genome editing. In particular the Cas9 enzyme, a variant from Streptococcus pyogenes, has been

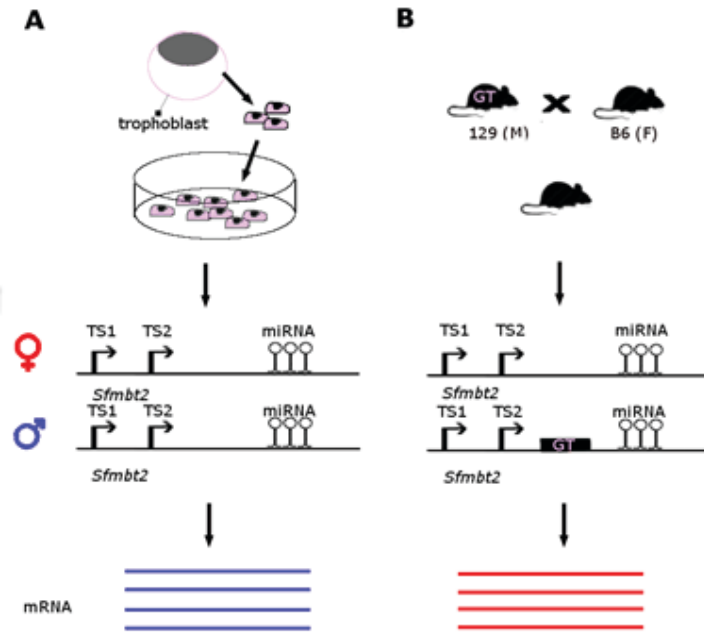

Figure 1. The Sfmbt2 gene trap construct terminates transcription after the first coding exon, which has been found to be correlated with re-activation of the maternal allele. A) In mouse trophoblast stem cells, Sfmbt2 is paternally expressed. (B) A gene trap (gt) mouse generated in a previous study was mated to produce offspring with a +/gt genotype. The gene trap construct is described in [16] and contains a splice acceptor, β-geo cassette and polyadenylation site, terminates transcription. The construct was inserted after the two transcriptional start sites and the first coding exon of Sfmbt2. During transcription of Sftmbt2, the gene trap construct splice site allows the mRNA to be spliced so that transcription is terminated early due to the polyadenylation site. When examining extraembryonic tissue from E7.5 and E9.5 of the +/gt, Sfmbt2 was found to be expressed from the previously silent maternal allele.

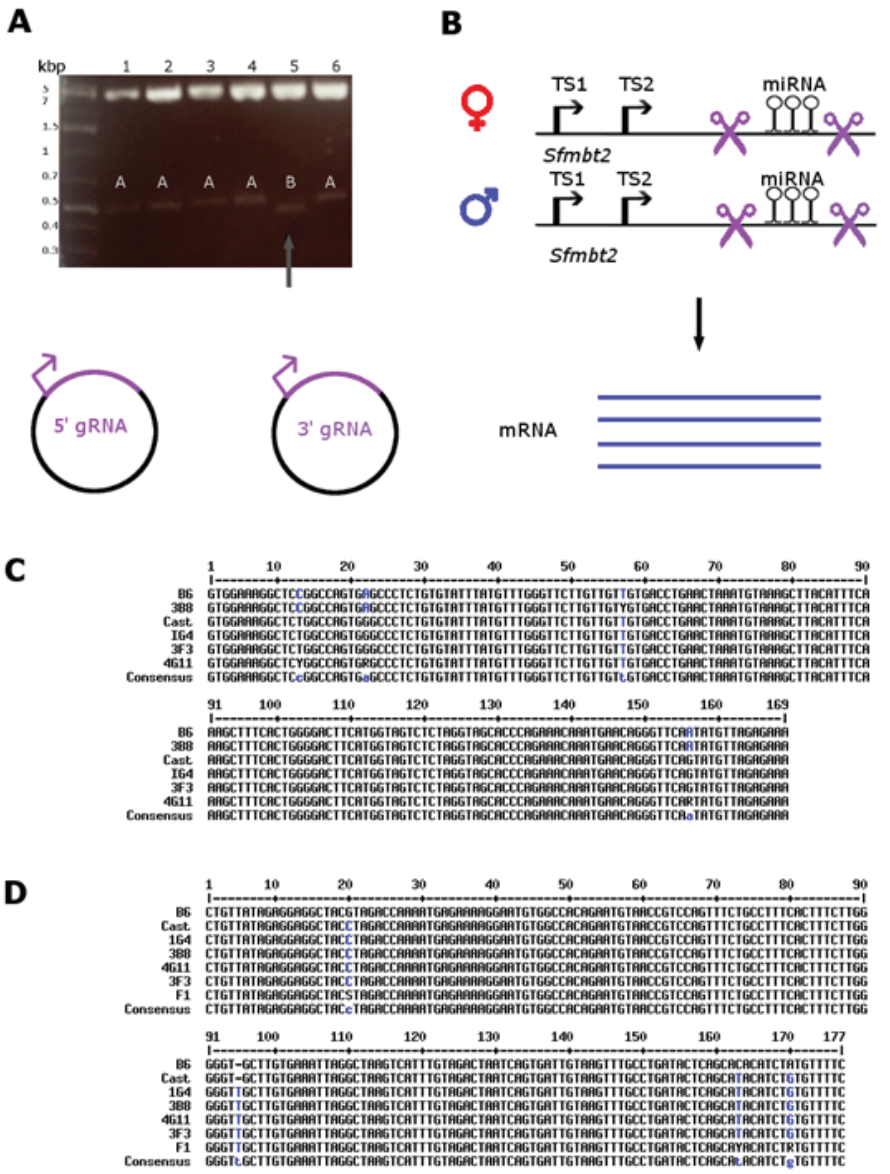

Figure 2. Treatment of trophoblast stem cells with gRNAs and Cas-9 deleted a region containing the miRNA block in either one or both alleles, but all treated cell populations expressed the paternal allele only. (A) Gel electrophoresis of gRNA constructs made by Gibson cloning and digested by EcoRI. The lanes represent different miniprep products from individual colonies grown after Gibson cloning. Each of lane 1-6 contains a thick band around 5kbp and a smaller band around 500bp. When the insert is successfully cloned into the gRNA backbone, we expect that the size of the smaller band will increase slightly (see Figure 4 which shows that the gRNA target insert increases the length of the region between EcoRI cut sites). When comparing the products A to the product B, we see that there is a difference in size between A and B of approximately 50bp. This suggests that product A (Lanes 1-4 and Lane 6) contain successfully assembled gRNAs. (B) Description of CRISPR experiment. The gRNAs were made to target regions around the miRNA block, but after treatment only the paternal allele was expressed. (C) and (D) are excerpts of alignments made using Multialign [23] for the gDNA and cDNA sequences of generated cell lines, respectively. Sequences of the parent F1 cell line, the maternal (B6) and paternal (Cast) alleles of Sfmbt2 are also present. Differences are highlighted in blue. (C) Among the cell lines, an extract of reads near the target sites of gDNA shows that cell lines are heterogeneous. Some cell lines have the predicted sequence for successful deletion of the miRNA block from the Cast allele only (IG4, 3F3), the B6 allele only (3B8) or both (4G11). (D) The mRNA content of all cell lines match the Cast allele sequence, indicating that although the cell lines have been successfully treated with CRISPR to delete the miRNA block from either one or both alleles, Sfmbt2 is only expressing mRNA from the paternal Cast allele only.

extensively used for genome engineering in mammalian cells [18]. In such applications, custom oligos called guide RNAs (gRNAs) serve as the crRNA with which Cas9 targets DNA for degradation based on homology [19].

MATERIALS AND METHODS

CRISPR Guide RNA constructs

A schema of the CRISPR method is available in Figure 2b. Two gRNA constructs were made with homology towards sites flanking the miRNA block on the 3' and 5' sides. The gRNA backbone vector, which contains a kanamycin resistance cassette, and a plasmid expressing Cas9 with an ampicillin resistance cassette were both purchased. Insert sequences for each gRNA were made by primer extension, whereby primers that partially overlapped the intended insert region were incubated with PCR reaction mix to produce an approximately 100bp product. The gRNA backbone was made by linearizing the gRNA construct and purified using a Qiagen MiniElute column. Lastly, the inserts and backbone were ligated by Gibson cloning to form each of the final gRNA vectors. These constructs were transformed into chemically competent DH5α. Candidate transformants were cultured in Kanamycin and plasmid DNA was harvested using a GeneAid Endotoxin-free Maxiprep Kit. The insert sequences for each gRNA were verified first by digestion with EcoRI (**Figure 2a**) and subsequently Sanger sequencing.

Trophoblast cell culture transfection

A mouse trophoblast stem cell line derived from blastocysts of a C57BL6 x Castaneus cross was used for performing the CRISPR-Cas9 experiment. The TSC were transfected with the constructed 3' and 5' guide RNA plasmids as well as a Cas9-GFP plasmid using lipofectamine. Transfected cells were selected for GFP signal, indicative of Cas9 expression, by fluorescence activated cell sorting into 96-well plates. The resulting cell lines were expanded for genomic DNA extraction.

PCR genotyping of genomic DNA

The genomic DNA extracted from cell lines was amplified using primers that flanked the miRNA block as well as primers that were located directly within. Each amplicon was sequenced to determine which allele(s) had been successfully edited on the basis of SNPs present in the amplicon. Cas9 targeting was not highly accurate, thus the amount and position of sequence deleted was variable between cell lines (**Figure 2c**).

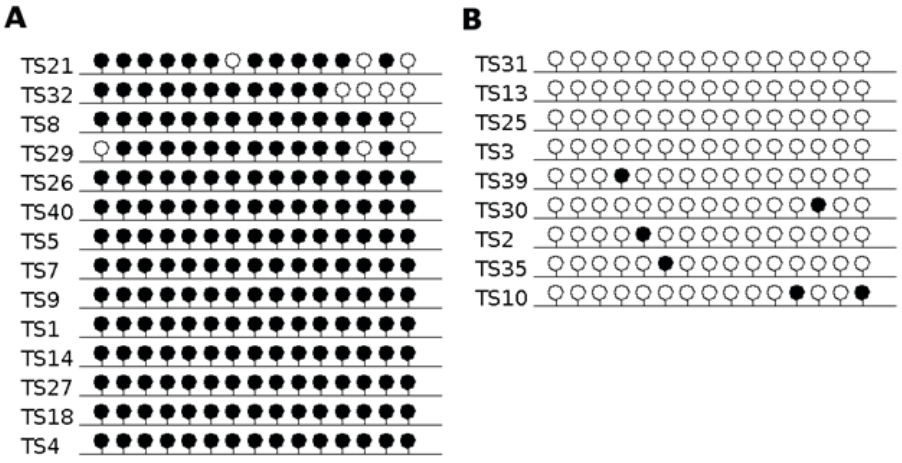

Figure 3. Bisulfite Sequencing results from the B6 and Cast allele of Sfmbt2 in Trophoblast Stem Cells show that the maternal allele is methylated around the promoter region whereas it is not methylated in vivo. The diagram represents the methylation status of the B6 (maternal) allele of a population of trophoblast stem cells around the promoter region, obtained by bisulfite sequencing. Each colored dot represents a methylated section, while the uncolored dots represent sequence regions that are not methylated. (A) The maternal allele was heavily methylated in this region of the CpG island, though it is not methylated in vivo. (B) The paternal allele was not methylated in this region of the CpG island in the majority of cell lines. It is also not heavily methylated in vivo.

RFLP Analysis of mRNA

Cellular mRNA was converted to cDNA by reverse transcriptase using random hexamers. Then, cDNA products were amplified by PCR using Sfmbt2 -specific primers. The sequences were analyzed and matched only the sequence of the paternal allele (**Figure 2c**).

Bisulfite sequencing of untreated Trophoblast Stem Cells

Genomic DNA samples from TSC were treated with sodium bisulfite, which mutates all of the unmethylated cytosine residues to uracil residues. Subsequently, regions of the CpG island in Sfmbt2 were amplified using primers that were specific to only one of the bisulfite-treated strands. The PCR product was subcloned into plasmids, which were transformed into competent DH5α. Randomly chosen individual clones were sequenced to distinguish between the Castaneus allele and C57BL6 allele by SNPs (**Figure 3**).

Methylation sensitive digest of genomic DNA followed by PCR amplification

Genomic DNA from trophoblast stem cells treated with CRISPR was digested with the methylation sensitive enzymes AciI and HpaII to determine whether the region was methylated. Three regions of the CpG island in Sfmbt2 were then chosen to be amplified by PCR for each cell sample. If methylated, restriction enzyme activity would be blocked and thus presence of a PCR product indicates that the region is methylated.

RESULTS AND DISCUSSION

PCR of the FACS-sorted TSC followed by sequencing after CRISPR showed that a variety of mutants had been generated which had deletions of the miRNA block from the maternal allele, paternal allele or both based on SNPs found in the genomic DNA sequence (**Figure 2c**). The cDNA from these clones, when amplified, was found to contain SNPs from only the paternal allele (Figure 2d). These data suggests that across these cell lines, the expression of Sfmbt2 remained imprinted regardless of whether the miRNA block was deleted in genomic DNA from either of the alleles or from both. This result suggests that deletion of the miRNA block in TSC does not result in reactivation of the maternal allele.

Methylation status of the transcriptional start site of sfmbt2

Most imprinted loci are regulated epigenetically by differential methylation of a central ICR, which as previously discussed is not true in the case of Sfmbt2. This gene is imprinted in a tissue-specific manner in mice, and tissue specific imprinting of genes in extraembryonic tissues is usually facilitated by methylation [7]. Previously the Varmuza lab had shown that there was no methylation of the CpG island at the TSS of Sfmbt2 in murine trophoblast tissue, suggesting that imprinting of Sfmbt2 is not determined by methylation marks [16]. To investigate whether this held true in the cell culture used, regions around the transcriptional start site of the gene were analyzed by bisulfite sequencing. Despite being derived from murine trophoblast, TSCs have been cultured in vitro over many generations. Bisulfite mutagenesis distinguishes methylated from unmethylated CpG sequence [20]. Bisulfite sequencing was performed on these cultures and the data shows that a section of the CpG island found at the transcriptional start site of the maternal allele of Sfmbt2 was methylated in TSC (Figure 3).

In order to confirm that this result holds true for cultures used during the CRISPR experiment as well, genomic DNA from the parental cell line as well as from cell lines generated from CRISPR were analyzed by digestion with methylation specific enzymes AciI and HpaII. Subsequently, three regions within the CpG island of Sfmbt2 were amplified by PCR for three selected cell lines 3B8, 4G11 and 1C4. Sanger sequencing of the PCR products revealed that the SNPs present corresponded to the maternal C57BL6 allele only. By this analysis, CpG islands on the C57BL6 allele were methylated in the majority of subclones, but the paternal allele was not methylated.

FUTURE DIRECTIONS

These results suggest that the maternal allele of Sfmbt2 is methylated at regions downstream of the transcriptional start site, supporting the hypothesis that there has been methylation of the maternal allele in TSC which distinguishes it from murine trophoblast cells in vivo. The next step in determining the role of the miRNA block is to apply the same genomic editing technique to study Sfmbt2 expression in vivo rather than in cultured cells.

The catalytic Argonaute protein family consistently associates with small RNA guides to target mRNA in all of these pathways [21]. Given that miRNAs interact with small RNA machinery, it may be possible to determine if this miRNA block has any effect on imprinting by knockdown of silencing proteins in pathways such as transcriptional and post transcriptional gene silencing. The effects of silencing during imprinting are exerted at the genome level

so transcriptional silencing is more likely to be involved. Though transcriptional gene silencing has been recorded in eukaryotes such as yeast, the role of small RNAs in such pathways is yet to be fully understood [22]. However, if small RNAs facilitate silencing of Sfmbt2, removing the small RNA silencing machinery from cells is likely to have an effect on imprinting at this locus.

It is also possible that small RNAs are only part of a larger system that is responsible for the imprinted expression of Sfmbt2. For instance, small RNAs may be responsible for the establishment of imprinting, while maintenance is through other mechanisms, such as methylation in this case. It has been mentioned that both cis and trans acting elements have been found to contribute to genomic imprinting. A combination of different mechanisms may help to explain how the imprint of Sfmbt2 is established in the germline and maintained in a tissue specific manner.

CONCLUSION

Sfmbt2 is a maternally imprinted gene for which the imprinting mechanism is not fully characterized. It is imprinted within extraembryonic tissues including the trophoblast. Correlational evidence suggests that the miRNA block present inside intron 10 of this gene is involved with imprinting. This miRNA block was deleted by CRISPR in one or both alleles within a trophoblast stem cell culture. Subsequent cDNA analysis revealed that Sfmbt2 remained imprinted after treatment. A methylation sensitive restriction enzyme digest of gDNA followed by amplification of regions around the transcriptional start site of this gene has shown that there is methylation of the maternal allele of Sfmbt2 in the trophoblast cell culture used. This distinguishes the cultures epigenetically from in vivo trophoblast tissues.

ACKNOWLEDGEMENTS

The author is grateful to the members of the Varmuza Lab, particularly Dr. Susannah Varmuza, whose support was invaluable during this project. This work was supported by an NSERC summer studentship.

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JULS REVIEWS

Inhibitory Interneurons of The Neo-Cortex & The Role of Parvalbumin-Expressing Cells in Schizophrenia

Tasneem Khan[1], Blake Richards[2]
[1]Department of Psychology, University of Toronto
[2]Depatment of Biological Sciences, University of Toronto

Corresponding Author: Tasneem Khan (taz.khan@mail.utoronto.ca)

Abstract

The cerebral cortex is involved in higher order processes such as thinking, planning, problem solving, executive functions, and organizing information. These processes involve sending feedback to other brain regions through signals that require synchrony. Such synchrony is controlled by oscillations in brain activity. Evidence suggests that inhibition is a key factor in maintaining synchrony and oscillations. There are many classes of inhibitory neurons involved in generating synchrony; however each class can have complex variations. Therefore, a vast literature is dedicated to understanding the molecular, physiological and functional characteristics of these neurons, often referred to as inhibitory interneurons. Furthermore, not only are inhibitory interneurons complex in categorical distribution, but they can interact with other neurons in various ways to allow for proper signaling. This paper provides an overview of the literature present on the major types of inhibitory interneurons in the cerebral cortex and their importance in signalling processes. Furthermore, there is emerging evidence that deficits in cortical interneurons could be the underlying cause of symptoms in neuropsychiatric diseases such as autism, epilepsy and schizophrenia. The second purpose of this paper is to highlight the physiology and the role of disarrayed signalling processes of the parvalbumin interneurons in schizophrenia.

Introduction

Neural circuits, no matter how complex, consist of two types of intermingled cells, namely neurons and glial cells [1]. Neurons are electrically excitable cells that process information and control behaviour, whereas glial cells are known to be responsible for homeostasis and support functions [2]. The canonical work performed by Santiago Ramon y Cajal showed the diversity of neuron sizes and morphology amongst various species and in different neural circuits [1]. Depending on the method used for classification, it can be argued that thousands of neuron types exist in the human brain. However, neurons can be broadly categorized based on their morphology, the projections that they make, as well as the neurotransmitter that they release from their synaptic terminals.

Neurons of the cerebral cortex remain of particular interest to researchers. The cerebral cortex is the outer gray matter over the brain hemisphere and is approximately 2 to3 mm thick in humans, with both gyri (folds) and sulci (grooves). The functions of the cerebral cortex include sensation and motor control as well as complex functions such as memory, decision making and consciousness (Swenson, 2006). Although the cerebral-cortex is primarily comprised of the neocortex, it also includes the hippocampus.

Neuroanatomists in the 20th century used staining methods to reveal that cortical neurons are uniformly arranged in multiple layers and connect to different cortical and subcortical regions consisting of various types of neurons [1]. Although these were the initial findings the neurons of the neo-cortex, they still remain fundamental to cerebral organization and continue to influence research in recent times [3]. General consensus amongst neuroscientists at present is that the neocortex is composed of six layers [3] (**Figure 1**).

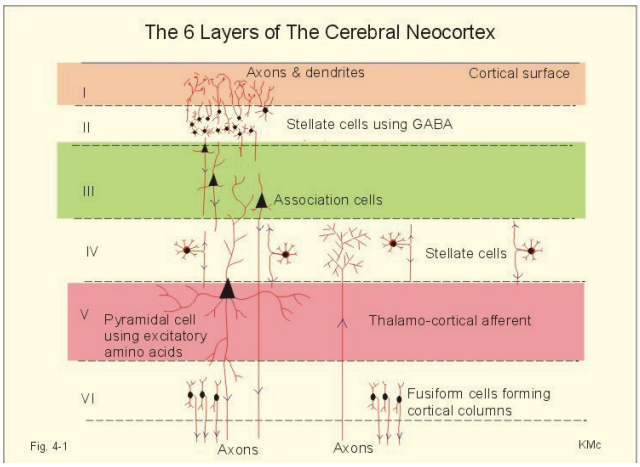

Figure 1. Layers of the cerebral cortex showing pyramidal, stellate, association & fusiform cells (Taken from Paulev, 2000, Fig. 4.1)

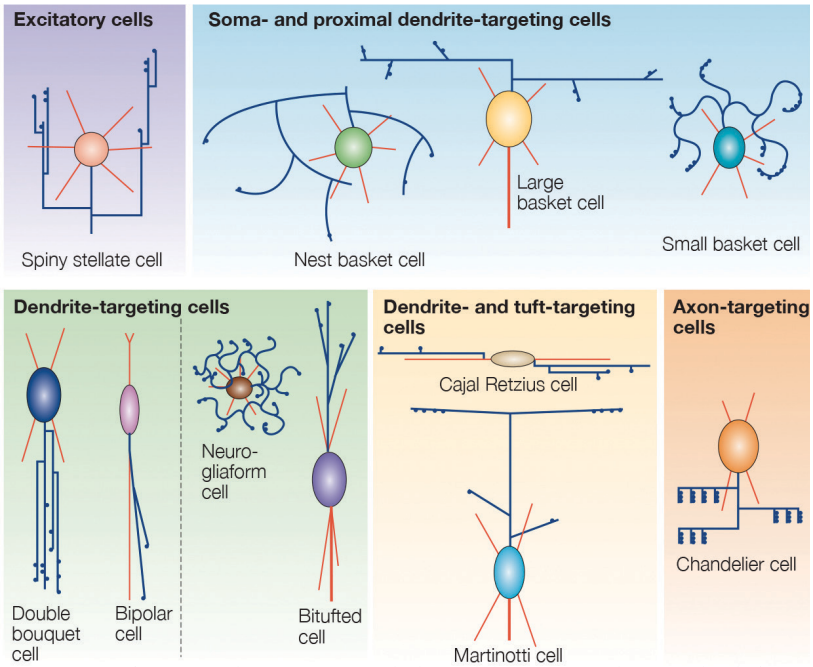

Figure 2. A reference to the various morphological descriptions showing the different types of somas, dendrites and axons of inhibitory interneurons (taken from Markram et al., 2004)

Each cortical layer of the neocortex contains neurons of different shapes, sizes and density as well as different organizations of nerve fibers. Furthermore, neurons in these layers are grouped into two major types; neurons which release either glutamate (the principal excitatory neurotransmitter) or those which release Gamma Amino Butryic Acid (GABA, the principle inhibitory neurotransmitter). It is GABA type neurons which are considered inhibitory, and are densely packed in layers I, II/III and IV, and have non-pyramidal somas [5]. These neurons do not project their axons outside of the local circuits that they belong to, hence they are referred to as inhibitory interneurons. Although approximately 80 percent of the neo-cortical neurons are excitatory and only 20 percent are inhibitory interneurons [6], researchers have focused their attention on the latter. This is due to the comparatively immense diversity of the physiological characteristics of these interneurons. Hence, it is important to provide an overview of the current state of knowledge on the inhibitory interneurons of the neocortex (which has been studied primarily in mammalian brains). This overview will be the first focus of this paper.

Inhibitory interneurons exhibit diversity in their somatic, dendritic and axonal morphology, as well as their electrophysiology and the molecular markers they express. The most common types of inhibitory interneurons include (1) Parvalbumin-expressing, fast-spiking Basket and Chandelier cells, (2) Somatostatin-expressing Martinotti cells, (3) 5-Hydroxytrptamine-3a (5HT3a) receptor-expressing Neuro-gliaform cells and (4) Vasoactive intestinal peptide-expressing interneurons. Of importance to this review are Parvulbumin-expressing interneurons which will be discussed in details.

We now know that each type of interneuron has unique functions in neocortical information processing. Moreover, proper sensorimotor and cognitive functioning is dependent on proper performance of these interneurons [7]. Consequently, research has also investigated whether specific interneurons are related to psychiatric disorders. This review discusses the properties of Basket and Chandelier cells which are known to be important in this regard. The latter part of this paper aims to explain the relation between dysfunction in inter-neuronal circuitry and schizophrenia. Typical symptoms of the disease will be outlined, along with malfunctions in inhibitory interneurons and how these malfunctions in particular brain areas associate with specific symptoms of schizophrenia.

Overview of Interneurons

Despite hundreds of years of research, interneuron classification still has not been standardized. This is mainly due to the variety in the phenotypical aspects of each interneuron type. To be able to understand the functional role of these interneurons in signalling and in neurological disorders, it is important to review the complex classification methods used to identify these interneurons.

In an attempt to overcome the nomenclature problem of interneurons, a group of scientists (the Petilla group) have agreed on the use of a common list of terms that describe the morphological, molecular and physiological features of neocortical interneurons [8]. According to Petilla terminology, morphological features that can be used to define an interneuron include the soma shape, size and orientation; arborisation and branch metrics of the dendrite; the terminal shape of the axon and its somatic targets. Molecular features that can be used to describe interneurons, according to the Petilla group, include neurotransmitters, structural proteins, neuropeptides and calcium binding proteins expressed by the interneurons [8]. Lastly, the Petilla terminology has also identified an extensive list of physiological aspects that can be used to identify an interneuron including action potential measurements, and post synaptic responses by other neurons [8].

Scientists have classified inhibitory interneurons into some major categories. Inhibitory interneurons named Large Basket and Chandelier cells comprise a major portion (50%) of all inhibitory interneurons [6], while 30 percent are Martinotti and Small Basket cells, 15 percent are Small Bipolar and double bouquet interneurons and a minority of 5 percent are Neurogliaform/ Mutipolar putative cells [9]. Research has also shown that interneurons are not always inhibitory; the spiny stellate cell (SSC) is an important type of excitatory interneuron. **Figure 2** shows the anatomical diversity of neocortical neurons.

Basket Cells Morphology & Projections

Early work on interneurons focused on the basic understanding of their structure and morphology. Research from this time period divided Basket cells into three types depending on the size of the cell body, the dendritic and axonal projection: Large, Nest and Small Basket cells [10] and established that side branches of basket cell interneurons terminate around pyramidal cell somata in a basket-like appearance. Notable studies in relation to the morphology of basket cells from this early period include DeFelipe & Fairén (1982), Fairén (2005), Wang et al. (2002) [11, 12, 13]. Work by Wang and colleagues is an often cited work on the types of GABAergic synapses in the neocortex [13]. More recent tech-

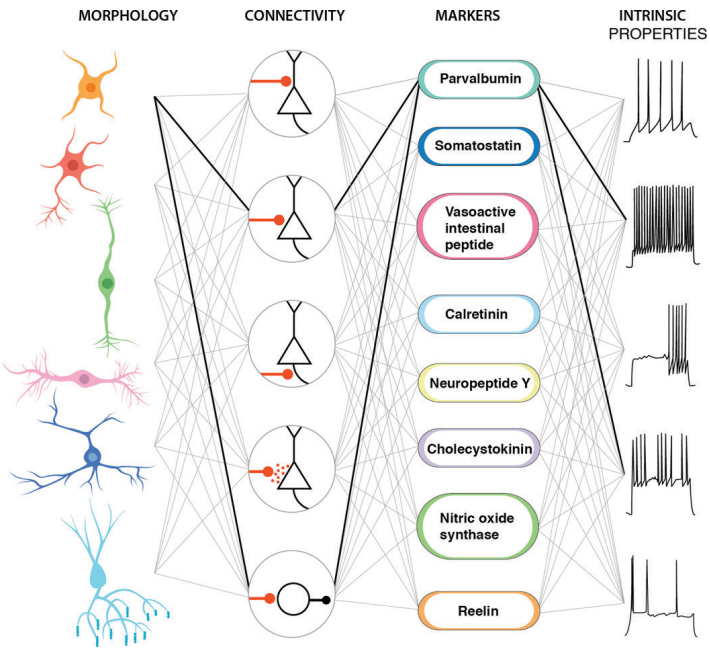

Figure 3. The above figure (adapted Kepecs & Fishell, 2014) displays the diversity of the inhibitory neuron types, their connectivity with pyramidal cells & the vast amounts overlap in somatic marker expression and the general electrophysiological patterns produced by them.

niques of classifying the morphology of interneurons include the Sholl Analysis, which is a technique which creates concentric circles in 3D around the soma of a neuron [14].

Basket Cells' Somatic Markers & Electrophysiology

Somatic markers expressed by interneurons are a key determinant of their signalling processes as they determine the various functions of these cells. **Figure 3** provides an overview of the connections and electrophysiology of the major interneuron types. All three types of basket cells are known to preferentially target proximal regions of pyramidal cells, although their functional properties, including their firing patterns and time course of outputs, differ according to the somatic markers they express [15]. Basket cells are now known to have distinct neurochemical profiles: Large and Nest baskets contain either Parvalbumin (PV) or Cholecystokinin (CCK) and Calbindin, but never express Vasoactive Intestinal Peptide [15]. Other authors have elaborated on this concept by stating that Basket cells that express PV are ones that project horizontally at great lengths, [16]. Keeping in line with this concept, Thomson & Lamy (2007) have shown that Basket Cells immunoreactive to PV Basket cells display a "Fast Spiking" (FS) firing pattern with narrow action potentials [15].

Chandelier Cells Morphology, Somatic Markers & Electrophysiology

Although Chandelier cells are a subset of interneurons with short vertical boutons on their axons, reminiscent of candlesticks, they also express the Parvalbumin somatic marker as well as Corticotrophin-releasing factor [18]. Research has shown that these cells target initial axonal segments of pyramidal neurons [19] and they are present in all layers of the neo-cortex, although they occur most abundantly in layer II [20]. Just like Parvalbumin-expressing basket cells, these cells have been classified as "fast-spiking". "Fast-spiking" is defined as a short action potential and high frequency without adaptation [15, 18]. Although both Large Basket Cells and Chandelier cells are PV expressing fast-spiking cells, recent work by [21] show that membrane properties differ in Basket Cells and Chandelier cells. This likely contributes to their distinctive roles in cortical circuitry.

The Connection of Interneurons to Psychiatric Disorders

There is a stream of literature which aims to find the role of inhibitory interneurons in psychiatric disorders such as autism, epilepsy, and schizophrenia, developmental disorders as well as intellectual disabilities. Due to the complexity of underlying pathology in each disorder, this paper focuses on schizophrenia and the alterations in interneuronal anatomy, function and circuitry that possibly cause it. Evidence suggests that Parvalbumin-expressing interneurons play a key role in schizophrenia through variations in their molecular, genetic and functional properties [22, 23]. An important functional property of the PV interneurons is their contribution to cortical oscillations, which is suggested to be defected in schizophrenia [24].

What is Schizophrenia?

Schizophrenia is a devastating mental illness that affects approximately 1 percent of the total world population. Schizophrenia is diagnosed based on a cluster of clinical symptoms, which are set forth by the Diagnostic and Statistical Manual of Mental Disorders (DSM). The current DSM in its fifth edition provides certain criteria for diagnosing schizophrenia [25]. For simplification, certain

Table 1. Summarizes the categorical information pertaining to all interneurons discussed (taken from, Vitalis & Rossier, 2010).

Markers	%/GABA+Cells	Morphology	Axonal Targeting	Firing Pattern[a]
		Large Barsket Nest Basket	Proximal dendrites/Soma Soma	FS Accomodating Nonaccomodating FS
Parv	~50%	Chandelier	Axonal initial segment	FS
SOM SOM/SC	~30%	Martinotti Small Basket	Distal Dendrite Proximal dendrite/Soma	Bursting Non-FS
VIP Bipolar CR	~15%	Small bipolar; Double bouquet	Proximal dendrites other GABA neurons	Adapting; Bursting
NPY "only"[d]	~5%	Multipolar putative Neurgliaform	Dendritic shaft blood vessels	Late spiking accomadating

[a]Firing pattern elicited from intracellular injections of depolarising currents: FS, fast spaking.

symptoms have been categorized into positive and negative symptoms in literature. Positive symptoms include excessive symptoms that are otherwise not experienced by normal individuals such as hallucinations, delusions, disorganized speech and behaviour [26]. Hallucinations involve disturbances such as smelling, tasting hearing or feeling something that does not exist in reality. Delusions on the other hand are false beliefs held by an individual, such being controlled or harmed by external forces [26].

Negative symptoms of schizophrenia involve the lack of experiences that normal individuals otherwise experience. Such symptoms involve lack of motivation, social withdrawal, and a reduced ability in cognitive functioning [27].

Molecular pathology of Interneurons in Schizophrenia

Gabaergic signaling is regulated in part by the enzyme Glutamic Acid Decarboxylase (GAD) which contributes to the synthesis of GABA. Schmidt & Mirnics [22] indicate that the deletion of GAD67 (isoform of GAD) reduces GABA levels by 97%. Levels of decreased GAD67 have been found in Dorso-Lateral Pre-Frontal Cortex (DLPFC), sensory, motor and limbic regions of schizophrenia patients [22]. Of primary interest in molecular research has been GAD67 pathophysiology in parvalbumin interneurons [23]. In particular, GAD67 mRNA is not observed in 50% of parvalbumin interneurons in this region of Schizophrenic patients. As a compensatory mechanism to GABA deficiency, a 100 percent increase in GABAa2 receptors is observed in postsynaptic pyramidal cells of these interneurons [22]. To reiterate, parvalbumin interneurons include Large Basket cells which target pyramidal cell somata and Chandelier cells which form projections on the axons of pyramidal cells. Another compensatory mechanism in response to this deficit has been established as the down regulation of GAT-1 (a reuptake transporter) in Chandelier cell axonal terminals [22].

There has also been a considerable examination of genetic abnormalities in the GABAergic system that may lead to behavioural symptoms of schizophrenia [23]. A review by Marin Oscar [23] highlights various mice model studies involving gene deletions in certain enzymes and proteins in PV interneurons. It was illustrated that up and down regulation of genes for certain enzymes lead to reduced numbers of PV-expressing interneurons. Lower numbers of PV+ interneurons caused these mice to display schizophrenia-related symptoms such as hyperactivity, and defective working memory [23].

Pathophysiology in Interneuronal Synchrony

Proper functioning of the interneuronal circuitry, which produces synchronous oscillatory activity is suggested to be responsible for maintaining higher order cognitive functions. This phenomenon has been studied in various brain regions [22].

What Are Oscillations?

Repetitive-rhythmic neural activity generated spontaneously, and in response to stimuli by neural tissue in the central nervous system, is known as neural oscillations [29]. When a large number of neurons produce synchronized activity, oscillations in the local field potential are produced, that can be recorded using electroencephalograms (EEG). Typical oscillatory activity spans five orders of magnitudes classified as Alpha (3-18 Hertz), Beta (18-25 Hz), Theta (3.5-7 Hz) Gamma (30-60 Hz), and Delta (0.5- 3.5 Hz) [29]. It is recently becoming more apparent that event-related oscillations (which are increases in oscillation strength in response to specific sensory, motor or cognitive event) are modified throughout the cortex in pathological brains, in particular for patients with cognitive impairments [29]. Work on Gamma oscillations has been proposed widely as a requirement for proper cortical functioning and the parvalbumin interneurons have been suggested to play a key role in producing gamma oscillations [28]. Furthermore, literature has established that Gamma Oscillations are related to cognition and are disarrayed in schizophrenic patients.

Role of Interneurons in Oscillations

Oscillations are formed by neural activity that has rhythmicity between action potential firing and synaptic inputs [30]. The repetitive nature of oscillatory cycles can be caused by the interneuronal cell circuitry. As discussed previously, interneurons play an important part in shaping the activity of the central nervous system by projecting to various areas of excitatory cells. Marin & Colleagues [23] state, "Interneurons are 'tailor-made' to influence the excitability of pyramidal cells or other interneurons in unique ways". Other researchers have also shown that networks formed exclusively by interneurons are capable of generating rhythmic oscillatory activity [31].

Parvalbumin Interneurons & Gamma Oscillations

Gamma (30-60 Hz) activity is associated with alternating current sources and sinks in the peri-somatic region [32]. This finding can be consistent with the involvement of basket and chandelier cells as literature shows these interneuron types innervate the per-somatic region of pyramidal cells. However, basket cells have gained more support for their involvement in generating gamma oscillations. Upon recording the electrical activity of a basket interneuron in a behaviour study on rats, researchers found higher number of spikes in the gamma frequency range [32].

PV-expressing chandelier cells activity is shown to be weakly coupled to Gamma oscillation rhythm [24]. One study even concludes that chandelier cell projections to pyramidal cell axon initial segments are excitatory [33]. On the contrary, it has been found that chandelier cells are implicated as a result of the upregulation of GABAa2 receptor [22]. As previously mentioned, GABAa2 receptors maybe increased in numbers in schizophrenia (Section 3B), meaning that Chandelier cells are of importance as well. Consequently, researchers propose that it is the GABAa2 receptor alteration that leads to increased excitation of post-synaptic pyramidal neurons by chandelier cells [22]. The different synopses presented, and the emerging importance of PV cells such as Basket and Chandelier cells in schizophrenia, calls for further research in this area.

Gamma Oscillation Models

Two models have been proposed for the production methods of gamma oscillations. An earlier model named Interneuron Network Gamma (ING) proposes that gamma oscillations are produced due to reciprocal inhibition between GABA neurons. In other words, GABA neurons are excited by a strong current, which is synchronized by the mutual inhibition of these neurons [34]. As the ING model continues to be tested, some studies favour the second model named Pyramidal Interneuron Network Gamma (PING). This model favours the recurrent interactions between ex-

citatory-inhibitory neurons for generating oscillations. The PING model depicts that gamma oscillations rely on excitatory input to PV basket cells, which are synchronized by the strong feedback inhibition to the pyramidal cells [34]. Although computational models of neurons generate gamma oscillations via ING or PING mechanisms, more evidence is developing in favour of the PING model and the role of PV basket cells involved in PING [24, 34, 35].

In conclusion to this section, it is clear that that pre and post synaptic abnormalities in the fast-spiking PV interneurons are said to result in impaired gamma oscillations [30]. Reduced numbers of PV neurons have also resulted in defective long term potentiation, defective gamma synchrony and impaired inhibitory function [27]. In particular to basket cells, it has also been hypothesized that decreased function in the N-Methyl-D-Aspartate (NMDA) receptor results in altered basket cell activity in Schizophrenic patients (NMDA is a receptor for glutamate, which is an excitatory neurotransmitter) [24].

Cognitive impairment in Schizophrenia via Gamma oscillations

Using Electroencephalography (EEG) recordings, it was demonstrated that cognitive functions including attention, learning and memory were related to gamma band range (30-60 Hz) [36, 37]. In studies requiring Schizophrenic subjects to perform cognitive tasks with simultaneous EEG recordings, dysfunctions are observed at 40 Hz (which corresponds to the Gamma band frequency) [38, 39]. Task demands with stronger pre-frontal cortex dependence have also revealed gamma deficits in patients while performing mental arithmetic tasks (measured by magneto-encephalography). Magneto-encephalography is a technique which measures electrical brain activity by the magnetic fields produced [40].

A certain form of cognitive ability that is impaired in Schizophrenic patients is known as cognitive control. Cognitive control refers to a collection of processes responsible for directing goal-relevant decision making [41]. Such processes include directing our attention to specific stimuli in the environment in a given situation and to maintain our performance in accordance to it, (requiring behavioural flexibility) [42]. The ability to modulate our behaviour in accordance to contextual needs is dependent on the direction and redirection of information processing in our brain.

A computer performance task on cognitive control has found increases in gamma band frequency in the prefrontal cortex. These increases were not seen in Schizophrenic patients [43]. Other studies have established specific areas involved in gamma band oscillations suggested to be implicated in Schizophrenic patients include the Prefrontal cortex (specifically Dorsolateral Pre-frontal cortex, & Ventro-Lateral Pre-Frontal cortex) along with limbic regions [42]. Impaired cognitive control is associated with a wide range of other cognitive processes and leads to an array of deficits in higher cognitive functions (attention, memory, language comprehension and emotional processing) in Schizophrenic patients [42].

This section concludes that interneurons are vital for proper functioning in the cerebral cortex as alterations at various levels in these neurons can result in devastating behavioural symptoms of disorders such as schizophrenia.

Summary

Neuroscientists have performed extensive studies to observe the inhibitory interneurons found in various layers of the cerebral cortex in the past decades [4, 7, 15, 30]. Through review of past and present literature, it has been affirmed that distinct classes of inhibitory interneurons exist in various layers of the cerebral cortex and they have been classified using various categories [5, 44]. With such a detailed analysis of inhibitory interneurons, this paper has established a clear reference point for understanding the complex aspects of each category used to classify inhibitory interneurons [9]. Apart from providing a summary of inhibitory interneurons, it has been demonstrated through a review of literature that alterations in interneurons at genetic, molecular and functional levels lead to Schizophrenic symptoms [22]. The involvement of PV-expressing interneurons in gamma oscillations is also clearly established [32, 34]. Specifically, evidence for the role of PV-expressing basket cells in generating gamma oscillations through the Pyramidal Interneuron Network Gamma model has been presented [24].

Along with debilitating symptoms in schizophrenia, underlying defects in gamma oscillations cause cognitive impairment [25, 38, & 43]. Moreover, through the observed case of defective PV interneurons resulting in cognitive control deficits, it is reasonable to state that other implicated interneuron classes may be playing a role in the underlying psychiatric symptoms in schizophrenia, autism, epilepsy and developmental Disorders.

Future Scope for Literature

The Petilla group has given a fine direction to the research on interneurons by defining the various features in each category [8]. This review incorporated elements from various categories listed by Petilla Interneuron Naming Group. However, most research studies have focused on one or few aspects of only one category (i.e. morphology, somatic markers, or electrophysiology). This is primarily due to the complex characteristics of each interneuron class and their connections to other neurons in the brain circuits. Earlier studies focused primarily on morphological aspects of interneurons [6, 20] while recent interest of neuroscientists has shifted to seek out the genetic, molecular and electrophysiological features of interneurons in detail [13, 16]

Although such detailed research has helped in advancing our knowledge about interneurons, there seems to be a lack of reviews that discuss the findings on the various categories of an interneuron type in one paper. With further review of the vast and complex categorization of interneurons, a common ground can be established which will help in using the important literature pertaining to these cells.

Multidimensional Approach Model

Graphing of neural activity using EEG began in the 20th century and is becoming a well-known technique overtime. A great amount of work is currently being performed to understand oscillatory activity, in particular on gamma oscillations in relation to PV interneurons as we have seen in Section 3C of our review [24]. Although the scope of this paper allowed us to focus on the functional role of basket interneurons in schizophrenia, other interneurons may be of critical importance in relation to schizophrenia or other neuropsychiatric diseases. Therefore, it is recommended that each class is studied for their functional significance. Future work is also required to establish a connection between the molecular aspects of interneurons and their functional significance. An emerging technique in this stream of research is known as optogenetics,

which offers control over neuronal activity by turning neuronal populations on or off [45]. Using this technique may broaden our horizons in understanding interneuronal role in psychiatric diseases through the activation and deactivation of certain neurons and brain areas that are known to be related to these diseases.

Next, I present a model for achieving a greater understanding of inihibitory interneurons and their relation to neuropsychiatric diseases. This general model represents the interconnection between the entities required for the proper understanding of a neuropsychiatric disease. As we have seen in the various sections of our paper, components such as interneurons, molecular pathology, and behavioural abnormalities are studied extensively and are critical for the understanding of psychiatric illness. However, they do not provide a complete picture on their own. Each branch in itself is complex, however more review papers are required to create a holistic view of information from various streams of research. Interconnection is important between these various branches to make use of the vast amount of knowledge present on inhibitory interneurons, which are evidently of great significance. In order to further our knowledge on the treatment of diseases such as schizophrenia, autism, epilepsy, a multi-dimensional approach such as this is required.

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JULS REVIEWS

Quality of life in kidney transplant patients: an exploration of instruments, group comparisons, correlates, and outcomes

Aarushi Bansal[1,2]

[1]Department of Biological Sciences, University of Toronto
[2]Department of Multi-Organ Transplant, University Health Network

Abstract

Quality of life (QOL) in kidney transplant recipients is an important indicator of disease burden and general wellbeing of patients. Research in this area can help guide clinical practice and identify areas of future study. This review explores the current literature on the topic of QOL in kidney transplant recipients, beginning by defining and discussing numerous instruments used to measure QOL. The quality of life of kidney transplant recipients is compared to the general population, other patient groups, and patients experiencing other renal replacement therapies. In addition, longitudinal assessments of QOL post-transplantation, outcomes and socio-demographic, clinical and psychosocial correlates of poor QOL are discussed. The purpose for this review was to identify potential gaps in the current literature and establish guidelines to aid in the development of future research studies in this field.

Introduction

The final stage of chronic kidney disease (CKD) is known as end stage renal disease (ESRD), which is a chronic illness requiring renal replacement therapy (RRT). Three treatment modalities include peritoneal dialysis (PD), hemodialysis (HD), and kidney transplantation (KT). KT is often the most desirable RRT for eligible patients as it offers better long-term survival outcomes [1]. Each treatment modality has advantages and disadvantages and can impact individuals' physical, psychological and social health [2]. Although successful transplantation offers many patients freedom from the time-consuming and uncomfortable demands of dialysis, KT requires lifestyle changes that are associated with undesirable side effects and pose future health risks. The most notable is the immunosuppressive drug regimen, which carries potential future risk of comorbidities including malignancy, cardiovascular complications, and infections [2].

Renal replacement therapies are disease management strategies for the underlying kidney disease. Since these are not cures, survival alone is considered an incomplete evaluation of the overall benefits of KT [2,3]. Measures of health related quality of life (HRQOL) provide a more patient-centered approach to the evaluation of treatment efficacy and outcomes.

The purpose of this review is to examine the current literature on the topic of quality of life in kidney transplant patients to develop a better understanding of the areas that have been investigated.

Search Methodology

A literature search was conducted on Ovid MEDLINE® using the following key words "Quality of life" OR "Karnofsky Performance Status" combined with (by AND) "kidney transplantation". The search was limited to the English language and from year 2000 to the current month and year, November 2015. The search yielded 642 articles which were screened for relevance by abstract and title. Additional resources used included PubMed and Web of Science databases through University of Toronto libraries.

Defining Quality of Life

There has been increased interest in evaluation of quality of life (QOL) in health care settings in the past few decades. The World Health Organization has defined QOL as "an individual's perception of their life status concerning the context of culture and value system in which they live and their goals, expectations, standards, and concerns."[4] The increased life expectancy from improved medical therapies and expansion of surgical technologies may possibly explain the increasing interest in QOL evaluations in healthcare [5]. However, due to the lack of consensus around the definition of QOL, it is often used as a generic term encapsulating numerous concepts including employment [6,7], long term graft function [8], health status, perceptions, impact of disease on overall activities, and physical, mental and social wellbeing [2]. These variations in definition lead to alternative measurements and operationalization of QOL [2].

Health related quality of life (HRQOL) can be defined as an individual's subjective perception of the effects of a disease or treatment on their health and overall quality of life [3]. It includes physical, psychological, and social measures of health and is an important marker of disease burden and measure of treatment efficacy and outcomes [3,9]. Despite the challenges associated with

defining and quantifying this subjective concept, many reliable and validated instruments are available and have been used in the assessment of HRQOL.

Instruments used to measure HRQOL

The two types of instruments used to measure HRQOL are generic and disease specific instruments. The most commonly used generic instruments include the Medical Outcome Study Short Form 36 (MOS SF-36), Illness Intrusiveness Rating Scale, Nottingham Health Profile, EuroQOL 5-Dimensional Questionnaire, and the Sickness Impact Profile [3,10]. These instruments allow for the assessment of individual HRQOL across many domains and can be used in medically ill and healthy populations, permitting comparisons between healthy and different diseased states [2,3]. Alternatively, disease specific measures are tailored to a population with a specific disease and capture dimensions relevant to the respective condition. Instruments targeted to kidney disease include the Renal Disease Quality Of Life, ESRD Symptom Checklist-Transplantation Module, and the Kidney Transplant Questionnaire.3 Some instruments that are used to evaluate HRQOL contain kidney disease specific domains, examples include: the Kidney Disease Questionnaire, Dialysis Quality Of Life Questionnaire, Kidney Disease Quality Of Life—Short-Form Questionnaire (KDQOL-SF), and the Choice Health Experience Questionnaire [3].

The most frequently used HRQOL assessment utilized among kidney disease and kidney transplanted populations is the KDQOL-SF, which includes the MOS-SF-36 and several multi-item scales [2,11]. Computed scores range from 0 to 100, with higher scores representing better HRQOL. The MOS-SF-36 measures physical and mental health across the following eight dimensions: physical functioning (PF), physical role functioning (RP), bodily pain (BP), general health perceptions (GH), vitality (V), emotional role functioning (RE), social role functioning (SF), and mental health (MH).

Quality of life in kidney transplant (KT) patients compared to other patient groups

Numerous studies have compared HRQOL of KT patients to different patient cohorts including (1) the general population, (2) other solid-organ transplant groups, and (3) across other ESRD treatment modalities.

Compared to the general population

According to the review published by Dobbels et al. few studies reported similar QOL between KT patients and the general population [2]. A recent publication by Hossain et al. reported similar QOL scores on all domains of the KDQOL-SF-36 questionnaire among KT recipients and healthy controls in Bangladesh [12]. However, as acknowledged by the authors, a pre-transplant assessment of QOL was not conducted [12]. Cornella et al. compared QOL as measured by the MOS SF-36 questionnaire, of a geriatric cohort (60 years of age and older) to the national age and gender matched norm and found no significant difference in physical functioning and emotional status [13]. However, significant limitations in other areas of patient lives included reduced social activities, lower psychophysical energy and greater pain perception [13].

A majority of studies have reported lower scores on physical functioning in transplanted patients when compared to healthy controls [2,13]. A recent publication in 2015 by Ay et al. reported lower physical functioning ($p=0.007$), physical role ($p=0.004$), and emotional role ($p=0.03$) in KT recipients three months post-transplant when compared to healthy controls [14]. Scores collected nine months post-transplant indicated significantly lowered physical role functioning ($p=0.002$) [14]. In 2014 Von der Lippe et al. reported significantly lowered SF-36 scores in transplanted patients when compared to the general population for all domains except bodily pain and mental health, even after adjustments for age and gender [15]. The most significant differences were identified in domains of physical functioning, physical and emotional role functioning, and general health [15]. However, hospitalization at the time of assessment was an exclusion criterion, therefore introducing a potential selection bias by excluding patients who may be experiencing post-transplant related problems. Kontodimopoulos et al. also found a better score in the bodily pain domain of the SF-36 in Greek patients [16]. In addition, it is important to note that most recent studies have been conducted in non-Canadian cohorts that often lack ethnic and cultural diversity.

Compared to other solid organ transplant groups

It has been reported that kidney transplant recipients have similar or slightly better scores in the domains of physical functioning and overall QOL perceptions when compared to other chronically ill populations [2]. Ortega et al. examined changes across the SF-36 domains at three and twelve months post-transplant in renal, liver and lung transplant patients [17]. KT patients had slightly better mental health scores after twelve months, while lung patients had the greatest improvements in all dimensions of HRQOL, after twelve months. In contrast, other studies have reported better QOL scores in renal populations within the first two years post-transplant, after which scores showed a decline [2,14].

Compared across ESRD treatment modalities

Many investigators have reported on the variations in QOL between patients on hemodialysis (HD), peritoneal dialysis (PD), and those that have received KT. It is generally reported that QOL is higher in transplant recipients than dialysis patients, specifically in physical, psychological and social relationship domains [18,19,20]. However, there is considerable uncertainty in these comparisons as Liem et al. suggest that underlying differences between transplanted and dialysis patients may be the root cause of QOL outcomes [20,21].

A meta-analysis conducted by Liem et al. reported lower MOS-SF-36 scores for patients on HD and PD when compared to transplanted patients ($p<0.01$) in all dimensions except for the mental health dimension, in which the PD and KT group were not significantly different ($p=0.019$, significance level set at $p<0.01$) [20]. After adjusting the MOS-SF-36 scores for age and diabetes, scores for the HD group increased, decreased in the KT group and did not change in the PD group [20]. Previous differences in the vitality dimension and social functioning dimensions between dialysis and KT groups disappeared. HD and KT patients no longer had differences in PF, BP and RP dimensions [20]. The authors acknowledged the need to adjust for independent demographic predictors of QOL (age, sex, ethnicity, socioeconomic status, education, employment, and income) and disease-associated factors (primary renal disease, treatment history, anemia, comorbidities) [20].

Kovacs et al. extensively adjusted KDQOL scores of transplanted and waitlisted patients on dialysis for socio-demographic, clinical and psychosocial characteristics including sleep disorders and depression. Their final multivariate regression model demonstrated an independent association of modality with general health perceptions, effect, and burden of kidney disease [11]. The authors acknowledged the lack of generalizability due to lack of an ethnically diverse population, low comorbidity and relatively younger population [11].

Long-term assessment of quality of life after kidney transplantation (KT)

Some studies have shown an increase in HRQOL after KT when compared to assessments before transplant or while on dialysis. In 2014, Oriz et al. evaluated HRQOL using the 15-dimensional (15D) questionnaire, which is a generic, standardized and self-administered instrument. The dimensions include mobility, vision, hearing, breathing, sleep, eating, speech, excretion, usual activities, mental function, discomfort and symptoms, depression, distress, vitality, and sexual activity [22]. Generally, HRQOL improved post-transplant in all dimensions, most notably in the dimensions of sleep, usual activities, discomfort and symptoms, depression, vitality, sexual activity, mental function and vision. However, patients reported lower scores in the dimensions of "mobility" and "distress" after transplantation [22].

Ay et al. found a significant change in only one dimension assessed by the MOS-SF-36, an increase in physical functioning (p=0.028) from three months to nine months post KT [14]. A study in Taiwan assessed long-term HRQOL with the MOS-SF-36 in KT patients fourteen years (SD =3.9) post-transplant and reported lower scores in all dimensions except bodily pain when compared to the general population [23]. However, this study lacks baseline assessments, making it difficult to draw conclusions on long-term changes in HRQOL within individual patients as a result of transplantation.

Recently, Schipper et al. employed a qualitative research design to assess HRQOL among KT patients (two to nine years post-transplant) through individual semi-structured interviews in an attempt to contribute to the understanding of clinical issues and patient needs and wishes [24]. A novel finding of this study was that patients with improved physical and practical functioning reported facing specific problems such as tasks of daily functioning, familiarizing with their roles and finding balance in relationships, all which contribute to an individual's QOL. Among the various perspectives of KT patients, they identified the presence of emotions like gratefulness, fear, and guilt after renal transplantation. Although the sample size was small (n=242), these are important considerations in assessing QOL as they ultimately impact clinical communication and understanding of long-term QOL in KT patients [24].

Correlates of health related quality of life

Factors associated with HRQOL can be categorized into three groups: (1) sociodemographic, (2) clinical, and (3) psychosocial [2,20].

Sociodemographic correlates

Female sex has been documented as a correlate of lower HRQOL compared to males [2]. Wei et al. reported female KT patients to have a lower mean score on the physical functioning dimension than male patients (76.2 vs. 85.1, p = 0.038) [23]. Female patients also reported more bodily pain, less vitality and poorer mental health status compared to male patients [2]. Older age is also largely associated with poor QOL at time of transplant [23]. Furthermore, several studies have found unemployment to be associated with lower QOL scores [2]. Wei et al. found that patients who were employed had significantly better mean scores in five (PF, RP, GH, RE, MH) of the eight dimensions assessed by the MOS-SF-36 when compared to unemployed patients [21]. Since higher education has been correlated with better HRQOL [2,14], it has been hypothesized that higher education might lead to lower levels of unemployment and higher income [2]. Tavallaii et al. showed that higher income was associated with better total HRQOL even after adjusting for age, sex, and time interval from transplantation [24].

Some studies have observed Asian ethnic background in KT patients as a correlate of poor QOL, specifically compared to whites, in the dimensions of physical and psychological functioning [2].

Clinical Correlates

Higher rates of comorbidity, including diabetes and heart disease, postoperative complications, and medication side effects, are associated with lower HRQOL in KT recipients [2]. Specifically, hypertension, diabetes, higher serum creatinine and lower hematocrit have been independently and significantly associated with lower scores for the MOS-SF-36 physical component summary [26]. Additionally, severity of renal dysfunction, immunosuppressive drug regimen, comorbidity, anemia and physical fitness have been implicated in poor QOL in chronic kidney disease patients [3]. Investigation of these factors in KT populations can help improve clinical care.

Psychosocial correlates

Psychosocial correlates of poor QOL include sleep problems, psychiatric disorders, negative body image, and sexuality related issues in KT and kidney disease populations [2,3,27]. Increased social support, measured by close relationships or marital status, has been associated with better QOL in KT recipients [28]. An important future consideration would be an investigation of psychosocial correlates of QOL in a culturally diverse population.

Outcome measures

Measures of HRQOL have been shown to predict objective measures of physical health in KT patients. Molnar-Varga et al. showed that unadjusted models of HRQOL predict mortality in KT recipients. Even after adjustment, an independent association between the physical composite score (PCS), physical functioning (PF), and general health (GH) perception subscale scores remained predictors for mortality [29]. Additionally, hospitalizations, mortality, graft failure, and other clinical factors have been reported as outcomes of poor HRQOL [1,20,30,31,32].

Conclusion

This literature review has examined the topic of QOL in the context of KT recipients. Most studies were conducted on non-Canadian patient populations and were limited by lack of an ethnically diverse population. The ethnically and culturally diverse patient population treated by the KT program at Toronto

General Hospital can contribute to the current literature by evaluating the longitudinal HRQOL post-transplant, identifying predictors of clinical outcomes and understanding treatment efficacy. Furthermore, the Comprehensive Renal Transplant Research and Information System [33] database provides a comprehensive set of patient variables and correlates to accurately investigate potential associations and to make the required adjustments as guided by the current literature.

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JULS REVIEWS

Guessing Glomerular Filtration: A Systematic Review of the Validity of EGFR Calculations

Sri Gaveen[1,2], Nishant Fozdar[2,3], Yanhong Li[4]

[1]Faculty of Arts and Science, University of Toronto
[2]Multi-Organ Transplant Student Research and Training Program, Toronto General Hospital, University Health Network
[3]Faculty of Medicine, University of Toronto
[4]Multi-Organ Transplant Program, Toronto General Hospital, University Health Network

Corresponding Author: Sri Gaveen (iliyanag@uhnresearch.ca)

Glomerular Filtration is the fundamental and most frequently employed assay of renal function, quantifying the unit volume of filtration occurring per unit time. Though precise and direct assays of the glomerular filtration rate (GFR) exist, such as inulin or other radiological tests, they are grossly impractical to perform in a typical clinical context, thus equations which estimate GFR are employed using naturally occurring biological markers like creatinine. This reviews sought to identify the best among the variety of equations (namely the Cockroft-Gault, the MDRD, the CKD-EPI and the Mayo Clinic equations) which most closely predicted a patient's true GFR. Despite exhaustive revision of the published literature, a clear 'best' equation is indeterminable. Instead it is evident that equations typically perform best in populations mimicking the samples from which they were derived. Further, customized equations tend to perform ideally for their specific population, but may produce wildly inaccurate estimates elsewhere. This review demonstrates that for diverse, multi-ethnic populations, an effort should be made to determine which equation best estimates GFR in that specific population as no equation hitherto formulated is a panacea.

Introduction

Assaying kidney function is amongst the most fundamental requirements in diagnosing and treating kidney disease. It also serves as a crucial benchmark for a variety of additional health parameters such as dosing of drugs, or determining candidacy for organ donation - among many others clinical applications [1].

One such assay of kidney function estimates the volume of filtration that occurs through the functional unit of the kidney: the nephron (specifically the glomerulus) - thereby identifying the Glomerular Filtration Rate or GFR [2]. Specifically, the GFR quantifies the volume of fluid filtered from the afferent glomerular capillaries into the Bowman's Capsule per unit time[2]. In a healthy individual, the GFR ranges from 90 to 120mL/min/1.73m^2 with progressively lower GFR values indicating less renal filtration and therefore less kidney function. [1,2]

Defining GFR

There is no direct method to ascertain precisely how much fluid passes through any given glomerulus, thus the GFR is typically gauged by the clearance of some substance (either introduced or endogenous) from the body. [1,2] The rate at which a solute is cleared from the body will equal the GFR presuming the solute in question is not metabolized or excreted from the body by any means other than the kidneys. [2] To measure this clearance in practice, the quantity of the solute in the urine (or filtrate of the kidney) must first be identified; this can be easily determined by multiplying the urine concentration of the solute by the total urine flow. [2] By comparing this output to the concentration of the solute in the blood plasma, the clearance of the substance and by extension the kidney function can be determined.

Mathematically, the GFR can be stated as:

$GFR = C_{urine} \times Flow_{urine}/C_{plasma}$; where C_{urine} represents the concentration of the solute in the urine (in mg/mL or mmol/L), $Flow_{urine}$ represents the volume of urine excreted per unit time (typically in ml/min or ml/24 Hours), and C_{plasma} represents the plasma concentration of the same solute (again in mg/mL or mmol/L). This calculation will yield the GFR as a volume per unit time, however to account for the fact that larger individuals necessitate a higher GFR to clear a given concentration of filtrate; GFR is normalized by the body surface area. Thus, a final GFR value is reported in mL/min/1.73m [2]; which can now be effectively compared among individuals. [2,3]

Measuring GFR

Despite this seeming simplicity, all GFR calculations are contingent upon the selection of an appropriate marker, and the accurate measurement of this in both blood and urine. The latter can be performed with relative ease for most markers using modern spectroscopic or chromatographic analysis. [3] However, the selection of an appropriate marker has proved difficult, as all

filtration markers have some non-GFR determinants[4]. The current gold-standard measure of GFR involves inulin: an exogenous plant based polysaccharide that is introduced to the body intravenously. It is not secreted, nor reabsorbed to any appreciable degree by the kidney, thus proving an optimal marker to track filtration [2]. A similar molecule, sistrin is also occasionally employed as a similar exogenous filtration marker. [5] However, both inulin and sistrin pose immense pragmatic limitations; in an extensive review of markers, Early *et al.* noted that inulin is significantly more costly than other markers, is in short supply and is difficult to dissolve and maintain in solution. [3] Thus, for daily clinical use, as well as most research, inulin is grossly impractical.

An alternative marker, identified in 1969 by HU Pixberg, was ^{125}Iothalamate, which already served as a popular angiographic medium [6]. This was inexpensive and readily available - however as early at 1980, it was known to be prone to renal tubular secretion [6,7,8,9]. In this case, the marker may enter the renal tubules directly from the blood stream, and not through the glomerulus, hence the filtration rate may be overestimated. In fact, Odlind *et al.* noted significant and marked renal secretion in chickens, rats and humans, which significantly altered GFR calculations.[7] However, despite these known inconsistencies, Iothalamate remains a popular assay of GFR, with over a third of investigations reviewed employing it as the reference standard of GFR. This poses a major source of error, especially in investigations seeking to validate other assays of GFR based upon the potentially erroneous estimates of Iothalamate. Other markers including Iohexol, EDTA (Ethylenediaminetetraacetic acid) and DTPA (diethylene triamine pentaacetic acid) are also known for similar propensities for absorption or secretion, yet they comprise the reference standard for the majority of investigations examined. [5] Though more practical, this consistent reliance on less accurate markers to assay GFR represents a major limitation in the current study.

Estimating GFR

Despite their various inaccuracies, all methods hitherto discussed allowed for the direct measurement of glomerular filtration, as a known quantity of a marker could be administered to the patient. In this way, the denominator of the GFR equation (the plasma concentration of the marker) can be known with certainty. However, all the these methods require the collection of urine for an extended period of time, in addition patients are required to endure an extended intravenous injection - both of which render any direct measurement of GFR hopelessly impractical in most typical clinical contexts.

Instead, clinicians far prefer to estimate GFR using endogenous filtration markers. These markers are naturally produced by the body, and therefore require no injection. However, one cannot simply measure the urine and plasma concentration to estimate GFR as there is a constant replenishment of the marker, by natural production from the body. Instead, to determine the estimated GFR (or eGFR), the plasma concentration of a marker is simply measured. If the concentration of the marker is higher than expected, it implies that there is less filtration from the kidney, thus implying a low GFR - and vice versa. However, endogenous markers are subject to a variety of physiological and biological determinants, and thus must be carefully adjusted to reflect a definitive and translatable GFR value.

The most common of these markers is creatinine, which is produced from the breakdown of creatine phosphate in muscles.[3] However age, sex, race, body weight and even certain genetic polymorphisms all directly alter the rate of muscle breakdown, and therefore directly affect the serum creatinine levels independent of any influence of filtration and GFR. [10,11] Thus, to employ creatinine to estimate GFR, one must account for factors effecting the plasma concentration. Extensive investigations, starting in 1973 have produced GFR estimating equations, derived from regression analyses where estimates from serum creatinine were compared to known reference standards (obtained by directly measuring GFR as stated above). [10] However, despite inordinate scientific investigation - the ideal equation to estimate GFR has remained undetermined. It has instead resembled a game of leap frog, where a superior equation periodically appears claiming better accuracy than their predecessor. This systematic review therefore seeks to explore which equation provides the greatest estimate of true GFR.

Methods

Two separate systematic searches were conducted employing the OVID Medline database (1946 to October Week 2 2015) and the Embase Database (1974 to 2015 October 10). For both, general search terms including "Glomerular Filtration Rate", "Kidney", ["Equation" or "Estimation"] were combined using 'AND' (see Appendix 1 for full search strategy). No date, language or type restrictions were initially applied.

Excluding duplicated investigations, a total of 3046 publications were identified. An initial screening was conducted by tittle, where 2615 investigations were removed from consideration as they were deemed irrelevant either for failure to address eGFR equations or for focusing on a unique population (e.g. pediatric patients). Of the remaining investigations, a further 212 were excluded upon abstract review, as they did not address the validity of eGFR equations. Of the remaining 219, only 23 investigations compared an eGFR measure to a direct reference standard, and were therefore examined in most detail. The remaining investigations either examined factors associated with high/low GFR, or compared the concordance of individual estimates of eGFR.

eGFR Equations

There are a variety of eGFR equations, and a plethora of subdivisions thereof all of which claim superior estimation of GFR in a particular population. This review will consider the most fundamental of these, namely the Cockroft-Gault (CG) Equation, the Modification of Diet in Renal Disease (MDRD) equation, and the Chronic Kidney Disease in Epidemiology Collaboration (CKD-EPI) as well as any notable iterative of them.

Assaying Equation Performance

The validity of an equation stems from its precision, the ability to minimize random variation, as well as its accuracy, the ability for it to predict the true value of GFR. No single metric can indicate an equation's precision and accuracy, thus investigations may report a variety of statistical metrics.[10] The most common, and perhaps most indicative of accuracy is statistical bias - which indicates how far the estimations of an equation were from the true value (as identified by some direct GFR measurement). [2,3,5,10] Accuracy may also be identified by 'P_x' values, which indicate what proportion of the estimations which were greater than x% from the true value. Typically the P_{30} value is reported, which identifies

what proportion of the estimations were more than 30% greater/less than the actual value of GFR. [3] It is noteworthy that such a difference of 30% in GFR can translate to a difference of two stages of kidney disease, thus a high P_{30} percentage represents a major failing of the equation. [4]

Likewise, there are several common measures of precision - most of which characterize the spread of the differences between the true value and the estimation. Simple measures such as the inter-quartile range, standard deviation or the mean squared error of the differences are commonly reported. [3] Other assays of precision quantify the quality of regressions among plots of eGFR vs measured GFR using R^2 or Kappa values. [3] Ideally, investigations should consider both the accuracy and the precision of their equation against a reliable measure of direct GFR. [10]

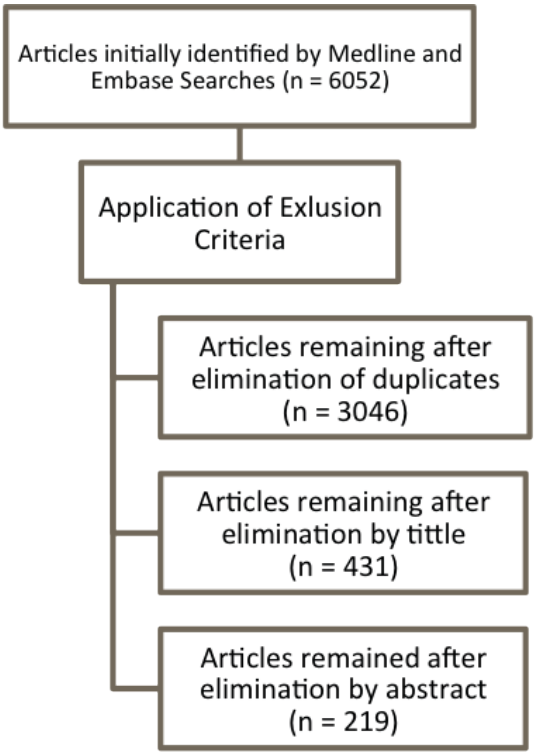

Figure 1. Study Flow Diagram

Summary of the Cockcroft-Gault Equation

The now famous Cockcroft-Gault (CG) equation represents the first cohesive and successful attempt to estimate renal function by creatinine clearance. Having been first published in 1973, it has been extensively examined in a variety of populations. However, it was first developed in a small population (n=249) of white males, with a mean age of 57. [10, 12] Amongst all modern equations, this is the most limited development dataset, and the applicability of the equation beyond middle-aged white males has been questionable. Furthermore, no data was collected on the patients' kidney health or whether they had received a transplant - thus the applicability of the equation to a general population is further indeterminate [12].

In a Chinese population, investigated by Kuo *et al.* the CG equation consistency produced a higher estimate of GFR with an over 17% bias. [13] It was also significantly different than estimations from the more modern MDRD equation, producing a Kappa correlation coefficient of 22% ($p<0.05$). [13] However, in an investigation of Polish transplant recipients by Chrobak *et al.* the CG equation presented the least bias (at -1.65mL/min) and had the best regression with the measured GFR values (r=0.46) [14]. Though the latter metric is hardly impressive, it does demonstrate the sporadic applicability of the CG equation, despite its age and developmental limitations. However, in an extensive review in 2010, and again in 2011 Stevens and Levey reported that the CG equation consistently overestimates GFR and is therefore of minimal utility in light of modern equations. [3,10]

The greatest failing, however, is that the CG equation was conceived before creatinine values were standardized for race and body surface area. Despite attempts to add coefficients to the equation, the CG equation cannot be re-expressed for standardized serum creatinine; thus investigators have generally concluded the CG equation is irrelevant in modern nephrology. [10]

Summary of the MDRD Study Equation

First published in 1999, the MDRD equation is a dramatic departure from the CG equation. [10,15] It was developed in a significantly larger patient population of 1,628 patients, who were comprised of both men and women. The population was also more diverse with a representative 12% of it being black - yet all other participants were categorized as white/Caucasian meaning this remains largely a racially homogenous cohort. However, most intriguingly - as the name implies (Modification of Diet in 'Renal Disease') all patients in this cohort had some degree of chronic kidney disease. In fact, the mean GFR calculated for the developmental database is approximately 39 mL/min/1.74m^2 - implying the average participant was in stage 3 kidney disease. [15] The applicability of this equation to a general, healthy cohort has therefore been consistently questioned.

In general, Levey and Stevens report that the MDRD is prone to underestimate eGFR below 60mL/min/1.73m^2 - however that it was more accurate and more precise than CG. [10] Yet, ample evidence including investigations by Chen *et al.*, Teo *et al.* and Chrobak *et al.* have demonstrated that the MDRD performs worse than all other equations in ethnic cohorts with dramatic over and under estimations of GFR being reported (bias ranging from -8.3 to + 9.7) [14,16,17]. It is likely that the use of an entirely diseased cohort of patients, each of whom had a compromised renal function from an innumerable number of additive factors may have confounded the predictive power of the equation in general populations.

Summary of the CKD-EPI Equation

In 2009, a large collaboration sought to address some of the inadequacies of the MDRD equation with an entirely new and arguably more representative equation. [10,18,19] It originated in a cohort of 5,504 patients, with representative proportions of men and women, as well as Blacks, Asians, Hispanics and White participants. [10] However, over 73% of the study cohort had some measure of chronic kidney disease, and the mean GFR of 67mL/min/1.73m^2 remains unrepresentatively small compared to the general public. [10] Also, nearly a third of the cohort had diabetes, whose effects on GFR remain unclarified and poorly understood. [19]

As it demonstrates many of the same concerns as the MDRD equation, which the CKD-EPI was seeking to replace, its perfor-

mance has also been varied and unpredictable. A particularly notable trend has been the underestimation of GFR at higher ranges (above 60mL/min/1.73m^2). Schmid et al. have even reported that the equation is most effective at diagnosing stage three kidney disease, but is of limited utility outside this range. [20] In brief, all three equations are less than ideal, and demonstrate noteworthy anomalies and exceptions.

Comparison of Equations

The performance of the equations among the investigations comparing a GFR equation to a direct measurement method is summarized below. Note that no consistent measure of precision was employed throughout the studies, thus the confidence interval about the bias will be considered (where applicable) as a proxy for precision. Furthermore, the bias of the equation as well as the P_{30} values will considered as measures of accuracy.

Summary of Comparisons

As with prior reviews, despite exhaustive efforts, this investigation cannot identify a definitive equation superior to the others in all contexts. However, a cursory examination of the bias reveals that of the investigations comparing the basic MDRD to the CKD-EPI, 10 identified the CKD-EPI equation as more accurate than the MDRD, while only 2 reported the opposite. This relationship is affirmed by the P_{30} values which identify an analogous outcome. Precision cannot be adequately considered as few investigations reported a consistent measure that could be compared among studies. However, from the data available it appears that the MDRD is generally more variable, and thus less precise than the CKD-EPI equation. Both these conclusions are corroborated by a prior review by Levey and Stevens. [10]
It is also exceptionally noteworthy that only one investigation examined considered the CG equation in a comparison to a definitive creatinine measure. Though widely agreed as outdated, the surprisingly strong performance of this equation in some studies as well as its ease and practicality merits further investigation.

An intriguing occurrence was also noted for the population specific equations. One would presume that these adjusted equations would perform the

Table 1: Summary of Studies Cosidering the Validity of eGFR Equations

Study Author and Year	Population	Measured GFR Standard	GFR Equation	Bias (mL/min/1.75m^2)	Confidence Interval	P_{30} Value
Chen et al. (2014)[16]	695 Adult Taiwanese Patients -µAge: 47 -µMen: 47.1%	Inulin Clearance (Gold Standard)	MDRD	-5.4	Not Reported	63.3%
			CKD-EPI	-8.0	Not Reported	60.4%
			Taiwanese CKD-EPI	0.17	Not Reported	73.4%
			Thai MDRD	-14.0	Not Reported	52.5%
Stevens et al. (2011)[22]	1022 Chinese, Japanese and Black (South African patients) -µAge: 49 -µMen: 52%	Iothalamate Clearance	CKD-EPI	12.4	7.6 to 18.3	55.6%
			CKD-EPI with Post-Hoc Race Coefficient	-1.3	-2.2 to -0.6	72.1%
Matsuo et al. (2009)[23]	350 Japanese Renal Transplant Recipients and Donors -µAge:54 -µMen: 58%	Inulin Clearance (Gold Standard)	MDRD	12.0	Not Reported	59%
			CKD-EPI	-7.9	Not Reported	73%
Horlo et al. (2010)[24]	350 Japanese Renal Transplant Recipients and Donors -Age: 54 -Men: 58%	Inulin Clearance (Gold Standard	MDRD with Japanese Coefficient	-1.3	Not Reported	73%
			CKD-EPI with Japanese Coefficient	-0.4	Not Reported	75%
Yao et al. (2010)[25]	102 Korean Kidney Transplant Recipients -Age: 42 -Men: 53%	Cr-EDTA Clearance	MDRD	-3.0	Not Reported	94.1%
			Japanese Modified MDRD	17.95	Not Reported	68.6%
Van Deventer et al. (2008)[26]	100 Black South African Patients -µAge: 47 -µMen: 51%	Cr-EDTA	MDRD with ethnicity factor	13.1	Not Reported	52%
			MDRD without ethnicity factor	1.9	Not Reported	74%
Teo et al. (2015)[27]	335 Multi-ethnic patients from Singapore -µAge: 53.5 -µMen: 49.1%	Inulin Clearance (Gold Standard)	MDRD	-3.0	-4.2 to -1.7	79.7%
			CKD-EPI	-1.2	-2.7 to 0.3	82.8%
Ma et al. (2006)[28]	230 Chinese patients with CKD	Tc-DTPA	MDRD Equation	-7.8	-21.5 to -1.8	66.1%
			MDRD with Chinese Coefficient	-0.9	-9.6 to 7.4	77.8%
Praditponslipa et al. (2011)[29]	100 Thal patients with CKD	Tc-DTPA	MDRD	-11.9	Not Reported	62.7%
			CKD-EPI	-10.9	Not Reported	68.0%
Murata et al. (2011)[30]	5238 American Adults who underwent renal testing -µAge: 56 -µMen: 55% -KTR: 28%	Iothalamate Clearance	MDRD	-4.1	Not Reported	77.6%
			CKD-EPI	-0.7	Not Reported	78.4%

Levey et al. (2009)[31]	3896 American adults (from other trials) -µAge: 50 -µMen: 55% -KTR: 29%	Iothalamate Clearance	MDRD	-5.5	-5.0 to -5.9	80.6%
			CKD-EPI	-2.5	-2.1 to -2.9	84.1%
Lane et al. (2010)[32]	425 American patients after Nephrectomu -µAge: 56 -µMen: 7%	Iothalamate Clearance	MDRD	-1.0	Not Reported	75%
			CKD-EPI	-1.7	Not Reported	80%
Michels et al. (2010)[33]	271 Dutch potential kidney donors -µAge: 44 -µMen: 44%	Iothalamate Clearance	MDRD	14.6	Not Reported	81.2
			CKD-EPI	12.3	Not Reported	84.5
White et al. (2010)[34]	207 Canadian stable Kidney Transplant Recipients -µAge: NR -µMen: 60%	Tc-DTPA	MDRD	-7.4	Not Reported	79%
			CKD-EPI	5.2	Not Reported	84%
Chrobak et al. (2014)[14]	215 Polish Patients at a single centre -µAge: 45.6 -µMen: 60.1	Cr-EDTA	MDRD	7.46	Not Reported	67%
			CKD-EPI	-1.65	Not Reported	71%
			CG	-1.0	Not Reported	77%
Eriksen et al. (2010)[35]	1621 healthy Norwegian patients participating in a survey	Iohexol	MDRD	18.2	17.2 to 19.5	93%
			CKD-EPI	15.4	14.5 to 16.3	95%

best in that specific population, however rather than consistently doing so, this review noted an exceptionally erratic performance of these equations. Yao et al. [25] and Vandeventer et al. [26] demonstrated that upon application of the appropriate ethnicity factor, the accuracy of the MDRD equation was reduced up to 6 times. Though Ma *et al.* [24] and Horio *et al.* [28] demonstrated the opposite, where they improved accuracy with the ethnicity factor - the remarkably varying performance of these equations is concerning. One explanation can be found in Chen *et al.*'s [16] investigation where among Taiwanese patients, the worst equation was the Thai MDRD. This may indicate that regional equations are highly specific to that region alone and may be wildly inaccurate in adjacent or nearby regions.

In light of this conclusion, it may be appropriate to dispense with the idea of the 'ideal single equation'. Given the diversity of results, it may be more prudent that each region or perhaps develop their own equation with best estimates GFR for their unique population. Centers with diverse patient cohorts, may even wish to consider the use of multiple equations for differing demographics. Though this would entail an increase in complexity, it would likely yield greater accuracy in estimating GFR.

Significance of eGFR Accuracy

It is crucial to note that differences in the prediction of eGFR due to equational variation have direct applications to patient care and treatment. In their investigation, Chrobak *et al.* demonstrated that there was generally poor concordance between the three major eGFR equations. [14] In their large, multi-centered study, Kuo et al. demonstrated that simply switching between the MDRD and the CG equations can raise the prevalence of stage 3-5 kidney disease from 1.9% to 25.5% which may have a marked difference in the care of these patients. [15] In a similar large-scale investigation, Matsushita *et al.* demonstrated that 43.5% of patients classified as having stage 3a chronic kidney disease under the MDRD equation were reclassified as having no kidney disease using the CKD-EPI equation. [21] The consequences of these discrepancies and the resulting under/over diagnoses of renal disease are profound and may include changes in the provision of treatment, changes in drug regimen and even eligibility for organ donation. Thus, centers must seek to identify the most prudent equation to estimate GFR in their particular patient population.

Conclusion

Despite extensive examination, there is immense contradiction and variation as to which equation best estimates GFR in a given population. This investigation identified the CKD-EPI equation as most accurate, and precise - though the immense variation strongly suggests that this may not necessarily be applicable universally. Ideally, a major centre should seek to identify which eGFR equations best models the true GFR in their specific patient population. A more concerted effort to rely on rely on accurate GFR measuring methods (such as inulin clearance) should also be made, rather than relying on less accurate Iothalamate methods - particularly for investigations seeking to validate other GFR estimating methods.

It is noteworthy institutions with comprehensive data systems, as well as those who perform regular direct GFR calculations can conduct such an investigation with relative ease. Toronto General Hospital in particular, with its extensive data on Transplant Recipients, Donors and Potential Donors, stored in such information systems as the CoReTRIS Database, or the Organ Transplant Tracking Record (OTTR) would be particularly appropriate for such an investigation. Furthermore, the patients of these institutions would benefit most from the identification of specific GFR equation which best models their true GFR.

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Appendix

Medline Search Strategy

1) exp glomerulus filtration rate/
2) exp kidney/
3) exp equation/
4) exp estimation/
5) 3 or 4
6) 1 and 2 and 5

Embase Search Strategy

1) glomerular filtration rate.mp. [mp=ti, ab, ot, nm, hw, kf, px, rx, ui, an, tn, dm, mf, dv, kw]
2) kidney.mp. [mp=ti, ab, ot, nm, hw, kf, px, rx, ui, an, tn, dm, mf, dv, kw]3) exp equation/
3) equation.mp. [mp=ti, ab, ot, nm, hw, kf, px, rx, ui, an, tn, dm, mf, dv, kw]
4) estimation.mp. [mp=ti, ab, ot, nm, hw, kf, px, rx, ui, an, tn, dm, mf, dv, kw]
5) 3 or 4
6) 1 and 2 and 5

Equations Referenced

The Cockroft Gault Equation

Cockcroft-Gault CrCl = (140-age) × (Wt in kg) × (0.85 if female) / (72 * Cr)

The MDRD Equation

GFR = 186 x SerumCr-1.154 × age-0.203 × 1.212 (if patient is black) × 0.742 (if female)

The CKD-EPI Equation

GFR = 141 x min(Scr/κ,1)α x max(Scr/κ,1)-1.209 x 0.993Age x 1.018 [if female] x 1.159 [if black]

JULS REVIEWS

Role of Sleep Deprivation in Glial Modulation of Memory

Zohra A. Ahsan[1], Zachariah Campbell[2]

[1]Department of Neuroscience, University of Toronto
[2]Department of Psychology, University of Toronto

Corresponding Author: Zohra A. Ahsan (zohra.ahsan@mail.utoronto.ca)

Abstract

The importance of sleep in physiological functioning has attracted scientific research for more than two decades. A variety of processes have now been discovered to require adequate sleep for ideal functioning including memory consolidation. While abundant research is available on the role of hippocampal neuronal activities in these processes as we sleep, it is important to consider the role of the more abundant neuronal counterparts in the Central Nervous System (CNS), the glia. Glial cells, especially astrocytes, have recently been established as an essential component of the process of long-term depression (LTD) as well as mediators of long-term potentiation (LTP), both of which are essential cellular correlated of memory. Given their important role in memory consolidation, it is not surprising that sleep deprivation (SD) may mediate some of its negative impacts on memory through glial cells. The following review discusses various glial factors that may explain how SD alters glial processes of memory, namely its effects on adenosine, Nitric Oxide (NO) and the metabolism of energy in the brain.

Moreover, emerging research on the effects of Brain Fatty Acid Binding Protein (FABP7) and cell proliferation within the hippocampus following SD is also discussed. However, given the abundance of changes observed in glial cells following SD, it is likely that these factors make up only a proportion of the overall influence that glial cells may have on memory following SD. Furthermore, a lack of available human research as well as a difference of methodologies employed for achieving SD in an experimental setting demand a better approach for advancing our knowledge of the role of SD in altering the various glial processes that play a role in memory consolidation. Based on the possibility of a connection that may exist between the various species of animals discussed, it is suggested that these connections be considered for future research.

Introduction

Role of Sleep in Memory Consolidation

Scientific research has seen a boost in research on the role of sleep in memory consolidation for over the past two decades. Memory consolidation is a process of stabilization of memories [1]. Such processes have widely been studied using Electroencephalography (EEG), which has enabled the correlation of various brain structures with specific forms of memory consolidation processes. For instance, an enhancement in a procedural learning task – the tower of Hanoi task– is observed only when an individual is allowed Rapid Eye Movement (REM) sleep, and not when the subject is prevented from REM sleep the night following training [2]. Likewise, EEG evidence from hippocampal dependant memory tasks shows increased hippocampal activation during SWS, which correlates with an overnight enhancement observed in the individuals in terms of task repetition [3]. Further, a temporal reactivation of hippocampal networks that were initially recruited in learning of a spatial task is observed during both REM and SWS stages [4], reflecting a process of memory consolidation occurring within the hippocampus across multiple stages of sleep. The sleep dependent enhancement of both hippocampal and non-hippocampal dependant learning tasks indicates that the processes of memory consolidation are particularly affected by sleep deprivation (SD). This factor, hence, becomes particularly important in light of the fact that approximately 30% of Canadian individuals report being sleep deprived due to various modern day factors such as longer work days and commutes [5]. Given that such SD may contribute to a decrease in health of individuals, it becomes necessary to thoroughly investigate the factors that may be negatively influenced by SD and cause observed decreases in memory.

Role of Glial Cells in Modulation of Memory Processes

Despite the fact that glial cells outnumber neurons by ten-fold, less research is being done on glial cells in the field of learning and memory. The first indication of a role for glia emerged from the findings that they mediating synaptic plasticity within the retinal ganglion cell (RGC's) cultures, where an increase was observed in the excitatory post-synaptic potential frequency (EPSC) upon glial cells' activation [6], indicating glial modulation of synapse strength. These results along with additional studies focusing on

electrophysiological alterations in response to glial activations [7] indicate the presence of certain mechanisms through which glial cells are able to modulate information processing in neurons. It should be noted, however, that studies determining effects of SD most often test for astrocytes, while research on the effects on other types of glial cells such as microglia is scarce in literature. Moreover, as will be discussed in detail below, the limited ways of inducing SD makes it difficult to reliably determine the effects on glial cells.

Another important contribution to the understanding of glial cells in memory consolidation comes from molecular studies. Experiments involving hippocampal cultures and slices have demonstrated the role of various cytokines that specifically effect glial cells, such as tumor necrosis factor-α (TNF-α) , in increasing surface α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor expression, and a decrease in EPSC amplitude and frequency following the blockage of TNF-α receptors [8]. The role of glial cells, more specifically astrocytes, has also been established in controlling the in vivo neuronal morphology by the release of Ephrin-A3 molecules [9]. Through their study, Murai, Nguyen demonstrated that the astrocytes surrounding hippocampal dendrites are able to release ephrins, which in turn prevent expansion of the spines [9].

In addition to releasing glial specific factors, glial cells also release "gliotransmitters" [10], such as glutamate and cannabinoids. These gliotransmitters are exactly like neurotransmitters, with the only difference being that they are released form glia. Glutamate, being the most common excitatory neurotransmitter within the central nervous system, is released from astrocytes upon excitation of pre-synaptic neurons. The glutamate released by the glia acts on the post-synaptic neuron, and results in an enhancement of long-term potentiation [11]. Likewise, astrocytes have been shown to have a critical role in potentiating Long-term Depression (LTD), albeit in a more complex way. LTD involves the long-term decrease in synaptic strength, and results from action potential induced influx of Ca2+ at the pre-synaptic terminal and co-incident opening of the pre-synaptic neuron's intracellular Ca2+ stores. These events rely primarily upon astrocytic release of glutamate [12]. Briefly, while high frequency glutamate release leads to post-synaptic action potential, glutamate release from the pre-synaptic terminal at low frequencies lead to the release of cannabinoids from the post-synaptic neuron. These cannabinoids then activate cannabinoid receptors on astrocytes, which in turn release glutamate into the synapse. The astrocyte-derived glutamate activates the metabotropic glutamate reception (mGLUR) channels on the pre-synaptic cell, resulting in Ca2+ release and efflux from intracellular stores [11]. The processes thus demonstrate the critical importance of astrocytes in LTD and, hence, in modulation of synaptic strength and plasticity following neuronal activity.

Glial cells are also found to indirectly and directly alter neuronal activities without input from nearby neurons. First, using human hippocampal slices, it has been shown that glial cells release glutamate onto kainite interneurons within the hippocampus [13, 14]. The interneurons have GABA-ergic projections on the principal cells, which cause mini-IPSPs in the hippocampal neurons [13]. Thus, glial cells indirectly effect activity of the principle cells through their effect on interneurons. Secondly, an important role of glial cells is exhibited in mediating heterosynaptic depression within the hippocampus of rats. This signalling starts with the release of Adenosine Triphosphate (ATP), which (after conversion into adenosine by Ectonucleotidases) acts on Adenosine 1 Receptors (A1R) and mediates heterosynaptic depression within the Schaffer collaterals [15]. Since A1R are G-protein coupled inhibitory receptors (Gi), they decrease the amount of cellular cyclic Adenosine Monophosphate (cAMP) and, hence, downstream processes within a cell, further leading to a further decrease in neuronal activity [16].

Role of SD in Altering Glial Modulation of Memory

Electroencephalography (EEG) studies have demonstrated a distinct brain activity in sleep states, particularly during Slow-Wave Sleep (SWS), as contrasted with awake states. Such a change in brain activity is also accompanied with changes in protein expression within the brain [17]. It has been found that, during both SWS and REM sleep, hundreds of genes are expressed within the brain, many of them in glia of mice [18]. Such increases in genetic transcription (compared to the awake state), coupled with the role of sleep in memory consolidation, point toward a critical role played by sleep in mediating memory consolidation through effects on glial cells. Following the same line of evidence, the role of SD in causing memory deficits is accompanied by a role of both acute and chronic SD in significantly changing the transcription of various genes in glia found in rat brains [19]. Hence, it is becoming increasingly evident that the effects of SD on memory processes are, at least to a certain extent, mediated by glial cells.

Mechanisms Involved in the Effect of SD on Glial Modulation of Memory

Glial Nitric Oxide production

Nitric Oxide (NO) has recently emerged as one of the unconventional neurotransmitters. It is unconventional in the sense that it is not stored in a cell upon production but rather, it diffuses out of the cell in order to affect functions in surrounding cells. Such NO release is reported to have an effect on various cellular processes, including memory formation within the hippocampus [20]. Given the abundance of glia within the hippocampus [21], it seems plausible that glial cells may be a major source of NO under processes of memory consolidation. Therefore, it also seems likely that the decrease in memory consolidation that is observed following SD arises from a reduction in the production and release of NO by glial cells. Indirect evidence of such an effect is observed through a study by Hsu and Lee which found a decrease in the amount of Nitric Oxide Synthase (NOS) within the rat hippocampus following SD [22].

It is also likely that SD may affect NO production in glia through the metabolic homeostatic responses to SD. Since SD is associated with increased adenosine within the basal forebrain, and the increased adenosine level following SD exerts its effects through both A1 and A2 receptors, it is possible that deprivation of NO production is mediated by activation of A2 receptor pathways [25]. Adenosine A2 receptors inhibit NO production in glial cells which explain the absence of iNOS induction in glial cells following sleep deprivation [26] [27]. However, a direct testing of such effects needs to be determined before the mechanism can be explained with certainty.

Glial Fibrillary Acidic Protein (GFAP)

Glial Fibrillary Acidic Protein is primarily expressed in astrocytes and aid in neuronal growth and survival. The study by Hsu, Lee [22] also found an enhanced expression of GFAP within the astrocytes of the rat hippocampus following SD, along with hypertrophied astrocytic processes. The hypertrophied processes are likely responsible for an increased coverage of synapses that these astrocytes surround. Since the degree of glycine occupancy sites on the N-methyl-D-Aspartate Receptors (NMDARs) is governed by the degree of neuronal astrocytic coverage, and thus the availability of neuronal activation and long-term synaptic changes [23], the hypertrophied astrocytic processes reflect another possible mechanism through which SD leads to deficits in memory consolidation. Additionally, increase in GFAP expression within glial cells following SD support findings of increase in astrocytic plasticity [24], thus further limiting available NMDAR sites for consolidation processes.

Adenosine

Among other important gliotransmitters released by astrocytes in response to SD, adenosine has recently received considerable attention. As mentioned previously, adenosine is one of the primary signalling molecules contributing to the increased homeostatic sleep drive within the basal forebrain (BF) [28] in response to prolonged wakefulness, via both A1- and A2 receptors. The role of these adenosine receptors in contributing to an increased sleep drive can be observed by the studies showing a significant attenuation of CNS responses to SD by pharmacological blockade in mice studies [29] or conditional knock-out in rat studies [28]. However, while cholinergic projections from the BF to the hippocampus (30) are inhibited as an indirect result of adenosine signalling [28], the direct effect of adenosine is also reported within the rat hippocampus.

One of the techniques frequently used to observe the effect of astrocyte-dependent adenosine release in response to SD within the hippocampus involves the use of transgenic dominant-negative domain of vesicular SNARE (dnSNARE) mouse model. The details of the technique are reported in Pascual et al. (2005) but are briefly presented here. The dnSNARE system targets vesicular exocytosis by expressing a dominant-negative cytosolic domain of synaptobrevin 2. Since synaptobrevin 2 normally mediates vesicular transport in exocytosis by mediating the formation of the SNARE complex between the plasma membrane and the vesicle, transgenic expression of dnSNARE leads to a competition of dnSNARE with endogenous synaptobrevin 2 [31], leading to a decrease in SNARE complex formation and hence vesicular fusion when dnSNARE expression is induced. The selectivity for dnSNARE's induction within astrocytes is achieved by using the "Tet-Off" system under the control of GFAP promoter [32].

A major contribution to understanding the role of Astrocyte-dependant adenosine release in effecting memory has come from Halassa and Colleagues [33]. Using the dnSNARE system, the group first demonstrated the importance of adenosine in modulating sleep pressure in dnSNARE-induced mice by measuring the time spend in compensatory sleep after a period of sleep deprivation, and by monitoring the slow wave activity (SWA) during Non-REM sleep using Electroencephalogram (EEG). Using these measures, it was reported that a decrease in astrocyte mediated adenosine release significantly reduces the build up of sleep pressure in sleep-deprived mice compared to control mice [33]. Further, the dnSNARE mice were also observed to be significantly superior to the control mice in a Novel Object Recognition (NOR) task following sleep deprivation [33]. Since NOR task is particularly sensitive to effects by sleep pressure [34], the group was able to demonstrate that sleep deprivation leads to a release of adenosine by astrocytes which play a role in decreasing efficiency in hippocampus-dependant memory tasks in rodents. Further, using the same model it has also been reported that adenosine released by astrocytes is responsible for a decrease in hippocampal synaptic plasticity following SD [35].

However, certain key issues remain to be unanswered before a sole role of astrocyte-derived adenosine in modulating synaptic plasticity and memory in response to SD can be determined. First, it has recently been discovered that the vesicular release is not only affected in astrocytes in the transgenic dnSNARE mice, but is also affected in neurons [36]. Since adenosine is also released from the BF projections within the hippocampus [37], this questions the possibility of whether the adenosine implicated in causing memory deficits following sleep loss may have been derived from BF neurons rather than from glia. Likewise, the study reporting reduced levels of adenosine following sleep deprivation in dnSNARE used direct biosensor-based measurements to report their findings [16], which does not necessarily reflect the actual adenosine amount in intact hippocampus in vivo. Earlier studies measuring adenosine's increase in hippocampus have found no rise following SD [38]. Future studies could solve this problem by using an in vivo microdialysis approach following SD in transgene dnSNARE mice compared to Wild Type mice.

Glial Metabolism

Na+-K+ ATPase pumps are responsible for establishing an electrochemical gradient across neuronal and glial membranes in order to maintain homeostasis for neuronal cell functioning. Under normal conditions, Na+-K+ ATPase function along with the Na+ coupled uptake of glutamate to trigger the uptake of glucose, which undergoes glycolytic processing within the astrocytes, results in the release of lactate [39]. With REM sleep deprivation, however, a decrease in glial Na+-K+ ATPase pump activity has been observed within the hippocampus of rats [40]. The decreased Na+-K+ pump activity within the glia leads to reduced glucose intake [41] and hence a reduced release of lactate from astrocytes [42]. As lactate is the primary source of glucose for neurons –¬¬according to the Astrocyte-Neuron Lactate Shuttle Hypothesis (ANLS)¬– lactate reduction in glial cells would cause a decrease in neuronal metabolism and functioning within the hippocampus following SD. While direct evidence needs to be collected, it is known that the glycogen stores in the CNS– located within astrocytes– are increasingly depleted with wakefulness [43]. Importantly, breakdown and utilization of these glycogen stores in order to supply lactate to the brain through the ANLS has been reported to be critical in the formation of Long-Term Memories within the hippocampus [44, 45]. Therefore, it is likely that the disruption of memory due to sleep deprivation is mediated by a reduction in glycogen contents within the astrocytes and a subsequent reduction in neuronal fuel.

The aforementioned glycogen depletion forms the basis of the Brain Energy Restoration Hypothesis. According to this hypoth-

esis, organisms need sleep in order to replenish glycogen stores within their bodies. Interestingly, a number of enzymes involved in glycogen metabolism are affected by neuroactive molecules involved in sleep regulation, as is found in mice studies [41]. One of these enzymes is Protein Targeting enzyme (PTG), a key enzyme in glycogen metabolism, which has been shown to be controlled by adenosine [46]. Therefore, an astrocyte-derived increase of adenosine [33] during prolonged wakefulness can potentially lead to an effect on the A2R receptors. Since the A2R is a G-protein coupled stimulatory pathway, and hence involves cAMP mediated signalling, it leads to an increase in transcription of PTG levels. Such an increase has indeed been demonstrated following sleep deprivation in mice and has been attributed to lead to metabolic conditions [47].

There are two possible ways of neuronal disruption due to adenosine release from the glial cells following SD. First, it can lead to an activation of A1R, which would lead to a reduced synaptic plasticity within the hippocampus [35]. Secondly, it can cause an activation of metabolic enzymes that alter the availability of energy within neurons [41, 47], leading to a decrease in neuronal function.

Notch

While adenosine's increase leads to an increase in cognitive deficiencies, expression of Notch receptors lead to factors that appear to reduce the negative consequences of sleep deprivation in Drosophlia. Notch receptor belong to a family of receptors that mediate interaction between adjacent cells and Notch is negatively regulated by its transcription factor, bunched [48]. Under normal conditions, sleep deprivation leads to an increase in the expression of bunched [49], which mediates cognitive deficits in flies via action on Mushroom Bodies (MB) [48]. However, expression of bunched protein's dominant negative allele within the MB also leads to a reduction in sleep homeostasis, demonstrating a role of bunched that is similar to that of adenosine in maintaining sleep homeostasis [48].

Since bunched is a negative regulator of Notch, it seems likely that bunched mediates the negative consequences of SD by reducing the levels of Notch. Using a Notch gain-of-function model, Seugnet, Suzuki [48] reported that Notch signalling is able to counter the negative consequences of SD, as measured using the Aversion Phototaxic suppression (APS) task. The APS task detects the presence of aversive conditioning in flies, as a measure of their ability to learn the relation between a neutral stimulus and an aversive stimulus. It was observed that Notch gain-of-function flies were protected from learning impairments following SD. Importantly, it was also discovered that these Notch receptors are only located on the glial cell membrane.

Interestingly, mice analogs of bunched, TSC22D3 and TSC22D, have also been reported in sleep deprived mice [50] and humans [48], respectively. Their presence indicates the likelihood of a phylogenetic conservation of the transcription factor, and hints at the possible presence of factors like Notch that may also be in effect within the human brain to counter sleep induced cognitive deficits but is affected by negatively regulating factors.

Potential mechanisms of glial cells' effect on memory following SD

Brain Fatty Acid Binding Protein (FABP7)

The large family of Fatty Acid Binding Proteins (FABP) consists of various forms of proteins including the epidermal type FABP, heart type and the Brain type FABP. The brain type FABP (or FABP7) plays a key role in the process of cell differentiation [51], and has been found to play an important role in the formation of fear memory [52]. Interestingly, FABP7 is astrocyte specific and as such may form a component of glial contribution to the formation of long-term memory within the hippocampus as well.

The indication of their importance in glial modulation of hippocampus dependant memory arises from a series of publications by Gerstner and Colleagues. Through a set of experiments, the lab has reported a diurnal regulation of the levels of fabp7 in the mouse hippocampus [53, 54]. Specifically, FABP7 has shown to be diurnally regulated within the astrocytes, with greatest levels of protein and mRNA reported during the dark phase of mice's daily cycle [54]. The release of adenosine¬, a molecule involved in mediating the cognitive consequences of sleep homeostasis, is also reported to be under the control of the circadian clock [55]. Thus, fABP7's circadian regulation within glial cells –in light of its effects on LTP and LTD- indicate a potential role of FABP7 in mediating the cognitive consequences of sleep loss by effecting the hippocampus. Moreover, the expression of FABP7 mRNA has shown to be regulated by Notch signalling in Dorsophila [56]. Therefore, with a SD induced decrease in the expression of bunched, a negative regulator of Notch, it appears likely that an increase in levels of FABP7 during the light phase will also be observed, corresponding to a decrease in negative effects of sleep loss.

SD effect on hippocampal cell proliferation

Both acute and chronic SD have commonly been implicated to stimulate neurogenesis within the hippocampus. However, since glial cells are also abundant within the hippocampus, it is also interesting to observe the effects of sleep deprivation on glial cells following SD. One such study by Roman, Van der Borght [57] used rotating drums to induce SD in rats by interfering with sleep for one night. Upon observing the hippocampus, the study demonstrated a decrease in cell proliferation within the hilus of the dentate gyrus with no decrease in number of differentiated neuronal cells. Given a consistent neuronal population following SD, and that a majority of cells within the hilus differentiate into astrocytes [58], it was suggested that SD causes a reduction in gliogenesis within the hippocampus. However, the study used the method of forced activity to induce SD in rodents, which can potentially lead to stress in these animals. Since the effects of stress hormones such as Corticosterone have been well document in reducing hippocampal volume in mice [59], the effect of SD can not be differentiated from the effects of the corticosterone based on the study. However, a similar reduction in hippocampal volume following SD has also been observed where the decrease could neither be attributed to a decrease in neurogenesis nor to the effect of stress [60]. The authors speculated the decreases, hence, to reflect a decrease in gliogenesis. There needs to be further testing in order to be completely accept this premise. Further, as glial cells are a source of release of essential molecules for e.g. the release of

D-serine to act as a co-agonist on NMDA receptors [23], it seems feasible that the reduction in gliogenesis is one of the factors contributing to a reduction in hippocampal activity following SD.

Limitations of Current Literature

While interest in the role of glial cells has begun to escalate since the turn of the millennia, research determining the role of SD in mediating glial processes is faced with several challenges. One of the most important questions that emerge based on the current knowledge is that of ecological validity. Since most of the studies reported in this review are either based on rodents or Dorsophila, it remains elusive whether glial cells within humans have similar roles in modulating memory processes following SD. Unfortunately, however, there is a lack of studies that replicate the known impacts of SD on human glial cells. A recent study by Cross, Lagopoulos [61] reflects an increased ratio of myo-inositol in the hippocampus of elderly individuals with sleep related disturbances. A hippocampal increase in myo-inositol, a glial marker of activity, can potentially reflect a connection between SD mediated glial expression of inositol and memory; however, the study being correlational in nature, does not allow us to determine whether the increased glial myo-inositol expression is a result of SD, or conversely, leads to sleep disturbances in the elderly.

Another important limitation found across the literature relates to the presence of very few methodologies employed by different studies accessing the role of SD. The limitations emerge due to both the techniques involved in depriving the animals from sleep, and because of the various durations of SD across the studies. The studies presented in this review have employed methods such as placing rodents on running disks to make them walk against it in order to avoid a forceful contact with water [22], gentle handling [16, 27, 33, 35, 62], forced activity by placing rodents on rods or levers [36, 57], or by the 'flower-pot' methods in which the rodent is placed on an platform made of inverted, small-diameter clay pots with water a little below the surface, such that once an animal starts losing muscle tone due to sleep they make contact with water which deprives them of sleep [63]. All the aforementioned techniques, although having been associated with a less potential of stress inducing effects of SD, may or may not selectively impair particular stages of sleep rather than total sleep. For example, the flower-pot method selectively impairs the REM sleep stages, while the disc-walking activity can impair either selective sleep stages or total sleep [64]. The differences in SD techniques make it harder to generalize the effect of SD on glial cells' release of specific memory reducing factors, as deprivation of the different stages of sleep may have different effects on expression of proteins in the glial cells.

In addition, the duration of SD also varied widely across the studies. Since the effect of shorter SD on glial cell's expression of various proteins can be very different from the effect of a longer duration of SD, such a difference in duration of SD adds to the difficulty of comparing the different studies with each other.

Conclusion and Future Directions

The current literature highlights the involvement of glial cells in mediating memory deficits that are observed in organisms following SD. Factors like adenosine that have previously been understood as sleep mediators may potentially be exclusive to glial cells, specifically astrocytes. Furthermore, an interaction between adenosine, NO and glycogen metabolism within the glial cells may also exist in ways that are yet to be discovered. The research field, as it stands, has many avenues to explore including different glial factors that are affected by SD, and importantly, the processes of SD that initially affect glia. Circadian clocks may be a part of the processes that mediate such effects of SD on glial cells, setting off the negative downstream negative consequences of sleep loss on memory. Additionally, it is also observed that the changes in gene expression observed between sleep and wakefulness emerge independent of the time of the day [17], which may indicate that the change in wakefulness can be independent of the circadian clock. However, direct evidence demonstrating whether the circadian clock affects SD remain undiscovered. Lastly, SD causes a disruption of biological rhythms within an organism [5], and is associated with medical pathologies such as diabetes, cardiovascular disorders, as well as psychological disturbances such as depression in humans [65]. Emergence of such physical and psychological disturbances following SD indicate the critical need of further elaboration of the effects of SD on glial cell processes.

Future studies should focus on standardizing the methodologies through which SD is achieved in rodents. The studies can benefit by first determining the specific sleep stage that needs to be disrupted in order to observe glial role in consolidating an associated memory process with that sleep state, followed by selecting a standardized method aimed at disrupting the respective sleep state. As a recommendation, the "flower-pot" method–being the more commonly used method for disrupting REM sleep [66]– can be adapted to all studies disrupting REM sleep in rodents. Once standardized methods such as the one recommended exist, studies that aim to determine the effect of a disruption of specific sleep stages on glial cells may become more comparable than they are at present, and will lead to a better appreciation of the importance of glial cells in memory processes following SD. Such findings can also further increase our insights into the possible mechanisms that may be disrupting memory processes in human of the modern-day society.

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JULS REVIEWS

A Clinical Review of the Treatment of Opioids Dependence by Methadone Maintenance Therapy, Naloxone and Buprenorphine

Sohrab Zand[1]

[1]Department of Human Biology, University of Toronto.

Corresponding Author: Sohrab Zand (sohrab.zand@mail.utoronto.ca)

Background

Opium derivatives, particularly heroin and morphine, have caused detriment to the lives of many in North America. Opioid drugs are the main source of drug-related dependencies in North America, causing high incidences of crime, child abuse and drug related mortalities [1]. On top of the economic instability that narcotic addiction brings, individuals suffering from opioid dependence have been reported to refrain from social interactions [1]. Additionally, a portion of individuals who suffer from opioid-related dependencies are pregnant mothers who give birth to babies with addictions to the opioids that the mother had abused [1-5]. The cost of opioid use is complemented by the high opioid demand observed in Canadians. Health Canada reported that 16.9% of Canadians use opioids in a 2012 study that examined a sample of 11090 Canadians older than 15 [6]. In addition, a study by Madadi *et al* revealed that opioids accounted for 58% of the all drug related deaths in Ontario in 2013 [7]. Deaths resulting from opioid overdose are also statistically higher than mortalities related to diseases, such as AIDS, which are highly funded across North America [7]. It is evident that individuals suffering from opioid addictions must be given optimal treatment in order to better themselves and the society they live in.

When an opioid enters the body it can affect four different G-protein coupled receptors (GPCR): μ-, δ-, κ-, and OPL-1 (opioid like-1) receptors [8]. In this review, we will focus on the effects of the μ-opioid receptor as it is the most effective analgesic and euphoric inducing receptor out of the four and thus, the most relevant in regards to opioid dependent patients [8]. Once an opioid is bound to an opioid receptor, the $G_{\alpha i}$ and the $G_{\beta\gamma}$ subunits of the GPCR dissociate which is followed by a release of beta-endorphins causing the feeling of euphoria [8]. The $G_{\alpha i}$ subunit reduces cellular signaling by inhibiting adenylate cyclase thus reducing cAMP, a secondary messenger in the GPCR pathway functioning to further amplifying a given signal in order to produce physiological responses associated with the receptor [8]. Further neural inhibitions result from the activation of G-protein gated inward rectifying potassium channels and the hyperpolarization of calcium channels, which inhibits spinal cord pain transmission [8]. μ-opioid receptors are commonly expressed in the central nervous system specifically in pain-modulating descending pathways [8]. Specific regions where the receptors are located include the medulla locus coeruleus, periaqueductal grey area as well as regions in the midbrain and limbic regions [8].

It is understood that the treatment of opioid dependency is composed of three stages: stabilization, which involves substituting the abused opioid with a safer and regulated one; detoxification, which involves the removal of the substitute; and maintenance, which pertains to the prevention of relapse [1, 9]. Common opioid substitutes are methadone and buprenorphine in conjunction with naloxone. Additionally, certain medications, like naloxone, involve resuscitation of an individual's life during a possibly fatal overdose. This review will examine methadone, naloxone and buprenorphine in conjunction with naloxone in a clinical aspect in order to identify their added benefits and costs to opioid-dependent patients.

Methadone Maintenance Therapy

Methadone is a μ-opioid receptor (MOR) agonist that, upon binding, produces various physiological responses specific to the MOR [2, 4, 11, 16]. Methadone can be prescribed in methadone maintenance therapy (MMT) to gradually reduce a patient's dependence on more harmful opioids such as heroine and morphine by acting as a substitute [2-4, 13-16]. In various studies, it has been reported that although methadone is an MOR agonist, it produces unnoticeable euphoria under the prescribed dosages moreover, one of the indications of an over prescribed dosage is the feeling of euphoria [2, 3, 12, 15]. This implication could have both positive and negative effects. Patients could have less incentive to abuse methadone under maintenance therapy as less reward is given to the patient per increase in dose. On the other hand, patients looking to obtain a high from methadone due to

the inability to obtain morphine or heroine could cause them to overdose on methadone. This topic is worth researching in order to gain more insight in the behavior of prescribed methadone users.

The effects of methadone vary between patients so contraindications must be considered before prescribing the medication. For instance, the metabolism of methadone in the body is dependent on the specific enzymes that target the drug. Genetic variation causes these enzymes to differ across patient cohorts, and cause inconsistencies in the metabolic rates [5, 12, 14, 15]. Methadone is metabolized in the liver; thus, patients suffering from liver disorders might expect inconsistencies in the metabolism of methadone relative to the expected metabolism [5, 11, 12, 14]. Furthermore, patients suffering from breathing disorders, such as asthma, should use caution when taking an opioid agonist, like methadone, as a treatment for opioid-dependence as it can cause respiratory depression in higher doses [5, 11, 12]. The respiratory depression is caused by the interaction between an opioid agonist and the MOR located on the pre-Bötzinger complex in the brain, a region responsible for rhythmic respiratory activity in our body. Opioid agonists inhibit the function of the pre-Bötzinger complex causing the cessation of breathing followed by respiratory depression, possibility leading to death if left untreated [17]. In high dosages, methadone can cause cardiac problems such as slight bradycardia and high blood pressure [3, 5, 10, 12]. While these concerns do not arise under the prescribed dosages [3, 5, 12], patients with long histories of heart problems should be regularly monitored under methadone maintenance therapy. Methadone also binds to various proteins in our body, ranging from plasma proteins to those found in organs and tissues, causing the level of methadone in the body to accumulate over time [12, 15]. Therefore, patients previously exposed to methadone should be carefully monitored due to an increased possibility of overmedication if methadone levels in the body have not declined in time [12, 15].

Methadone relieves withdrawal symptoms in patients who have a long history of frequent opioid use; the patient may prefer methadone treatment to partial MOR maintenance treatments such as buprenorphine maintenance treatments [2, 5, 16, 18, 19]. Partial MOR produce less effective MOR responses giving a less efficient means of easing withdrawal symptoms, which provides a less comfortable treatment [5, 12, 16, 18, 19]. Comfort in therapy is vital to the overall treatment process as it reduces the treatment's opportunity cost, which is the effect felt by the patient from refraining from using their desired opioid. However, it has been reported in various studies that individuals find it difficult to transition from MMT to a normal lifestyle [1, 3, 4, 10]. This effect comes from the dependence of methadone over long treatment duration. Often patients undergo MMT for many years; hence, it causes individuals to enter into an addiction cycle alternating between being addicted to their original opioid and their therapy opioid [1, 3, 10]. Studies have shown reduced intravenous use of opioids after MMT [1, 3, 5, 10]. As such, individuals who carry blood-vectored pathogens such as HIV should be advised to partake in MMT in order to reduce the potential infection rate of those pathogens [1, 3, 5, 10]. In cases were the patient is pregnant, MMT has been shown to be good choice in treatment for opioid dependency as it shows minimal effects towards the fetus provided that the dosage is carefully monitored [1-5]. Although there have been cases were infants born under MMT experience withdrawal symptoms, these cases should be examined further as multiple factors can contribute to the development of infant addiction. One of these factors is how early in the pregnancy MMT began, as previously abused opioids such as heroine and morphine could have taken a toll on the infants body. Furthermore, patients who are addicted to street opioids such as heroine or morphine have been known to continue to abuse those opioids while on MMT [1-5, 20]. These can all factor in to why an infant may suffer from withdrawal symptoms. While MMT is effective in controlled environments, there have been reports in several studies that show that when patients leave the controlled setting, they become susceptible to relapse [1, 3, 5, 10, 18, 19, 21-23]. Patients suffering from opioid addiction oftentimes feel depressed and in the worst circumstances suicidal; since this treatment eases the patient throughout their withdrawal, it may be best for those individuals.

Naloxone Treatment

Naloxone is a MOR antagonist that blocks the binding site of MOR [16, 24] upon entry via intravenous, intramuscular or intranasal route. Naloxone has a high affinity to the MOR causing it to outcompete frequently overdosed opioids such as heroine and morphine resulting in a reversal of a possible overdose [24]. Due to its short half-life, naloxone is quick acting and is often used in emergency situations [5, 10, 16, 17, 24]

Prior medical conditions can factor into the use of this medication. Although there have been incidents of heart arrhythmias and seizures, these cases have been reported to be rare and thus should not stop individuals from using naloxone [3, 5, 10, 24]. There is a commonly misidentified alternative to naloxone, called naltrexone. Despite the similarity in names, naloxone and naltrexone should not be used as substitutes for one another. While naltrexone is also an opioid antagonist of the MOR, it has a much longer half-life, and thus results in a longer acting effect [3, 16, 25]. During time sensitive emergencies, using naltrexone can negatively affect an individual's chance of survival. There is no indication of tolerance to either naloxone or naltrexone [5, 16, 25, 26].

Naloxone's biochemical pathway does not allow it to be particularly effective in the long-term treatment of opioid dependence. Individuals looking to reduce opioid dependence should look towards methadone maintenance therapy or Suboxone. However, almost every opioid user can benefit from naloxone, as it provides an emergency safety net in case of overdose. The environments of patients differ greatly; some patients may have individuals who support them during their recovery while other patients are surrounded by negative influences that encourage the use of opioids. Patients in the latter case should be advised to have naloxone on hand. Since the majority of opioid related deaths are seen in patients who acquire illegal opioids, these individuals may refrain from contacting EMS when a peer is overdosing due to fear of possible incarceration [24]. The review by Kim *et al.* referenced three studies that examined the behavior of illegal opioid users [24, 27-29]. The results showed that individuals in the referenced studies contacted EMS in emergency overdose situations "only 10-56% and only as a last resort" [Kim *et al.*, 403] [16, 24, 27-29]. Furthermore, in an attempt to save their peers life, individuals used intravenous injection of salt, improper CPR, cold baths and other improper or inefficient techniques in order to avoid police confrontation [16, 24]. By promoting naloxone to individuals

who are using illegal opioids, the overdose mortality rate can be lowered. The review by Kim *et al.* also found that most users of illegal opioids indicated that they wished to partake in a training program designed to train users for naloxone, and the majority stated that they preferred their peers to administer the drug to them [24]. The preference to have naloxone is apparent in the patients who have the highest susceptibilities to have overdose mortalities and as such, naloxone should be advised to individuals who use illegally obtained opioids. This recommendation is not meant to protect the identity of illegal opioid users as much as it is meant to preserve the life of overdosed individuals whose situations are time sensitive. Kim *et al.* mentions an argument in opposition to advertising naloxone to street opioid users [24], and it stems from the idea that naloxone can provide illegal opioid users more reason to abuse high amounts of opioids as they can just use naloxone as a preventive measure [24]. A counterargument to this is that naloxone gives less incentive to opioid users to abuse opioids as the medication provided undesirable withdrawal symptoms [5, 24].

Although naloxone has a low tolerance rate, a concern regarding naloxone includes the fact that it reduces tolerance to opioid agonists; therefore individuals may be subjected to overdose by taking their usual opioid (agonist) dosage again [3, 16, 24]. Thus, patients who are prescribed opioids, or use them illegally, should be advised to use naloxone only in cases of dosage emergencies. However, if the patient is subjected to an overdose due to a decrease in tolerance to their normal opioid, the patient could take another dose of naloxone to prevent another overdose [3, 5, 16, 24]. Similar to illegal opioid users, naloxone will give prescribed users less incentive to use opioids due to the withdrawal symptoms it produces. Nevertheless, because prescribed individuals have little to fear from police, they may be more inclined to contact EMS under emergency situations. Coupled with safety training individuals could learn how to administer naloxone safely and effectively reducing any inherent risks [5, 24]. As a result, naloxone is a strongly recommended medication with the only marginal drawback being a withdrawal response that it produces upon entry to the patients system, lasting only a few hours [5, 16, 24, 26].

Buprenorphine in Conjunction with Naloxone

Buprenorphine in conjunction with naloxone is offered in a 4:1 ratio dose, known as Suboxone [18, 30]. In Canada, buprenorphine is prescribed alone in rare circumstances and is most often available in conjunction with naloxone [18]. The purpose of combining buprenorphine with naloxone is to discourage buprenorphine abuse, as naloxone produces unwanted withdrawal effects in patients [8, 19, 30, 31]. Buprenorphine is a partial MOR agonist, showing a similarly strong affinity for the MOR as methadone; however, buprenorphine does not produce as efficient a response [18, 19, 21, 22, 30-34].

The biochemical interactions of buprenorphine causes a ceiling effect in which, after a certain dosage, the activity of the MOR with buprenorphine is independent of the dosage [20, 21]. One such effect that becomes dose independent is respiratory depression [32, 33]. Despite the plateau of respiratory depression, individuals who suffer from respiratory related diseases should be carefully monitored under Suboxone, as the ceiling effects occur beyond the recommended dosages [32, 33]. This diligence comes from the fact that buprenorphine can inhibit the pre-Bötzinger complex leading to respiratory depression [17, 19]. As the liver metabolizes buprenorphine, individuals subjected to liver disorders should be carefully limited to their prescribed dose [18, 19, 34]. Since Suboxone contains a partial opioid agonist, it can be subjected to similar variances in metabolism as methadone. No papers mentioned in this review directly compared the differences between the metabolic rate in patients under methadone and buprenorphine; however, one can conjecture that due to buprenorphine being a partial opioid, it would be subjected to a similar variability in patient's metabolism. More research should be conducted to determine whether genetic variation in metabolic enzymes significantly affects individual's metabolism to Suboxone, and whether this difference should be considered when prescribing dosages.

Buprenorphine is inefficient at activating the MOR therefore; patients who are high-frequency opioid users would experience withdrawal symptoms, as buprenorphine cannot reduce cravings as well as commonly abused opioids such as heroine [18, 19]. Based on the aforementioned information, patients may prefer methadone to buprenorphine as methadone produces a more efficient MOR response, reducing their cravings [18, 19]. In addition, because naloxone causes unwanted withdrawal symptoms, patients may be discouraged from continuing the Suboxone treatment [19, 21, 31, 34]. In fact, naloxone effects are strong enough that patients often cannot tell the difference between the combined buprenorphine-naloxone mix and naloxone alone [18, 19, 21, 23, 34].

Despite MMT providing less withdrawal symptoms and therefore, less marginal costs, it is not universally accepted that patients remain in MMT significantly more than Suboxone. Three studies were performed to compare the differences in the proportion of patients who continued with MMT and Suboxone therapy. The Neumann study and the Kamien study showed no differences between MMT and Suboxone; yet, the Saxon study showed a significantly smaller proportion of patients continued with Suboxone treatment compared to methadone (46% and 74% respectively) [19]. The statistics behind the results, however, could be due to sampling error as well as non-sampling error due to a very small sample size and possibilities of exogenous variables [19]. Because of the presence of naloxone, Suboxone is subjected to less abuse in comparison with other treatments such as methadone [18, 19, 21, 30, 31, 34]. To expand the data on this point, a possible research experiment could be conducted in this area to examine the effects of patients subjected to MMT overtime by gradually transitioning to Suboxone therapy in order to minimize the its withdrawal effects as well as the chance for methadone misuse. The efficiency of MMT in comparison to Suboxone is varied and is therefore inconclusive in providing any evidence for either medication being more effective in reducing opioid use [1, 9, 18, 19, 21-23, 30, 31]. Despite this, patients who are prescribed Suboxone produced higher cognitive abilities in comparison to MMT when measuring visual and verbal memory [19]. It is worth noting that the review done by Canadian Agency for Drugs and Technologies found that Suboxone users showed improvements in social life as well as continuation of education [19]. Nevertheless, the result was not expanded upon and an explanation for this behavior was not concluded [19]. Similar to MMT, individuals leaving therapy in controlled environments are at risk to high relapse rates. Like MMT, it is also due to a variety of factors ranging from stress to

reintegration of negative influences [18, 19, 21-23, 30, 31].

Conclusion

Each of the treatments examined in this paper provides their own benefits and drawbacks. MMT provides patients with a natural substitute for the opioid they are addicted to, but presents the possibility for abuse. Suboxone combined with naloxone reduces the rate of abuse although the patient is subjected to withdrawal effects. Nevertheless, there was not a significant difference in retention to treatment in Suboxone compared to MMT. Yet, one has to question the accuracy of the studies in case they were subjected to sampling error due to a small sample size, or whether they were subjected to non-sampling error due to systematic differences between the sample and population, biased estimates, lower response rates, or systematic lying. These statistical issues must be resolved in future studies so as to develop a more accurate analysis of both MMT and Suboxone in order to identify an optimal treatment. Naloxone cannot be directly compared to either MMT or Suboxone as the fundamental purpose of the medication differs from the former two as treatments. Based on the studies observes, naloxone appears to have no substantial consequences but provide marginally higher benefits for both the illegal and legal opioid users; therefore, naloxone should be strongly recommended during the course of any treatment plan.

MMT and Suboxone have high relapse rates upon exit in treatment in controlled environments. High relapse rates can be due to the strong external factors that persuade individuals to continue abusing harmful opioids. An approach that deals with these underlying exogenous factors should be derived to better analyze their effects on relapse. A possible solution that is not mentioned in any of the studies examined in this review is introduction of an appropriate opioid mimicking substance in a regulated market similar to alcohol and tobacco. This hypothetical drug would have to give users incentive to use the drug by producing a moderate euphoric response, while possessing a type of ceiling response similar to buprenorphine. Understandably, this will be faced with political, ethical, economical, and medical constraints. In addition, many biochemical, physiological, and pharmaceutical factors have to be carefully considered, which is beyond the scope of this review. However, it can shift the demand from harmful and volatile opioids and other narcotics to a drug that is both carefully regulated and monitored. It might be time to influence the environmental factors in a profound way rather than continuing with the approach that is still producing disappointing results in relapse rates in patients.

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JULS REVIEWS

The epidemiology of *Clostridium Difficile* infections in kidney transplant recipients: an analysis of incidence, risk factors, management, prevention, prognostic determinants and outcomes

Jia Qi Li[1]

[1]Faculty of Arts and Science, University of Toronto

Corresponding author: Jia Qi Li (lig@uhnresearch.ca)

Abstract

Kidney transplantation is the ideal treatment for end-stage renal disease. However, post-operative maintenance required to preserve the transplanted organ includes a lifetime of immunosuppression as well as frequent antibiotics, leaving kidney transplant recipients (KTRs) particularly vulnerable to infections. Clostridium difficile, a gram-positive bacterium, is one opportunistic infective agent that affects KTRs. C. difficile infections (CDIs) occur in the gut, where disruptions in the natural microbiome often caused by wide-spectrum antibiotics allow the species to expand and produce toxins, leading to significant diarrhea and colitis, and in some cases even toxic megacolon or death. Literature in the area of CDIs in the KTR population is sparse and plagued by inconsistencies and inadequacies in the diagnostic methods used. Due to this, the reported incidence rates vary between lower than 1% to as high as 8%. Common risk factors found included bacterial colonization, blood and human leukocyte antigen (HLA) incompatible transplants, antibiotic usage, and certain immunosuppressive medications such as anti-thymocyte globulins and mycophenolate mofetil. Management usually begins with withdrawal of any potentially causative antibiotics, and subsequent replacement with targeted antibiotics such as metronidazole and vancomycin – this approach was found to be effective in the KTR population, however prophylaxis was suggested as an area that needed more research. Relationships between CDIs and graft outcome have been poorly studied, with no reports CDIs leading to higher numbers of graft failure or rejection – this may suggest, at least in the short term, that CDI has little impact on graft outcome, however longer term studies are needed.

Introduction

Kidney transplantation is most often the desirable treatment for end-stage renal disease. It offers advantages such as improved quality of life and lower rates of mortality compared to other renal replacement therapies[1]. Despite these advantages, the postoperative maintenance required to maintain allograft function renders the kidney recipient population vulnerable to post-transplant infections. In particular, immunosuppression and antibiotic usage, both of which are ubiquitous in the kidney transplant population, are established risk factors for one possible infectious agent, *Clostridium difficile* [2,3]. Within the current literature, there is immense variability in the incidence and risk factors of *C. difficile* infections in kidney transplant recipients. The methods used to diagnose *C. difficile* vary, with the majority of studies using diagnostic methods that are considered suboptimal, especially within the context of epidemiological studies [4]. Although kidney transplant recipients appear to be at high risk, the epidemiology of *C. difficile* infections in this population has not been characterized well in the current literature.

Clostridium difficile

C. difficile is a Gram-positive bacterium that colonizes the human gastrointestinal tract [3]. It is transmitted via the fecal-oral route, and is able to form spores that are capable of resisting harsher conditions than the bacterium itself. This makes it particularly amenable to transmission in a hospital setting [3]. Symptoms of the infection only occur if patients are colonized with sufficient amounts of toxicogenic strains of *C. difficile* which produce toxin A or B (tcdA/B) [3]. Even then, patients colonized with these strains may never become symptomatic as, under normal circumstances, any *C. difficile* proliferation is limited by competition with other microorganisms of the gut microbiota [3]. However, pharmaceuticals such as broad-spectrum antibiotics often disrupt this balance, facilitating the colonization and expansion of *C. difficile* into

pathogenic colonies, which may have resistance to these drugs [4]. Symptoms traditionally include frequent diarrhea and potential colitis, though in severe cases, infections may lead to toxic megacolon and even death [5].

The incidence of *C. difficile* infections (CDI) in the general population has risen significantly over recent years. Cohen et al. revealed in 2010 that CDI was the chief cause of infectious diarrhea in hospitals [4]. Moreover, Reveles et al. reported an increase in the incidence of CDI from 4.5 discharges/1000 discharges (0.45%) in 2001 to 8.2 discharges/1000 (0.82%) discharges in 2010 [6]. CDI creates large economic burdens on hospitals, with an estimated total incurred cost in the United States of over 3 billion dollars per year in 2012 [7].

Clostridium difficile Infections Post-Kidney Transplant

Kidney transplantation involves the transfer of a donor kidney into a recipient to replace the function of the recipient's dysfunctional native kidneys. Due to natural variations in tissues and immune systems between individuals, immunosuppressive medications are required during and post-transplant to prevent immune rejection of the donor organ [8-11]. Broad-spectrum antibiotics are also often given to kidney transplant recipients pre- and post-transplant prophylactically to minimize the chances of infection due to the operation [8]. In addition, kidney transplant recipients often have increased exposure to the hospital environment due to the need for other renal replacement therapies prior to transplantation. Patients requiring hemodialysis often visit the hospital frequently, greatly increasing their chances of contracting nosocomial infections such as CDI. Immunosuppression, administration of broad-spectrum antibiotics, and increased hospital exposure are all established risk factors of CDI [2,3,12] – this makes kidney transplant recipients a particularly at-risk population for CDI.

Literature Search Methodology

In recognition of its increasing prevalence and potential risks, a literature search on *Clostridium difficile* infections in kidney transplant recipients was conducted using Ovid MEDLINE® with results from 1946 to September week 5 2015. Keywords used included "kidney transplantation", "clostridium", "*Clostridium difficile*" and "clostridium infections". The initial search resulted in a total of 46 articles. Two articles were duplicates, and review of titles and abstracts resulted in a final total of 16 relevant articles. Articles on other bacteria in the Clostridium genus were excluded. Articles focusing on multi-organ transplants were kept due to the lack of literature. Out of these articles, 11 were case reports and 7 were retrospective chart reviews.

Incidence and Detection

The reported incidence of CDI in kidney transplant recipients is not well-documented, and varies greatly in the current literature. The reported incidence rates range from lower than 1% to as high as 8% [5,8,13]. Nevertheless, this data shows that the incidence of CDI within the kidney transplant population is indeed higher than the reported incidence of CDI in the general hospitalized population [6]. A single center retrospective study by Neofytos et al. in 2013 reported a mean incidence rate of 6.1% in a total of 603 kidney transplant recipients, where the CDI diagnosis was included if it occurred from 7 days pre-transplant to 6 months post-transplant [8]. The median time of diagnosis was 9 days post-transplant, with 51.8% being diagnosed within a week and 73% within a month [8]. Lionaki et al. reported a similar incidence rate of 5.4%, but with a much longer mean time from transplant to CDI diagnosis of 84.05 ± 63.9 months [26]. In contrast, Altiparmak et al. in 2002 reported that out of 308 kidney transplant recipients, only 2 were diagnosed with CDI (0.6%) [5]. The increase of CDI prevalence over time does not appear to account for this variation, as West et al. had previously reported a 3.5% CDI incidence rate in their adult kidney transplant population in 1999 [14]. Instead, this large variation in incidence may be attributable to the methods used by these studies to diagnose CDI.

A variety of diagnostic techniques are available for the detection of CDI. The three most commonly used techniques are enzyme immunoassays (EIA), real-time polymerase chain reaction (PCR) and bacterial stool culture for *C. difficile* [15]. The EIA technique, which can detect tcdA and tcdB, is most commonly used as it is the fastest and most cost-effective method [15]. However, EIA is considered the least sensitive method, with reported sensitivity rates falling between 32% and 73% [10]. PCR and stool culture directly detect the presence of the *C. difficile* bacteria itself, making them more specific and sensitive [15]. As such, EIA results are not recommended for usage in epidemiological studies according to guidelines established in 2010 by the Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA) [4]. Instead, it is suggested that epidemiological studies use stool culture for diagnosis [4]. Monteiro et al. in [2014] found that there was a high level of discrepancy between all three methods of detection for CDI, but that PCR was the most sensitive test, and that PCR and stool culture were both more sensitive than EIA [15]. However, the overwhelming majority of studies in the current literature used the EIA toxin test to diagnose for CDI, with the exception of one study that used a combination of EIA and stool cultures, and another that switched to PCR midway through, and saw an increase in CDI incidence after the switch [8]. Therefore, the method of CDI detection used is a major limitation that potentially rejects the validity of the majority of currently published studies. Usage of the more sensitive PCR and stool culture techniques should be considered in further epidemiological studies.

Risk Factors

While risk factors for CDI in the general population are well documented, there have been very few studies published in the context of kidney transplantations, with risk factors varying greatly. The study conducted by Neofytos et al. analyzed risk factors for CDI by comparing kidney transplant recipients that developed CDI to ones that did not. They found that the major predictors of CDI were vancomycin-resistant enterococci colonization (OR 3.6, P=0.03), high-risk transplants (OR 5.9, P=0.006) and the use of high-risk antibiotics (OR 6.6, P=0.001) [8]. High-risk transplants were defined as being blood group and human leukocyte antigen (HLA) incompatible, and high-risk antibiotics included broad-spectrum antibiotics such as clindamycin, fluoroquinolones, carbapenems, antipseudomonal penicillins, and third- and fourth-generation cephalosporins [8]. A study by Keven et al. also found that 88.6% of their kidney transplant patients with CDI had a history of antibiotic usage in the month prior to their

diagnosis [13]. However, no matched control group was presented in this study, and only univariate analysis was used, meaning potential confounding variables were not addressed. The usage of anti-thymocyte globulin for induction of immunosuppression was also reported as a significant risk factor (P=0.02), however only univariable analysis was used [8]. Immunosuppressive agents such as tacrolimus and mycophenolate mofetil (MMF) have also been implicated as possible risk factors through case reports [9,10], however no studies were available to support this. On the contrary, a study conducted by Apaydin et al. found that antibiotic therapy and immunosuppression were not significant risk factors for the presence of tcdA (P>0.05) [11]. However, these opposing results may again be due to the methods of diagnosis used in each study. As the study by Apaydin et al. exclusively used EIA to test for tcdA, which not all strains of *C. difficile* produce, there may have been false-negatives due to both the sub-optimal sensitivity of EIA, and lack of tcdA production by certain strains of *C. difficile*. One study which used PCR was conducted by Shah et al. in 2013, where they found that being male (P=0.003), receiving a deceased donor transplant (P=0.045), having a gastrointestinal procedure 3 months prior to CDI (0.038) and being more likely to have leukopenia (P=0.038) put patients at higher risk for CDI [25]. However, the number of CDI events in this study was very low (n=28), limiting its applicability to the wider population.

One possible risk factor that is not well documented in the literature is the asymptomatic colonization of the patients pre-transplant. There is no pre-transplant screening for asymptomatic *C. difficile*, as it is not considered clinically relevant [4]. All cohort studies found were retrospective clinical chart reviews, so data on asymptomatic colonization could not be collected for epidemiological purposes. However, the current literature suggests that asymptomatic colonization with *C. difficile* may be a significant risk factor for the development of CDI [8]. Therefore, further epidemiological studies should screen for asymptomatic carriers of *C. difficile* pre-transplant and identify any possible related risk factors.

The majority of the conducted literature search consisted of case reports, a large number of which reported the incidence of a CDI with a co-morbidity. These co-morbidities included hemolytic uremic syndrome [16], cytomegalovirus [17,18], and toxoplasmosis [19]. This suggests that these morbidities may be associated with CDI, though the precise causation and statistical significance is unclear.

Management and Prevention

The management of CDI in the general population has been well-characterized in the literature. As set out by the SHEA and IDSA, the standard of care involves withdrawal of the causative antibiotic regimens and subsequent replacement with targeted antibiotics such as metronidazole and vancomycin [4]. This approach appears to be effective within the context of kidney transplant recipients as well. Patients in studies by Neofytos et al. and Keven et al. were all treated with combinations of metronidazole and vancomycin [8,13]. In the former study, all patients were still alive 6 months after their CDI diagnosis, and 10.8% had a recurrent CDI infection [8]. The latter study reported that one kidney transplant recipient died from recurrent CDI due to inanition, while two others developed fulminant colitis, requiring colectomy. Within these two patients, one survived, while the other died post-colectomy [13]. It is generally recommended that CDI should be detected early and treated aggressively to prevent development of complications [4,13]. Recently, fidaxomicin, the first in a new class of antibiotic drugs which has been shown to be effective at targeting CDIs, has also been used in kidney transplant recipients [24]. However, literature on this is limited to case reports from Poland that are not available in English. Keven et al. suggested that metronidazole should be administered prophylactically post-transplant in order to reduce the incidence of CDI. Indeed, after implementing prophylaxis with metronidazole in their transplant population, preliminary evidence showed a significant reduction in the incidence of CDI (1.1% versus 5.5%, P=0.009) [13]. However, it was noted that further studies should be conducted in this area to investigate the efficacy of prophylaxis. No studies looking at preventative strategies against CDI in solid organ transplants have been published currently. Generally, studies have shown that hospitals which practice infection control strategies such as early detection, isolation and enhanced cleaning have reduced CDI rates by as much as 20% [27]. However, community health care settings such as nursing homes often do not participate in such strategies, increasing the risk that patients contract the infection in the community. Various vaccination methods are also being explored, in addition to the administration of probiotics [28]. However, the efficacy and safety of these methods for the transplant population is questionable due to their highly immunocompromised state.

Increasingly, fecal microbiota transplantation (FMT) is being used as an alternative therapy for CDI, in particular to prevent recurrent CDI [12]. The procedure involves the transfer of gut flora isolated from the stool of a healthy donor to the affected patient gut, which can provide a source of healthy gut flora that restores non-pathogenic levels of *C. difficile* [20]. Preliminary evidence from van Nood et al. suggests that using FMT as a therapy for recurrent CDI has enhanced efficacy over the use of vancomycin alone (P<0.001) [21]; however, this procedure is a relatively recent innovation, and its effects on kidney transplant recipients with CDI have yet to be studied.

Prognostic Determinants and Outcomes

The prognostic determinants of CDI in kidney transplant patients have yet to be fully elucidated. However, within the broader context of solid organ transplantation, a 2014 retrospective cohort study looking at 170 patients reported possible prognostic determinants. Recurrent CDI (OR 0.21, P=0.0128), treatment with vancomycin (OR 0.27, P=0.011), vasopressor support (OR 0.23, P=0.0161) and treatment before 2004 (OR 0.44, P=0.0446) were all predictors of clinical cures not being achieved after 2 weeks [22]. The finding that suggests vancomycin may be related to longer treatment times is problematic, as vancomycin is one of the drugs recommended for CDI therapy [4]. However, since the study was not a randomized trial, vancomycin might have been given preferentially to patients who were originally diagnosed with more severe cases of CDI. Another study of 1446 general inpatients reported that old age (OR 1.1, P=0.029), high leukocyte count (OR 2.2, P<0.0001), increased serum creatinine (OR 1.6, P<0.0001), gastric acid suppression (OR 1.8, P=0.0002) and narcotic usage (OR 2.1, P<0.0001) were all prognostic determinants for severe-complicated CDI, resulting in ICU admission, surgical intervention or death within 30 days [23]. Further studies to determine the prognostic determinants of CDI specifically within the kidney transplant pop-

ulation should be conducted in order to optimize care for affected patients. Increased serum creatinine as a prognostic determinant of CDI in this population is a potential area of increased interest, as it is commonly used as a measure of renal health.

The effects of CDI on graft function have also been poorly studied. There were no reports of graft failure or rejection correlating with CDI in the current literature. This appears to suggest, at least in the short-term, that a CDI has little impact on the outcome of the kidney graft. However, no studies have been published on the long-term effects of CDI on graft outcomes. In recent studies, death due to CDI in kidney transplant recipients has also been extremely low, with a less than 1% mortality rate [5,8]. Again, within the broader context of solid organ transplantation, a study by Hsu et al. found that in their cohort of 170 patients, 13 patients (8%) died during hospitalization and 49 patients (29%) died within 1 year of their CDI diagnosis [22]. Although none of these deaths were directly attributed to a CDI [22], this suggests that CDI could be further explored as a potential marker for other co-morbidities that could negatively affect graft outcomes and patient survival. Future studies should investigate this, as well as any possible correlation between CDI, recurrent CDI and renal health in the long term.

Conclusion

Although kidney transplant recipients appear to be at a high risk for *Clostridium difficile* infections, its epidemiology in this population has not been well-characterized in the current literature. All studies reporting the incidences of CDI in kidney transplant recipients consistently use methods of CDI diagnosis that are known to be insensitive and yield inaccurate epidemiological data. Risk factors, prognostic determinants and outcomes in the context of kidney transplantation have also been poorly described. All studies found were single-center retrospective cohort studies and case reports, with inherent limitations in patient population and availability of data. There was also an absence of studies on preventative measures, possibly due to the lack of conclusive risk factor data in the kidney transplant population. However, prevention of CDI in the transplant population presents a challenge, as they often require antibiotics to prevent or treat other infections due to their immunosuppressed state. This, in turn, paves the way for opportunistic infections such as CDI. Therefore, studies looking at prevention should ideally look to reduce other transplant-related risk factors. An optimal retrospective study to identify such factors would include a large cohort along with comprehensive and accurate clinical data on patients. One database that may allow for this is the Comprehensive Renal Transplant Research and Information System (CoReTRIS), which allows for the curation of many transplant related clinical features [27]. With its large number of data domains, CoReTRIS could be used to analyze relationships between CDI and a host of other transplant-related variables that have not been well studied. This system has the potential to eliminate many of the inconsistencies and confounding variables in published datasets and provides a relatively large cohort for clinical investigation. Future studies should make use of this resource to better characterize the epidemiology of *Clostridium difficile* infections after transplantation. Further studies should also aim to use accurate and sensitive diagnostic methods such as PCR or stool culture. This may facilitate better management of the infection, resulting in decreased costs overall and better patient outcomes.

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JULS REVIEWS

The Enigmatic Relationship of Bone Mineral Density and Cardiovascular Disease in Kidney Transplantation

Shanna Gu[1,2], Martha Ghebreselassie[1,2], Olusegun Famure[2,3], S. Joseph Kim[3,4]

[1]Faculty of Arts and Science, University of Toronto
[2]Multi-Organ Transplant Student Research and Training Program, University Health Network
[3]Kidney Transplant Program, University Health Network
[4]Faculty of Medicine, University of Toronto

Corresponding Author: Shanna Gu (shansan.gu@uhnresearch.ca)

Abstract

Currently, the main cause of transplant graft failure is chronic complications, among which cardiovascular disease (CVD) and low bone mineral density (BMD) are frequent culprits. Therefore, understanding CVD and BMD is critical to the improvement of overall graft survival. In order to properly ascertain this, five distinct literature searches were conducted in three databases, Ovid MEDLINE(R), Embase and Google Scholar. After reviewing current and relevant study articles, the relationship between CVD and BMD within kidney transplantation was determined to be inconclusive. Most of the studies neglected important potential confounders, presented contradicting results, and used small samples unrepresentative of the transplant recipient population. In addition, when some studies adjusted for risk factors of BMD and/or CVD, no significant relationship was produced. Lastly, rarely any study produced an association between low BMD and CVD; rather, most studies suggested that low BMD correlated to increased vascular calcification. This novel yet ambiguous area of research requires clarification which could be provided by a future retrospective study conducted at the Multi-Organ Transplant Student Research Training Program at Toronto General Hospital.

1. Introduction

Overview

With high long-term survival and improved quality of life, kidney transplantation is now recognized as the treatment of choice for patients with end-stage renal disease [1]. Over the last two decades, due to advances in immunosuppressive therapy, the main cause of graft failure has shifted from acute rejection to chronic complications, such as cardiovascular and bone diseases, potentially that often lead to the death of the recipient [2,3]. Therefore, examining the causes and associations of post-transplant complications is important in improving overall graft survival. Both cardiovascular disease (CVD) and low bone mineral density (BMD) are amongst the most prevalent postoperative complications [2,3]. However, the direct relationship between CVD and BMD in adult kidney transplant recipients remains undefined; even the causality between the two has yet to be ascertained - thus posing a novel area for research [4, 5]. The objective of this review is to enhance the understanding of CVD and BMD within kidney transplantation by discussing current findings on possible associations of all three or any two of the three factors. Illuminating this potential relationship could lead to changes in the procedure of pre-transplant cardiovascular and BMD testing, the medical eligibility to be placed on the transplant waiting list and improve long-term clinical outcomes.

Defining Low Bone Mineral Density

BMD is the measurement of the amount of minerals such as calcium, phosphorous, magnesium, sodium, bicarbonate in a certain volume of bone [6]. As a marker for increased fracture risk and osteoporosis, low BMD after kidney transplant can result from pre-existing renal osteodystrophy, increased parathyroid hormones, immunosuppressive therapy, and the effects of chronically-reduced renal function prior to transplant [4,5]. According to the World Health Organization, osteopenia (low BMD) is defined by a BMD more than 1 but less than 2.5 standard deviations below the young adult female reference mean, and osteoporosis is diagnosed by a BMD of more than 2.5 standard deviations below the mean6. The WHO measurements are taken by dual-energy x-ray absorptiometry (DXA), which is the common method to measure BMD because it allows for measurements of the lumbar spine and the femoral neck4. However, other means to define BMD, such as broadband ultrasound attenuation that measures the calcaneus, were also used in recent studies [7].

Defining Cardiovascular Disease

One great challenge of assessing an independent correlation between BMD and CVD within kidney transplant recipients is the vast pool of shared risk factors. Among numerous potential confounders, age, sex, body mass index, lifestyle, systolic blood

pressure, estrogen level, diabetes mellitus and hypertension are the most significant ones affecting all three variables [4, 5, 7, 9, 10, 11, 17, 21, 22].

CVD, when combined with chronic kidney disease, is frequently associated with increased vascular stiffness and calcification (7). There seems to be an inverse relationship between arterial calcification and BMD7, but no exclusive association between CVD and BMD. Without a standard definition for major adverse cardiac event (MACE), individual studies vary in their definitions of CVD8. The general spectrum of definitions for MACE in the literature includes heart failure, stroke, acute coronary syndrome, and malignant dysrhythmia. The broad range of CVD presents an obstacle in determining its role within the triangular relation BMD, CVD, and kidney transplant.

Literature Search Methodology Flowchart

Literature Search Methodology

Search Methodology

A total of five databases were searched to properly review the literature regarding the relationship or relationships between kidney transplantation, BMD and CVD. Two searches via Ovid MEDLINE(R) <1946 to October Week 1 2015> & Embase <1974 to 2015 October 16> focused on a link between BMD and CVD in kidney transplantation. The exclusion criteria was lack of the terms "bone mineral density"/"bone metabolism" and "kidney/renal transplantation" and "vascular/aortic calcification" /"cardiovascular disease/events" in the title and abstract. 5 articles were included in the review.

Embase <1974 to 2015 October 16>, focused on the relationship between BMD and CVD. Due to the high yield, only the first 100 articles were screened. The exclusion criteria was lack of the terms "bone mineral density"/"bone metabolism" and "vascular/aortic cal-

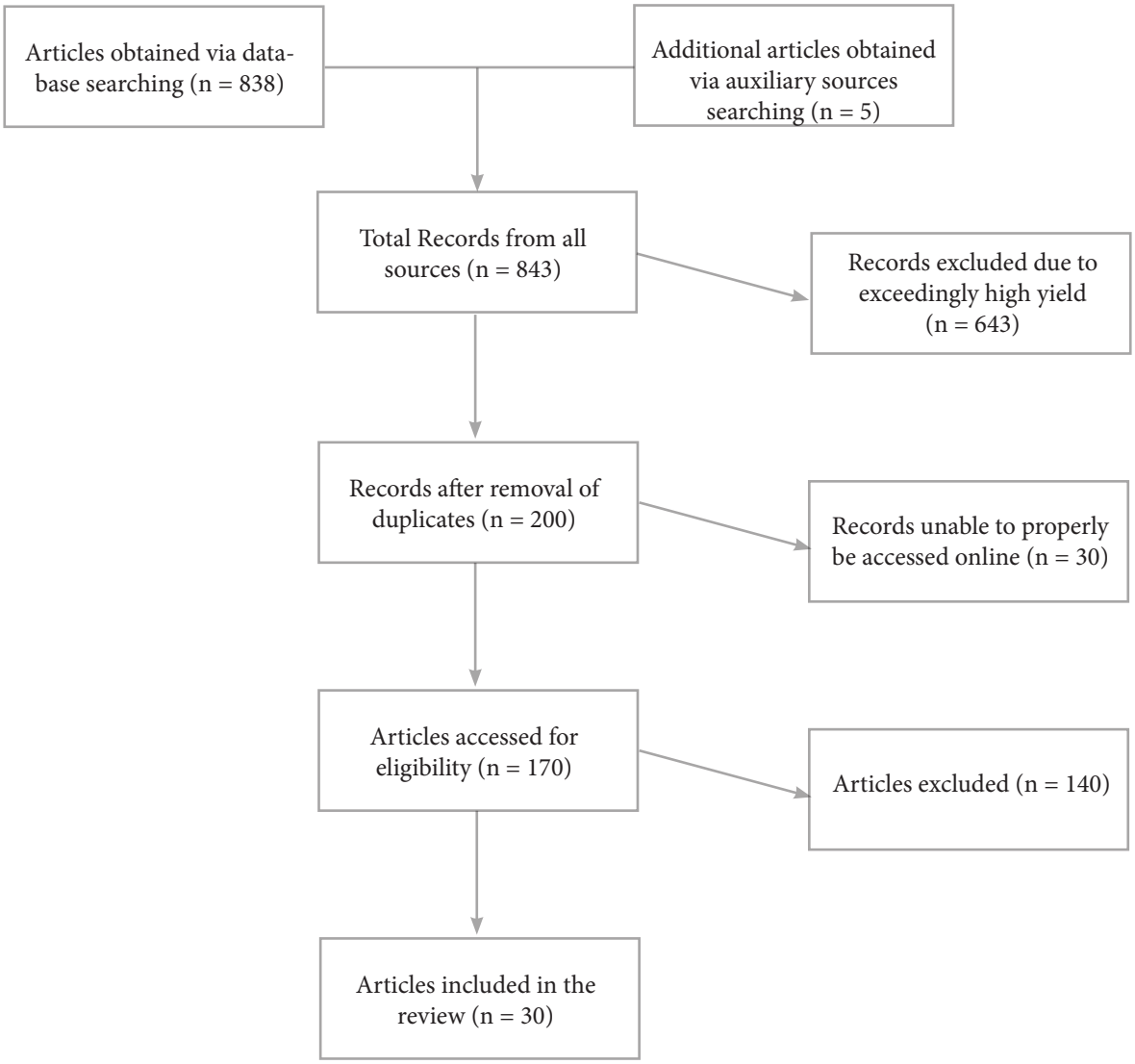

Figure 1. Study Flow Diagram

cification" /"cardiovascular disease/events" in the title and abstract. 13 articles were included in the review. Google Scholar <2005 to 2015 November 6>, focused on the relationship between BMD and kidney transplantation. The exclusion criteria was lack of the terms "bone mineral density" and "kidney transplantation" in the title and abstract. 4 articles were included in the review.

The last search via Google Scholar <2005 to 2015 November 6>, focused on the relationship between CVD and kidney transplantation. The exclusion criteria were lack of the terms "cardiovascular disease" and "kidney transplantation" in the title and abstract. 3 articles were included in the review.

Critical Examination of BMD, CVD and Kidney Transplantation

Due to the complext nature of this relationship, the review will consider the bilateral association between each two variables first, and then the three as a whole.

Cardiovascular Disease and Bone Mineral Density

One great challenge of assessing an independent correlation between BMD and CVD within kidney transplant recipients is the vast pool of shared risk factors and potential confounders [4, 5, 7, 9, 10, 11, 17, 21, 22]. A direct association between BMD and CVD in the general population has not been established in current literature. It is difficult to examine the independent relationship between the two variables due to numerous shared risk factors, and possible common pathophysiological mechanisms during disease development [7]. Many studies failed to show a significant correlation between BMD and CVD after adjusting for risk factors such as age, sex and fat mass [4, 5, 9, 10, 11]. However, despite the lack of a direct relationship, many studies have identified secondary relationships between BMD and risk factors for CVD. These include arterial calcification [14,15,16], lifestyle [17], renal function [18], and serum Dickkopf1, a peptide that aids in bone growth [19].

In a retrospective study of postmenopausal women, Atci et al. found a significantly higher prevalence of osteoporosis ($P < 0.001$) in the group with breast arterial calcification (BAC) compared to the group without BAC14. Intriguingly, the study also found low BMD to be more prevalent in the group without BAC ($P < 0.001$) [14]. Oversight of confounders like age, postmenopausal duration, mean number deliveries, and hormone replacement therapy could account for the discrepancy in the results. Fedichkina et al. compared bone metabolism status in patients over 65 years of age with idiopathic mitral annulus calcinosis (MAC). After conducting a multivariate regression analysis that attempts to see if the predictor is related to multiple outcome variables, they concluded that the presence of MAC was associated with a higher femoral neck bone loss ($P = 0.039$)(16). Nevertheless, the authors failed to account for risk factors such as cardiovascular history, medications, and lifestyle. Also, the study was restricted to an elderly population with a mean age of 71.5, therefore this cohort was at a tremendously elevated risk of bone metabolism because age is a predictor of high bone metabolism and low BMD. In addition, the size of the cohort was 165 people which is very small and most likely unrepresentative of the public at large.

Lastly, Pfister et al. attempted to determine a correlation between BMD and the risk of developing heart failure in their prospective study. They found a 23% decrease in heart failure risk with an increase in every standard deviation away from the mean BMD in healthy middle-aged individuals [7]. After adjusting for risk factors, such as age, sex, life style, systolic blood pressure, presence of diabetes mellitus, cholesterol concentration, and body mass index, the association of low BMD to high risk of heart failure was statistically significant ($P = 0.002$) [7]. Nevertheless, with a population of 42 to 82 year-old men and women, the investigators neglected the potential confounder of estrogen level in pre- and post-menopausal women when adjusting for the hazard ratios. Also, the study claimed to have a mean follow-up period of 9.3 years, but it only included incidental reports of heart failure events from the participants and a consistent follow-up for BMD measurements was also missing. Oddly enough, instead of using dual-energy x-ray absorptiometry like most studies, the investigators used the old-fashioned method of broadband ultrasound attenuation to measure BMD which is not widely accepted as the best method of measurement. In total, despite the lack of studies on how all CVD are directly related to BMD, agents of CVD, such as arterial calcification and heart failure, seem to be positively associated with BMD [7, 14, 15, 16]. Regardless, the relationship between BMD and CVD has yet to be clearly defined, which is partially because the correlation is clouded by multiple shared risk factors and confounders.

Cardiovascular Disease and Kidney Transplantation

Due to the high incidences of CVD in kidney transplant recipients, this relationship could be confounded, therefore it must be analyzed bilaterally. As a result, the correlation between kidney transplantation and CVD has yet to be definitively proven in addition to the relationship between BMD and CVD. There is a general agreement that kidney transplantation populations had a higher occurrence of CVD [20, 21, 22] but the causality behind this varied greatly.

Yong et al. claimed that this prevalence might be caused by immunosuppressive medications which may increase arterial stiffness possibly leading to CVD and ultimately death20. Participants not given calcineurin inhibitors experienced lower cardiovascular outcomes (risk ratio = −5.14; 95% CI = −9.99 to −0.28; $P = 0.04$) after fifteen months than their counterparts that did receive the inhibitor, suggesting a possible correlation (20). Another study claimed that their kidney transplantation population observed a higher prevalence of CVD as a result of previous cardiovascular history, diabetes and dialysis length (21). Lastly, Bignelli et al. claimed that traditional CVD risk factors increased in kidney transplantation recipients, suggesting that the high incidence of CVD may actually be directly linked to receiving a kidney [22]. However, they did not specify if the results had been adjusted for previous cardiovascular history, end-stage renal disease, chronic kidney disease, dialysis length, or diabetes [22].

To summarize, the relationship between kidney transplantation and CVD remains unclear and may involve multiple variables such as immunosuppression, dialysis length and previous CVD history [20, 21].

Bone Mineral Density and Kidney Transplantation

The correlation between kidney transplantation and BMD seems to be confounded by many other variables. There were unanimous observations between a few studies regarding BMD reduction within the first six months post-transplant [23, 24,

25] and partially consistent BMD reduction within the first year post-transplant [23, 24]. Subsequently, a resurgence of BMD was recorded after the one-year mark [23, 24]. This pattern may suggest the correlation between BMD and kidney transplantation is only temporary. Madeira et al. noted that older age, lower BMI and length on dialysis were major risk factors for lower BMD levels within the kidney transplantation population [23]. Strikingly, long dialysis treatment may be linked to both a decrease in BMD and a high occurrence of CVD [21, 22, 23], thus confounding this relationship.

Parathyroid hormone (PTH) seemed to be crucial to understanding this relationship. Evenepoel, P. et al. observed an initial fall in PTH levels after kidney transplantation, similar to BMD levels, then a steady increase in PTH levels, again similar to BMD levels [26]. Also, an elevated level of PTH was correlated to an increase in calcium and phosphate, which are the major bone minerals (26). Extrapolating from these results, it could be possible that high PTH correlates to low BMD. This conclusion matched the results of other studies [23, 25]. However, Marcén et al. observed an insignificant correlation between PTH and BMD thus contradicting those studies [24].

The consensus among the articles was that the relationship between kidney transplantation and BMD was not straightforward. In addition to numerous factors that may impact BMD in kidney transplantation recipients, short follow-up periods posed an issue [25] and studies on PTH produce contradictory results regarding its relevance to BMD in kidney transplantation [23, 24, 25, 26].

Bone Mineral Density, Cardiovascular Disease and Kidney Transplantation

This area presents a great paucity in scientific research in regards to the relationship between BMD and CVD in a population of kidney transplantation recipients. This review of the literature seemed to suggest a possible correlation between lower BMD and CVD occurrences [26, 27, 28, 29], and yet the source of this relationship has not been conclusively validated. The earliest primary article obtained from the literature search was from 2009 [26], meaning that this is still a very new and emerging area of investigation.

One of the first published article that explored this was by Alfieri, C et al. who claimed that in their population of kidney transplantation recipients, a progressive decrease in femoral BMD after the kidney transplantation ($p = 0.01$; $p = 0.03$) was predictive of an increase in aortic calcification (T0: AUC 0.65–$p = 0.02$; T12: AUC0.69-$p = 0.005$) [26]. Also, eight patients experienced a cardiovascular disorder (CD), defined as a major disease affecting the heart or vascular system, during the study. Unfortunately, this article had many inconsistencies [26]. The results were not adjusted for the large range during which transplantation occurred (nine-year variation) thus the follow-up periods varied greatly [26]. They also claimed that age could determine BMD, possibly presenting a bias in the data [26]. The full article could not be obtained so the average age was unknown. The claim that participants who had a CD also had different BMD values could not be verified or analyzed.

Cseprekal et al. observed a correlation between high bone turnover and aortic calcification, however, this was seen for only two years after kidney transplantation ($r=0.53$; $r=0.69$) [27]. Furthermore, the population size was only 47 participants and primarily consisted of minors (average age: 15.5) [27]. Thus it was not representative of the general population and the follow-up period varied greatly from 3 - 191 months, meaning that some participants did not withstand the two-year threshold [27], which could be a possible source of error in the data. Most importantly, bone turnover does not equate to BMD and aortic calcification does not equate to CVD. Hence this study did not directly explore the relationship of BMD to CVD in kidney transplant patients.

Lastly, Kinsella et al. claimed that vascular calcification correlated to low BMD, but their data stated otherwise [29]. Their population observed a correlation between high aortic calcification and low forearm T-score ($r = -0.300$, $p = 0.012$) as well as low femoral neck T-score ($r = -0.254$, $p = 0.009$) [29]. However, there was no significant relationship between aortic calcification and lumbar T-score [29]. Therefore, there was no concrete relationship between aortic calcification and T-scores. In addition, high PTH correlated with low forearm T-score ($r = - 0.300$, $p = 0.012$) [29], which possibly interfered with the correlation between aortic calcification and forearm T-score. The follow-up period was not specified so the progression of the relationship was unknown.

As a whole, there was critically no true consensus of whether the relationship between BMD and CVD existed [29]. Interestingly, PTH [23, 24, 25, 26, 28, 29] seemed to play a role in the relationships between BMD and CVD in kidney transplant recipients, and between BMD and kidney transplantation. Immunosuppression [20, 23, 27] seemed to affect the relationships between BMD and CVD in kidney transplant recipients, and between CVD and kidney transplantation. Notably, all of the discussed studies contained follow-up periods as short as one year [27, 28, 29, 30], populations that were not reflective of the general public [28, 29], insufficient definitions of CVD [29], and most alarmingly, presented inconsistent results on elements that could be confounding to one or more relationships [20, 23, 24, 25, 26, 27, 28, 29].

Conclusion

As the immediate patient survival rate continues to improve, more emphasis should be placed on studying the post-transplantation complications of kidney transplant recipients. CVD and low BMD seem to appear together and are among the most prevalent complications. However, the current understanding of an association between CVD and BMD in the unique population of kidney transplant recipients is inadequate. The few studies that attempted to examine this vastly confounded relationship [16, 17, 18, 19, 20, 23, 24, 25, 27, 28, 29, 30] produced contradictory results [5, 14, 23, 24, 25, 26, 27, 29] from unrepresentative samples that were mostly too small16, 18, 19, 28, 29, 30. Many studies concluded a positive association between low BMD and vascular calcification in both general and kidney transplant populations, [14, 15, 16, 27, 28, 29, 30] but few associated BMD directly with CVD [7, 26]. The relationships between BMD and CVD, BMD and kidney transplantation, CVD and kidney transplantation, and among all three variables remains unproven after adjusting for risk factors such as age, medical history, lifestyle [4, 5, 10, 11, 22]. Major limitations of the studies include inconsistent and short follow-up [7, 11, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30], negligence of potential confounders [16, 17, 18, 19, 20, 23, 24, 25, 26, 27, 28, 29, 30], ambiguous and conflicting definitions of CVD, and small sample size uncharacteristic of the target population [16, 18, 19, 28, 29, 30].

The Multi-Organ Transplant Student Research Training Program (MOTSRTP) at Toronto General Hospital (TGH) provides an opportunity to conduct a retrospective study using CoReTRIS to contribute to the current literature in the novel field of relating BMD and CVD in the context of kidney transplantation. The study will survey the adult kidney transplant recipients at TGH from 2004-2014 to investigate whether low BMD, as defined by a T-score less than -1 is associated with the risk of developing a major adverse cardiovascular event (MACE) over the time period of 2013-2015.

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Dr. Andrew Baines

Dr. Baines MD, PhD, FRCPC, is a professor emeritus with the Department of Laboratory Medicine and Pathobiology at the University of Toronto. Formerly, he was Biochemist-in-Chief of the University Health Network, Vice-Dean of Medicine (Faculty of Medicine, U of T), the Principal of New College (U of T) and the Chair of Clinical Biochemistry (U of T). He is currently the coordinator of the Augusta Stowe-Gullen stream of the ground-breaking ONE program at Victoria College. In his research roles, Prof. Baines' interests included ascertaining the fundamentals of kidney function including fluid electrolyte transport, catecholamine influences, and functional effects of renal oxygen delivery. Latterly he investigated hemoglobin based blood substitutes as an alternative medium for transfusions.

Interview conducted by JULS

"You've got to be prepared to make use of the serendipitous events that happen to you, be prepared to change course, change emphasis and not get stuck in a rut."

JULS: Why did you choose to study life sciences?

AB: I was drawn by the diversity and the fact that it dealt with life. I was accepted into math, physics and chemistry, as well as pre-med. It shows a certain ambiguity into my thought processes at the end of grade 13, but my recollection is that I flipped a coin. My judgment in retrospect was that I was probably balancing risks and saying, if I'm going to become a productive scientist in math, physics and chemistry, I've got to be really smart and really good in math, and my chances are fairly low. The other reason I chose medicine was the association with the humanities, dealing with humans and being part of a long tradition of working with humans, health and disease.

JULS: That takes us to our second questions, why medicine?

AB: Prof. Baines: For many of the reasons I went into life sciences – it enabled one to be part of a long but evolving tradition, which dealt with human achievements, suffering and enabled one to provide some help in this.

JULS: Your major clinical interest was nephrology – why did you choose this particular specialty?

AB: There were a multiplicity of reasons in that. One was a fascination with the structure, at the nephron level, where you could see the incredible architecture of the kidney – I loved the way it looked under a microscope. I was also fascinated with the way it worked, although we didn't really know how it worked back when I was a medical student. Next, when I was doing my internship, I was frustrated by our ignorance when it came to managing problems associated with fluid balance, blood pressure and so forth. I was inspired by a very effective clinical teacher, by the name of Abe Rapaport, who was an internist, as nephrology wasn't a sub specialty at that time yet. He was very interested in laboratory medicine and diagnosis, and captured my interested with trying to understand fluid electrolyte balance, regulation of body fluids, pH, blood pressure and so on. At the heart of all these issues was the kidney. Lastly, a family member also had kidney disease. With the advice of Abe Rapaport, I went on to do a master's degree in what was then called clinical biochemistry. The Department of Clinical Biochemistry was the only one that was both clinical and research based at that time, and is now the Department of Laboratory Medicine and Pathobiology. I did my masters with a supervisor who was an expert on the kidney. When I got there, I was put in charge of teaching the medical students clinical biochemistry, which was one of the major third year courses that had

both a lecture and laboratory component. As a graduate student, I was initially in charge of the laboratory. Eventually as I became more knowledgeable about the kidney, I became responsible for teaching the kidney lectures when my supervisor decided to go to Africa and become a dean of medicine in Nigeria. He toddled off leaving me without a supervisor, and at this point I was engaged in my PhD. I then found myself practically acting as my own supervisor, because there was no one else in Toronto who knew anything about the kidney at the level of research I was doing. I was actually working with another supervisor who was a specialist in liver function, so that was interesting. Through this experience, I discovered that I enjoyed teaching and seemed to be reasonably good at it.

"You've got to really understand the subject, and you have to be imaginative."

JULS: Let's talk about your early work. When you first started working in the life sciences as a PhD recipient, what were the first things you began looking at?

AB: This is actually really amazing and sad. I worked on the mechanism of recovery from acute injury. To do this, I would use toxins, which attacked the lower part of the proximal tubule, and then study the regeneration of cells using enzyme biochemistry, which was in its infancy at that point. What came out of that was a PhD thesis and at least one paper, but also a fascination with how the structure of the kidney influenced its function. During this time, a colleague and classmate who was working at SickKids and doing her PhD there, was associated with a pair of twins, who were subsequently diagnosed with Bartter syndrome. I initially got the biopsy material from those twins before any diagnosis of the syndrome could be made, and saw fascinating evidence about changes in enzyme distribution in the kidney, which pointed to a defect in the distal tubule. Unfortunately, I never pursued it any further – if we had done so, we would have gotten to an understanding of the biological defect in Bartter syndrome much sooner than it had occurred. I missed the opportunity because I was focused on writing my PhD at that point, and said, 'Well, these are very interesting, but I've got to finish this PhD', rather than going ahead and exploring this serendipitous result that I had been given.

JULS: That's very interesting, almost all of the professors we've spoken to over the past few years have touched on serendipity. How did you get into researching haemoglobin-based blood substitutes?

AB: That came much later in my career. I was really interested in looking at how the structure of the kidney informed the function – I did a lot of work looking at structural functional changes at the level of the nephron, tubule, and brush border. The technique that I used was micropuncture, which interested me because I was able to work with a living kidney in a living animal, see how the nephrons within the kidney actually functioned, manipulate them and induce interesting effects. This led to a desire to have an even more controlled circumstance, so with the help of a colleague at Oxford, we developed an isolated perfused kidney system. That's where I got into the blood, because I discovered at that point that if you were doing an isolated perfused kidney, you needed something that could provide a good oxygen supply, but not get mashed up by circulation pumps as red blood cells would be. The colleague I was working with was looking at toxic effects of conjugated hemoglobin molecules, where I would look at their effects on the kidneys. That's what led to us working with a hemoglobin based substitute.

JULS: It's funny that you mentioned that, because there is currently a revival in the blood based substitute industry, in order to find ways to preserve transplant organs.

AB: That's some of my work, looking at the metabolic needs of isolated perfused kidneys, so we were actually working on a system that could be used to look at maintaining perfusion to transplant organs. The hemoglobin based blood substitute industry was initially driven by the United States military, because they wanted a blood substitute that could be easily used on the battlefield without the need for refrigeration or cross-matching. They had a very intense interest throughout the late 60s and early 70s. Then, they discovered the bloodless war, where Americans don't get hurt, so they lost interest – the funding then came from commercial companies. The idea was that this would be the panacea for all blood substitutes – we'd get over the problem of infectious diseases from donated blood being transmitted and so on. It didn't pan out, and didn't work as well as it should have and there's a whole long story about that. Though that died out, it always struck me that the preservation of organs was going to be something that needed to be done, but it was too early for it to happen while I was working on it.

JULS: On a different matter, you are the coordinator of the Stowe-Gullen stream of the Vic One program. Could you tell our readers what this is?

AB: It is a pair of courses, part of the Vic One program, which are designed to provide first year students with a challenging base and background for the area or discipline that they hope to pursue as an undergraduate and possibly in graduate studies or professional school. It provides a background for people who want to go on in the life sciences and/or professional schooling in the sciences.

JULS: What is special about this program, professor?

AB: What's special about Stowe-Gullen is that first of all the students who are there are prepared to meet a challenge, and come in knowing they are going to be challenged. They are students who, while interested in the life sciences, have a much broader sphere of interests. I think the majority of them also have an interest in the effective use of language and communication. You can find all sorts of wonderful things, but if you can't communicate them, they're useless. If you want to keep in a highly competitive field, if you want to keep funding for the sort of research that you're doing, then you're going to have to communicate not only with other scientists, but also the government, industry, and the general public.

JULS: Switching gears now, you held the positions of Biochemist-in-Chief, the Principal of New College, and the Vice Dean of Medicine.

AB: While I was at New College, I was working at the hospital in biochemistry for 10 years. I was chair of the department, which became part of the Department of Laboratory Medicine and Pathobiology. I was actually involved with the undergraduate educational program of clinical biochemistry, which is the basis of the LMP program now.

JULS: On this note, how exactly did you become the heads of these departments?

AB: I guess nobody else was around at the time, I don't know! I've made no major discoveries, missed at least one, but I've always been fascinated by the aesthetics of life. I'm obviously very interested in teaching, which I began as a graduate student, and continued when I was involved with the structuring of the medical curriculum as chair of the curriculum committee. I was always involved with medical and graduate education, and also in arts and science teaching, even as a graduate student. Later, when I came back first as an associate professor, I had taught 4th year and 3rd year courses in arts and science, and I had very great interest in the selection of medical students and design of the medical curriculum. That got me interested in New College, which saw itself as being an arts and science college with a professional bend to it. How I got to be the Biochemist-in-Chief – I guess there weren't that many other MDs – I did have some clinical practice up until I became Vice Dean, so I was seeing patients. I represented something unusual within the clinical biochemistry department, in that I was a researcher, a teacher and a clinician.

JULS: To conclude, what advice do you have for undergraduates today?

AB: I should preface my answer by saying I was born basically with a silver spoon in my mouth. I was born when universities weren't flooded, so my experience cannot be repeated by anybody anymore, and relatively speaking it was easy to do all these things. Since university populations were expanding, the need for professors was exploding, so you could pretty much do what you wanted to to an extent that's not possible now. I was lucky to always be funded up until I shut my lab down in 2006 – I usually had 2 technicians, 1-3 graduate students, and an occasional post-doc. Now? It's much harder. The research now is very much a team process. It's big business, you can't do it on a shoe string. If you're going to do it seriously, you've got to do it in a big way and you've got to have a lot of collaboration, teamwork. I guess what you need if you want to be successful in this, is the capacity to work with other people and also to be a leader. Young people who are looking for that challenge should take every opportunity they can with working with others and leading those enterprises and teams. One thing I think possibly helped me was that I was captain of the UofT rugby team for 3 years. You have to learn how to work with, and ultimately guide people in the direction that you think will be useful. There are certain life skills that go along that aren't in textbooks and journals, and they are going to be very useful. You've got to be very persuasive in selling yourself, so you get the positions to start with, and then selling your ideas so you get the funding. There's a lot of salesmanship involved, and you've got to really understand the subject, and you have to be imaginative. You've also got to be prepared to make use of the serendipitous events that happen to you, prepared to change course, change emphasis and not get stuck in a rut.

JULS: Thank you very much, Professor!

Dr. Janet Rossant

Dr. Rossant PhD, FRS, FRSC is the former Chief of Research at the Hospital for Sick Children and is currently a Senior Scientist in the Developmental & Stem Cell Biology program at SickKids. She is also a Professor in the Department of Molecular Genetics and the Department of Obstetrics and Gynaecology at the University of Toronto.

Dr. Rossant's research interests centre on understanding the genetic control of normal and abnormal development in the early mouse embryo using both cellular and genetic manipulation techniques. Her work in the early embryo have led to the discovery of a novel placental stem cell type, the trophoblast stem cell. Dr. Rossant is also the Director of the newly formed Ontario Institute for Regenerative Medicine, and was the recipient of the Canada Gairdner Wightman Award for 2015.

Interview conducted by JULS

"As scientists, when you enter the field and you decide that you want to go down the academic pathway, that is a big enough decision in the first place. It is a tough road, but you have to have passion and commitment."

JULS: Every JULS' interview starts off with this question – why the life sciences?

JR: My interest in the life sciences really goes back to high school. I was always interested in science, just the discovery aspect and the fascination of trying to understand the world around us. I was interested in chemistry, not physics, biology, but I really got turned on to biology because of a very inspirational high school teacher. She recognized that I had potential and she gave me lots of books to read, she took us all on field trips into nature and various places that really engaged us in the fascination of life.

JULS: So how did you get into developmental biology?

JR: She encouraged me to apply to Oxford and Cambridge because nobody in my family had ever been to university – I was certainly going to go to university – but I wasn't really thinking of the elite universities because at that stage you have to do special exams. So she encouraged me to apply and I did and I got into both of them. I went to the University of Oxford and read zoology, the study of animal life. One of the courses was developmental biology and it was taught by John Gurdon, who won the Nobel Prize in 2012 for cloning frogs. He was doing that work, published his first paper, and was still doing additional papers showing that you could take adult cells from a frog and put them back into an egg [to] have them reprogram development, which at the time was used to show that the DNA code has to be the same in all cells. What makes cells different is how the [genetic] code is read out. Since then, it also is the underpinning of reprogrammed cells – to make stem cells – so that was why he won the Nobel Prize with Shinya Yamanaka. But, he was really the person that turned me onto developmental biology. He also had a whole group of people working in the lab at Oxford, and the way that the training works in Oxford is that you get 'tutorials', where you have one-on-one meetings, where you go in depth in particular areas. I had tutorials from members of his group and so that really enhanced my interest in the developmental biology field. It is still a fascinating and simply stated problem – how do you get from a fertilized egg to us? Whether it is a frog, a mouse, or a human, it is a fascinating and amazing problem. Here, at SickKids, we deal with some of the problems when it goes wrong; but generally, most of us are just fine, yet it is just a complicated process that it is amazing that it doesn't go wrong.

JULS: Speaking of that quite important question, if someone were to ask, how does a single cell become life, what would the developmental biologist say?

JR: Well, the developmental biologist, I guess, really would start with that fundamental finding that you start with a single cell – it has, within the nucleus, a genome that encodes all the information that can be used to be read out and turned into an organism, and the challenge is, how do you read it out in the right order, at the right time, and the right place? And that is the fundamental question of development – how do you read out of a single cell as it grows and turn on the right genes at the right time and place. We understand more and more about that and we could probably sit down and design an embryo. You could work out that if we turned on this gene – we know, for example, that this is the gene that is absolutely critical to make muscle cells – so if we turn it on, all downstream genes are going to turn on the muscle. It is not quite that simple, but we are beginning to understand the hierarchy of gene control. But that is what we have to understand – how do you read out that information in time and space – that is the fundamental difference between studying developmental biology as opposed to studying cells in culture, where you are looking at the fundamental aspects of how a cell looks. It is that time and space aspect. We are asking how do cells and tissues develop and bringing those two additional components, which make it that much more complicated.

JULS: Just on the issue of gene control, in simple layman's terms, what is gene control?

JR: So, here we have the DNA, it encodes proteins but there is a whole lot of DNA there that doesn't encode proteins and does other things, and among the other things it does, it has DNA sequences that can bind other proteins and be modified and changed such that if you bind proteins to this area, you will turn on or off genes. So, the particular coding sequence will be read out in one cell and not another. In a blood cell, the genes that make globin are turned on, and in a nerve cell, there are other proteins that bind upstream in the DNA and turn off that gene. It is that complicated dance of making sure that you have these networks of transcription factors that can regulate gene expression, again in time and space.

JULS: You are famous for discovering the trophoblast. Would you be able to tell me a little about it?

JR: So, I have always been interested in the very early stages of development in the mammalian system, but we focused a lot of our attention on the mouse. The first stage of development where cells make any special decisions in the mouse is called the blastocyst and it is a nice model system to address the problem of how cells read out gene expression because in about four cell divisions, they go from an egg to three different cell types that are expressing different genes. I've been working on this for about 30-40 years and we are just beginning to really understand the molecular pathways. One of the first cell types that forms is called a trophoblast, and it is the outer cells that the embryo uses in a mammal to interact with the uterus, so it is what makes mammals special – we don't have egg shells, we have a placenta and the first thing an embryo does in a mammal is make the cells that are going to make the placenta. It is quite special and very specific to mammals, which meant that we couldn't look to flies or worms to find analogous systems, and so we had to study it in a mouse. We have been studying trophoblasts for a long time and one of the things that helped us study it was when we were able to take that trophoblast from the blastocyst and culture them into stem cells (cells which have grown and would divide indefinitely). Now we have lots and lots of cells, [so] we can begin to look at the genetic pathways to make them become trophoblast cells and make them continue to grow as stem cells. So they are a nice model system to understand how a placenta works. Interestingly enough, we have not yet been able to make the same cells from humans, which is telling us that things are not quite the same in a mouse and human. We think what is going on there is that in the mouse, the early trophoblast is very proliferative so you can capture it and grow it in a petri dish. In humans, the first trophoblast actually invades right into the uterus and only later do you get a proliferative cell type, so we have to find other ways, which we are trying to do.

JULS: Regarding your work on the mouse embryo, if one speaks to a person on the street and says that we are spending one million dollars on embryo studies, it may not get the best response, so how would you justify the expense and effort we are putting into mouse studies versus human studies?

"Why the mouse? There are many reasons, but perhaps most important is that it has been a system in which we share about 95% of our genes."

JR: Why the mouse? There are many reasons, but perhaps most importantly is that it has been a system in which we share about 95% of our genes. We don't look like a mouse, it's true, but many of the functions we should have are shared with the mouse, so in many ways, you can study disease and general processes in the mouse and use it as the first path to understand how the human develops and grows. Importantly, you can make mouse models of disease. The other important thing about the mouse is that we have incredibly powerful tools to manipulate the genome. So first of all, we were able to use gene targeting in embryonic stem cells to make all sorts of alterations and make them in mouse. And more recently, with the whole CRISPR-Cas gene editing that has been applied to mouse zygotes, we can model very subtle alterations in humans and ask if it causes the same disease in the mouse. And, of course, in the mouse, we can do experiments that you can't do in humans. So if the mouse models the human disease, we can really dig into mechanisms. Having said that, there are differences. In many cases, you do the mutations in a mouse and it is very similar to what would happen in a human. But not all cases. We are not mice and there are differences. So, we have to also be able to complement the in-depth, whole-organism things we can do in mouse with the increasingly power to study human disease directly in a petri dish using induced pluripotent stem cells. Because we have complimentary tools now, we can tweak the genome of a mouse and model human disease in a whole mouse to study the physiology. We can now even take human cells from people with disease and turn them into stem cells in culture, and then make them differentiate into cell types that can

be used to help understand the disease as well. It is a dance. We can go from mouse to human, but we shouldn't forget the other model organisms – the fly, worm, zebrafish, xenopus. Because genetics pathways that control development, like organ formation, how brains work, how nerve cells work are highly conserved across evolution, we can get at the fundamental underpinning and find new pathways by going into flies and then take them up into humans. Or, you can go the other way around and find a human disease gene and start to study it in zebrafish because you can do more imaging analysis. Every system has its uses, so we never say the mouse is going to be absolutely the same as a human, because that would be foolish, but we can learn an awful lot from the mouse and many other model organisms.

JULS: Where do you see the field of developmental biology, specifically your work, being applied in the future?

JR: The fundamental issues of developmental biology is to try and understand how organisms develop in time and space, so that in itself satisfies our curiosity of where we came from. It also provides direct information that is relevant to birth defects and many of the kind of developmental syndromes that we see here at the Hospital for Sick Children, for example. Understanding normal development helps us understand what goes wrong in genetic diseases and developmental syndromes, so that is the most obvious direct applications. Also, we should remember that exploring development is how people identified important signalling pathways that can also be disrupted in disease. Perhaps one of the most famous signalling pathways in development from flies to man – and it's not my work in this case – is the Hedgehog signalling pathway, which was first identified in the Drosophila. [Drosophila researchers] like silly names, and mutations in this gene made the embryo look prickly. Hedgehog is a signalling molecule that is absolutely critical for patterning many different parts of the developing body, including limbs, skin, and nervous system, and it turns out that alterations in this signalling pathway is involved in a number of different diseases, including cancer. Some forms of skin cancer are caused by mutations in Hedgehog pathway, and some forms of brain cancer. This is a pathway that was first identified in Drosophila developmental biology and now is identified as a key pathway in cancer so, of course, researchers are directing drugs to block this pathway. From my own work – we worked years ago and still do work on how the blood vessels develop in the embryo. It is an early pathway because the embryo needs to a have circulatory system early on, so the heart, blood vessels, and blood develops as early progenitors. We identified a pathway involving a molecular called VEGF (vascular endothelial growth factor) and its receptor, and showed that when knocked out in embryos, no blood vessels could form. That was fundamental information that suggested that this is a really key pathway, and over time, it has become clear that VEGF is very important for normal development. If you have too much or too little VEGF, you will get abnormal vessels. Cancer cells actually produce this signal and attract more blood vessels to a tumour. So people have developed drugs to block the development of blood vessels and the VEGF receptor pathway. We didn't develop the drugs, but some of our most highly cited work is on this pathway because it demonstrated that this was a key signalling pathway. And if you disrupted it, you could block blood vessels – not just in the embryo but in the adult as well. It is a fundamental discovery and you never quite know where it is going to go, but developmental pathways, in general, are often re-activated in adult systems and in disease, so this is how you find new pathways and explore in disease.

> ***"The fundamental issues of developmental biology is to try and understand how organisms develop in time and space."***

JULS: Speaking of VEGF, JULS spoke to Professor Titia de Lange last year, who discovered the telomere abnormalities in cancer cells. When JULS asked her how she made the discovery, she said she just looked and happened upon it. How did you discover VEGF?

JR: The way we got at the VEGF pathway was because we were interested in early patterning. This was early days, going back to the 1990s, and we were interested in trying to find genes that might be involved in gastrulation. After the blastocyst, the next important phase is when you make the ectoderm, endoderm and mesoderm. Lewis Wolpert famously said that the most important time in your life is not birth, marriage, or death – it's gastrulation. So we were trying to find how to get into the genetic pathways, and one of the ways we could find them was to look for particular gene families, by PCR, so we looked for kinase receptors, receptors that had a particular domain that related to their signalling capacity, and we did a screen. One of the receptors was this VEGF receptor, and when we looked to see where it was expressed, we found it in all blood vessels. At the same time, Napoleone Ferrara at Genentech discovered VEGF, and had also identified the receptor from cells in culture. We had seen its embryo expression and he had seen how important it was in cells in culture. We made a mutation and it was clear that it was very important. It was because we looking for developmental patterning genes, and because blood vessels develop early, it came up as in gastrulation, where VEGF is expressed. It marks precursors which go on to form blood vessels, smooth muscle cells, and heart muscle cells.

JULS: You were the Chief of Research at SickKids. If an undergraduate wants to go beyond the principal investigator level, and go to something like the Chief of Research, how would they work out for them?

JR: As scientists, when you enter the field and you decide that you want to go down the academic pathway, that is a big enough decision in the first place. It is a tough road, but you have to have passion, and commitment. As you go down the pathway and you are starting to enjoy the science, there comes a time when you want to give back. First of all, anyone who is going into research should focus their first few years entirely on their research because that is what is most important. But as you go forward, we do need people who can help direct and support research, and mentor young scientists, and to go down that path one step at a time is what I would say. Absolutely always get involved; you always want to be engaged with your fellow investigators, graduate students, and university department. Be

useful, be on committees – but don't over commit – and see what you like. Not everybody, by any means, is destined or should end up as an administrator. It is another set of skills that scientists are not generally trained to do, but maybe more of them should be trained. I never had any formal training, but maybe these days, at some point it is worth having an MBA along the way, if that is really the path you want to take. I would say, take it a step at a time and it is not a necessary step. Most Nobel Prize winners have never run anything in their lives and just stuck with their science, so it really is a matter of what you like to do and what you find your skills are appropriate for.

"Explore, and take the opportunity, while you are an undergraduate, to explore as many different areas as you can and find out what you have a passion for and follow your passion."

JULS: What advice would you have for any undergraduate in life science?

JR: My advice for any undergraduate in life science is just to remember that we are in an amazing time for life science research. The tools and the technologies to explore nature, the applications of life science and medicine in new and exciting ways are amazing. Explore and take the opportunity to explore as many different areas as you can. Find out what you have a passion for and follow your passion. Your passion doesn't necessarily have to take you in the academic route or the medicine route for that matter. Just exploring a scientific education is really important. We are in a technological age and we need an educated public who understands the issues of science and technology. We need an educated public service, we need educated health politicians, and we need educated journalists. We need to have an environment in which we, as a country, are educated about these strengths, challenges, and the applications of technology. Too often we hear, even apparently educated people, not understanding some of the implications of science. My favourite hobby is vaccinations. In Canada and elsewhere, there are many people who are highly educated, who do not want to vaccinate their children, despite the fact that the science is extremely strong, and the idea that this is dangerous has come from completely discredited work, and yet the highly educated people still are not able to evaluate the scientific evidence properly. Science is training in being critical and being able to evaluate evidence. I'm very excited about the Liberal government's mandate letters to their ministers, many of which talk about science, innovation, evidence, and that I think is what we want to see our politicians do, and we need to be making sure that the public, also is as far as possible, able to weigh the evidence and make the right choices.

JULS: So, the undergraduate must learn the sciences, essentially?

JR: Yes. If you leave as an undergraduate and never directly use your science in whatever you decide to do, your training in being critical and evaluating evidence is really important. Use it. Talk to your neighbours, talk to whomever. When we look at global warming issues, stem cell tourism issues, vaccination issues, [there is] so much misinformation [that] you have to be able to sort out what is valid and what is not, and that is probably the training of being a critical scientist.

JULS
Journal of Undergraduate Life Sciences
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The *University of Toronto Journal of Undergraduate Life Sciences (JULS)* is looking for submissions that showcase the research achievements of undergraduate life science students. We welcome manuscripts in the form of Research Articles or Reviews. Submissions must come from University of Toronto undergraduate students or undergraduate students outside of U of T who have conducted research for at least three months under the supervision of a faculty member at U of T.

Research articles should present original research and address an area of the life sciences. Mini-reviews should focus on a specific scientific topic of interest or related to the research work of the author. Research articles should be between 2,000-3,000 words and mini-reviews between 1,500-2,000 words. All works must not have been previously submitted or published in another undergraduate journal. The deadline for submissions for each issue will appear on the JULS website at http://juls.sa.utoronto.ca.

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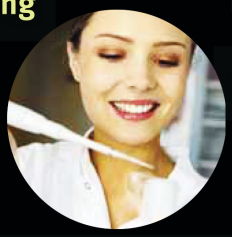

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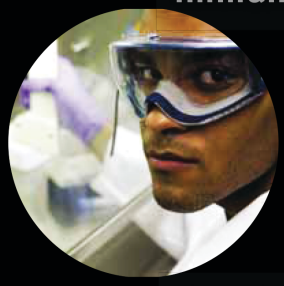

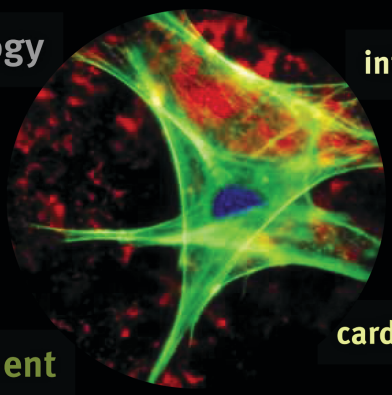

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