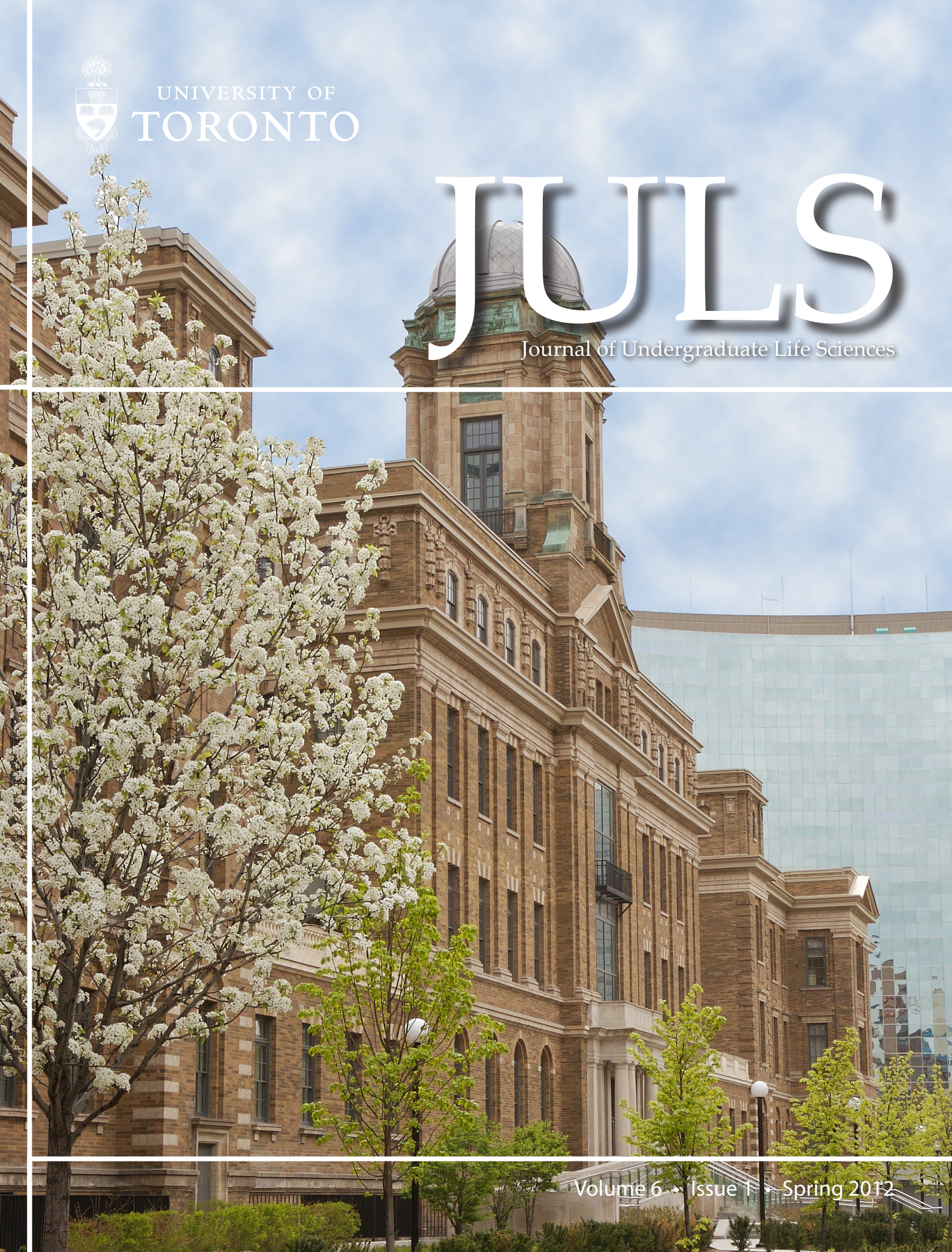
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Call for Submissions

The *University of Toronto Journal of Undergraduate Life Sciences (JULS)* is always 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.library.utoronto.ca.

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Acknowledgements

We thank Dr. Avrum Gotieb in the Faculty of Medicine and the Principal of Victoria College, Dr. David Cook for their support in the creation of this year's issue of JULS.

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About the Cover
In 1921-1922, Frederick Banting, Charles Best, John Macleod and Bertram Collip discovered and purified insulin in the Toronto General Hospital where the present-day MaRS Discovery Centre is located. The MaRS Discovery Centre continues this legacy as a centre for scientific, technological, and social innovation.

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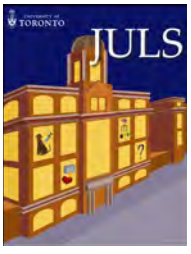

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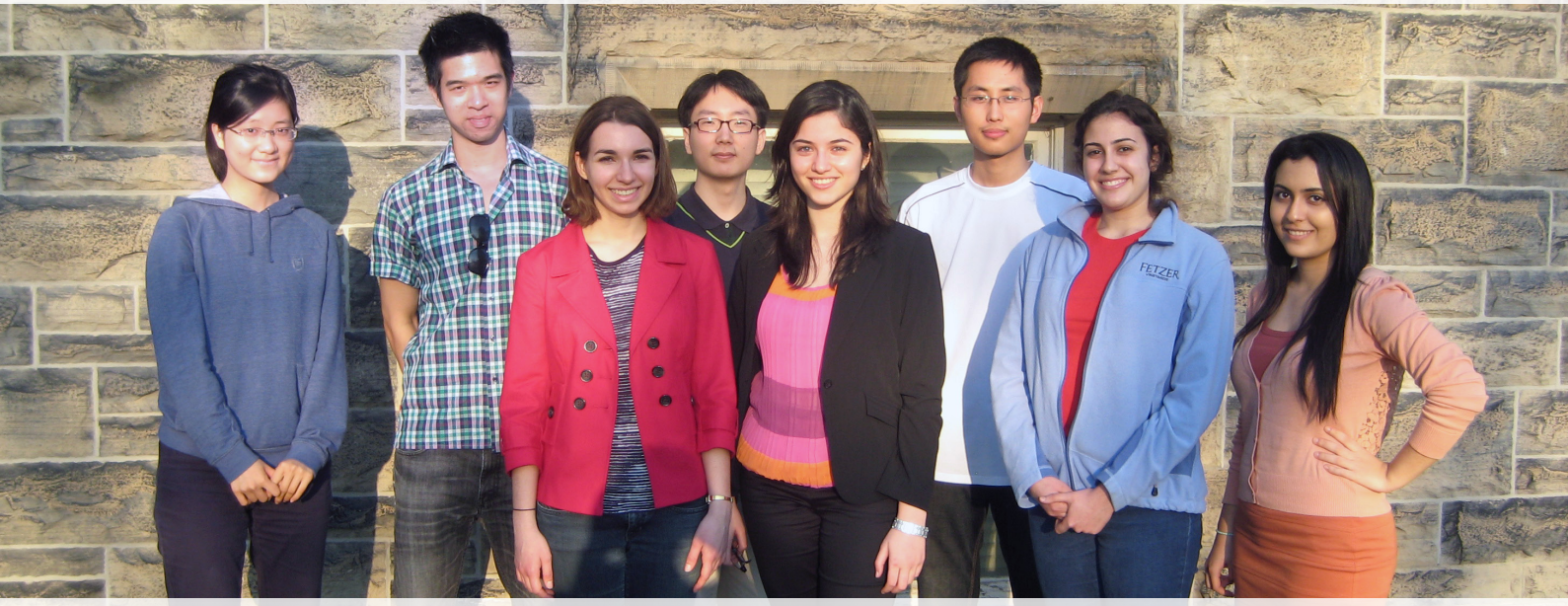
Letter From the Editors



Dear reader,

It is our pleasure to present you with the 2012 issue of the University of Toronto Journal of Undergraduate Life Sciences (*JULS*). This year we continue to feature the work of undergraduate students who have conducted research in a variety of disciplines in the life sciences. Promoting the innovative work of undergraduate students has always been and remains central to *JULS*' vision. The publication of this issue would not have been possible without the dedication and skills demonstrated by our staff and executive team, and for this we thank them.

Our theme for this year's issue honours the University of Toronto's long-standing tradition of excellence, leadership, and innovation in life sciences research. We were inspired to adopt this theme by the celebration of the 90th anniversary of the discovery of insulin at the University of Toronto. In honor of this remarkable discovery which has revolutionized the quality of life of diabetic patients worldwide, we are delighted to feature an interview with Dr. Mladen Vranic, Professer Emeritus from the Department of Physiology and a pioneer in diabetes research. As a post doctoral student, Dr. Vranic himself was mentored by Dr. Charles Best, one of the co-discoverers of insulin. It is clear from Dr. Vranic's interview that originality remains the hallmark of a successful research program, and that while the research environment at the University of Toronto continues to change, it does so for the better.

The discovery of insulin is only one of many groundbreaking discoveries originating at the University of Toronto, with a variety of research programs having been pioneered at the university and associated research centres, including stem cell research by Drs. Ernest McCulloch and James Till, and the development of the first artificial pacemaker by Dr. Wilfred Gordon Bigelow and his colleagues. The research articles published in *JULS* exemplify the spirit of innovation present at the University of Toronto and recognize the unique potential and enthusiasm that young scientists often bring to research environments. In addition to celebrating the longstanding tradition of scientific ingenuity at the University of Toronto, we are pleased to present you with interviews conducted with some of the Canada Gairdner International Award Laureates, who are pioneers in their respective fields and embody the innovative spirit which we are honouring in this issue of *JULS*. One of these interviews features Dr. Jeffery Friedman, the discoverer of leptin, whose work continues to reshape the field of diabetes and our understanding of the role of adipose tissue in metabolism.

We find ourselves in a truly remarkable era when research in the life sciences is extremely fast-paced. As undergraduate students, there is no better time to embark on an exhilarating journey into the world of research and innovation. We encourage you to pursue your scientific interests beyond the lecture halls and to partake in the unique research opportunities which the University of Toronto has to offer.

Sincerely,

Tina Binesh Marvasti and Ana Komparic
Co-Editors-in-Chief, 2011-2012

NOTE: All articles in this issue as well as supplementary information are freely available online at http://juls.library.utoronto.ca/. If you would like to join the JULS team, submit an article or have any comments or suggestions, please feel free to contact us as at juls@utoronto.ca.

Tumour Suppressor Genes, MEN1 and DAXX/ATRX, Discovered in Pancreatic Neuroendocrine Tumours

Ishita Aggarwal

Pancreatic cancer has the highest mortality rate of all major cancers, which can be attributed to the lack of diagnostic methods available for detecting the cancer in its early stages. The pancreas is involved in the digestive and endocrine systems of vertebrates and is responsible for making pancreatic juices that contain digestive enzymes. Pancreatic juices flow down the pancreatic duct into the duodenum (in the small intestine) where they aid with the digestion of food. Although most pancreatic cancers originate in the ducts that carry digestive fluids (causing pancreatic ductal adenocarcinomas or PDACs), rarer forms can originate in pancreatic cells that secrete hormones, resulting in pancreatic neuroendocrine tumours (PanNETs) [1].

In a recent study published in *Science*, Jiao *et al.* determined the nucleotide sequences of approximately 18,000 protein-coding genes in a set of 10 PanNETs taken from patients undergoing medical intervention. 157 somatic mutations were found in 149 genes among the 10 tumours. Jiao *et al.* compared the commonly mutated genes in PanNETs with the commonly mutated genes in PDACs to explore the genetic basis of these two major pancreatic tumour types. First, 60% fewer genes per tumour were mutated in PanNETs than in PDACs. Second, the commonly mutated genes in PanNETs were rarely altered in PDACs and vice-versa. Finally, C-to-G transversion mutations were more common in PanNETs, while C-to-T transition mutations were more common in PDACs. Jiao *et al.* suggests that the mutational differences between PanNETs and PDACs are due to exposure to different environmental carcinogens or to the inactivity of different DNA regulatory pathways [2].

Next, Jiao *et al.* sequenced specific genes that are components of pathways that are commonly altered in pancreatic tumours. Somatic mutations in tumour suppressor genes *MEN1*, *DAXX*, *ATRX*, *PTEN*, *TSC2*, and *PIK3CA* were identified in 44.1%, 25%, 17.6%, 7.3%, 8.8%, and 1.4% of PanNETs, respectively. Patients whose tumours consisted of mutations in *MEN1* or *DAXX/ATRX* survived nearly 5 years longer than patients whose tumours lacked these mutations. Jiao *et al.* explain the prolonged survival rate by proposing that *MEN1* and *DAXX/ATRX* categorize a specific biological subgroup of PanNETs that is governed by its own genetic processes [2].

By sequencing PanNETs, Jiao *et al.* were able to identify previously unknown tumour suppressor genes. Furthermore, their ground-breaking research identified the genetic differences between the two major carcinomas of the pancreas. The new information may aid in the diagnosis of pancreatic cancer and may provide a way to prioritize patients for treatment [2]. Future research in this area may focus on ways to increase the specificity and sensitivity of detection tests. Technological innovation will be instrumental in improving diagnostic capabilities for pancreatic cancer and in determining the best treatment options for individuals testing positive for PanNETs versus those testing positive for PDACs [2, 3].

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Investigating the Link Between Inflammation and Drug Disposition

Erik J. Bracciodieta

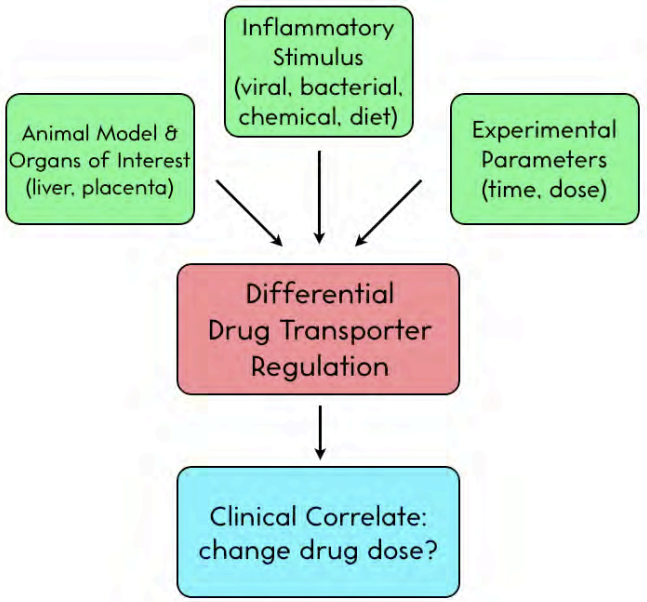
Based on an interview with Dr. M Piquette-Miller, Professor, Leslie Dan Faculty of Pharmacy at the University of Toronto.

The Piquette-Miller Laboratory studies the effect of disease on drug disposition. This link is important because many diseases have an inflammatory component in which cytokines are released into the bloodstream and alter the gene expression in many tissues. Diabetes, viral infections, malaria, bacterial infections and various cancers have an inflammatory component and they are usually treated with pharmacological products. It is key to understand the impact of disease on the expression and activity of drug metabolizing enzymes and membrane transporters because inflammation can alter the efficacy and toxicity of drugs [1, 2, 3, 4].

Over the past several years, the Piquette-Miller lab has found that many important drug transporters and metabolizing enzymes are altered in the presence of inflammation. In other words, tissues may have an altered accumulation of xenobiotics and their metabolites [5], which may cause a drug to be more toxic or less therapeutic. Accordingly, dosing regimens may need to be adjusted to account for the body's altered drug disposition in a variety of disease states.

In the Piquette-Miller laboratory, mostly rodent models of disease are used to study altered drug disposition during inflammation. Rats or mice are often injected with a disease stimulus such as lipopolysaccharide for bacterial infection, double stranded RNA for retroviral infection, malaria infected blood cells or streptozotocin to induce diabetes. RNA and protein levels of genes of interest are analyzed from important tissues involved in drug disposition including the liver, kidney, brain, intestine and placenta. Advanced studies use mass spectrometry or radiolabelled substances to track changes in xenobiotic concentration throughout the body's tissues, offering important *in vivo* information on how diseases affect drug disposition.

Alex M Cressman, a graduate student in the Piquette-Miller laboratory, is currently studying the impact of malaria infection on the drug disposition of pregnant mice. One group of mice is infected with malaria protozoa and the infected red blood cells are isolated and injected into pregnant mice. The symptoms of the mice and the fetal outcomes parallel what is observed in human malarial pregnancies [6]. For instance, the fetus in this experimental model are less vascularized then control fetuses and have lower survival rates [6]. There is little known about drug distribution in pregnancy models and pharmacological effects on fetal development. This study examines the expression of CYP enzymes and ABC drug transporters in the maternal brain, liver, kidney and placenta along with fetal brain and liver. Few, if any, studies have investigated altered gene expression and its involvement in drug disposition in fetal organs. It would be interesting to see what genes are differentially changed *in utero* and if they can explain clinical observations of poor fetal outcome in the malaria disease state. This experiment is in progress and will produce a paper later this year.



A flow diagram showing what factors to consider when studying differential drug transporter regulation.

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An ISO Domain in KCC2 Confers Isotonic Chloride Ion Transport in Hippocampal Neurons

Michelle Yuchen Huang, Jonah Chevrier

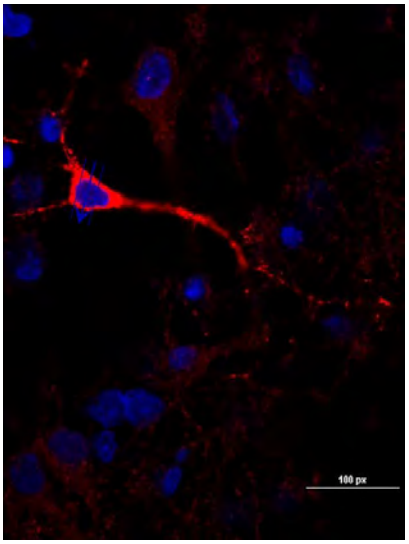
KCC2, a potassium-chloride co-transporter, is a transmembrane protein exclusively found in the central nervous system, in particular the hippocampus, brainstem and hypothalamus1. It has the unique role of driving Cl- out of the cell under physiological isotonic conditions using energy from the ion gradient created by the Na^+/K^+ pump. Conversely, other cation chloride co-transporters in the KCC family are only activated by osmotic stress [1]

KCC2 is an essential protein owing to its ability to extrude Cl^- from neurons allowing $GABA_A$ inhibitory synaptic transmission in the nervous system [1]. Nerve signals stimulate the pre-synaptic neuron to release GABA neurotransmitters into the synapses [1]. GABA then binds to post-synaptic $GABA_A$ receptors, opening the channel and enabling the influx of Cl^- into the cell; this induces a hyperpolarization response1. Mouse knockouts of the KCC2 protein display severe motor impairments, epileptic activities, and usually die within a few weeks due to respiratory failure [2]. Mice expressing only 17% of wild-type levels of KCC2 show a decrease in response to thermal and mechanical stimuli, higher susceptibility to epileptic seizures and decreased spatial learning and memory [2]. Despite the obvious importance of KCC2, the molecular mechanism that is conducive to its isotonic Cl^- transport remains unknown.

Dr. Melanie Woodin, the lead researcher of inhibitory synaptic plasticity in the Department of Cell & Systems Biology at the University of Toronto, investigates KCC2 through a combined approach using gene modification and electrophysiology to assay neuronal chloride levels.

Recently, several cDNA constructs of KCC2 tagged with GFP were transfected into mouse hippocampal neurons in order to observe their effects on isotonic Cl^- transport. To test the requirement of a unique 15 amino acid ISO domain in isotonic transport, a construct called KCC2ΔISO was used to transfect neurons. In this construct, amino acids 1022-1037 of KCC2 are replaced with the corresponding sequence from KCC4, a protein not found in hippocampal neurons. Doing so generated a construct incapable of isotonic Cl^- extrusion as evidenced by insignificant hyperpolarization. To confirm the function of the ISO domain, a "rescue" experiment was conducted in which researchers introduced the KCC2 ISO domain into KCC4: KCC4-ISO. The results conclude that only partial rescue was achieved, meaning that the ISO domain of KCC2 is required to extrude chloride ions, but insufficient for isotonic transport.

A medical application of this research involves the treatment of neurological disorders. Knowledge of the molecular mechanisms of KCC2 may allow for more effective treatments and prevention strategies to target neurological disorders associated with irregular chloride regulation. This information is important to understanding the mechanisms underlying disorders such as neuropathic pain and epilepsy. Furthermore, research in Dr. Melanie Woodin's lab will include determining other functionally important residues in KCC2 which may provide insights on how chloride regulation is conducted and its effect on morphology, osmolarity and polarity of neurons.



An immunostain image of a KCC2ΔISO construct compared to untransfected background hippocampal neurons. This image was provided by Brooke Acton.

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A Negative Inheritance: Children Mimic Parents With Bipolar Disorder in Cortisol Overproduction

Nothando Swan

Slowing the increasing incidence of mental health disorders has been difficult in part because no single etiological agent is responsible for their onset. However, researchers have reported that in people suffering from affective mood disorders, the relay system between the hypothalamus, anterior pituitary gland, and adrenal glands (HPA axis) is hyperactive [1]. Normally, the HPA axis releases cortisol in response to stressful situations. Thus, abnormally high cortisol levels indicate dysregulation of the HPA axis' response to stress.

Recently, Ostiguy and colleagues have linked high levels of interpersonal stress with increased cortisol levels among children whose parents suffer from bipolar disorder [2]. Interestingly, their work assessed how both chronic and episodic stress might affect the probability of affective disorder progression, whereas most research to date has focused only on the effects of episodic "negative life events" [2]. Ostiguy et al.'s findings indicate that HPA axis abnormalities may accompany other symptoms of affective disorders. Furthermore, they suggest that these abnormalities may be markers for the future onset of mental health disorders given stressful lifestyles.

In their longitudinal study, Ostiguy et al. recruited parents suffering from bipolar disorder and parents with no previous history of mental illness from the same Québec neighborhoods. Ten years later, 62 of the offspring of parents with bipolar disorder (OBD) and 60 offspring with no family history of affective disorders (OFH-) agreed to be interviewed and to produce salivary samples. Clinical psychologists conducted UCLA Life Stress Interviews in order to grade each participant's levels of chronic and episodic interpersonal stress.

The associations between these stress scores and cortisol levels after waking were particularly noteworthy. Radioimmunoassays of the salivary samples showed that cortisol response after waking and daytime cortisol levels were constant among the OFH- group, regardless of their levels of either type of stress. In contrast, OBD with high chronic stress scores had higher cortisol responses after waking than OBD with low chronic stress scores. In terms of episodic stress, OBD who scored in the severe range had higher daytime cortisol levels than OBD who scored lower, although this result was measured with 92% confidence of statistical significance, rather than the conventional 95%. This shortcoming cannot be ignored in light of work by Havermans and colleagues who reported no significant difference in daily cortisol levels between people suffering from bipolar disorder and those who are not [3].

The link between interpersonal stress and high cortisol levels identified by Ostiguy et al. indicates that children of parents who suffer from bipolar disorder have a pronounced physiological response to stress. This increased response supports genetic-based research which reports increased HPA axis reactivity in offspring with a short allele for the serotonin transporter gene [4]. Recognizing increased sensitivity to stress as a marker for increased susceptibility for developing an affective disorder opens the door for new epidemiological and public health research. Public health campaigns aimed at educating youth about the effects of stress might help to decrease the incidence of youth, and subsequently adults, who need treatment for mood disorders. This research also justifies the increased emphasis on behavioural modifications such as meditation and mindfulness to prevent stress and increase overall well-being. These interventions, and others like them, may be effective strategies in preventing the onset of affective disorders, particularly among those who are most vulnerable to developing them.

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Researchers and Public Health Staff Working to Address the Challenges of *Clostridium difficile*

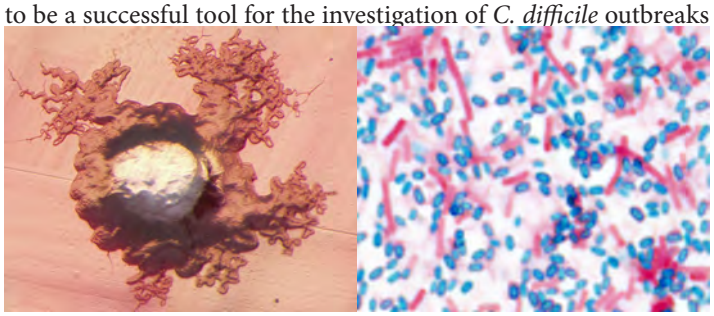
Yvonne Uyanwune

Clostridium difficile presents a complex challenge to health care providers in terms of prevention, management, and control. Recently the Niagara Health System (NHS) experienced significant issues with *C. difficile* infections and declared an outbreak at their Greater Niagara General site in December [1]. This followed a previous *C. difficile* outbreak in the summer.

C. difficile, a Gram-positive bacterium commonly found in hospitals, is a particularly opportunistic type of bacteria that colonizes the human digestive system and causes severe diarrhea and consequently, dehydration[2,3]. People suffering from *C.difficile* infections are often older individuals in nursing homes, patients in long-term inpatient care in hospitals or individuals undergoing antibiotic treatment [3]. *C. difficile* infections are commonly caused by the hypervirulent NAP1 (North American Pulsotype 1) strain [2,4].

The increase in *C. difficile* outbreaks in hospitals in the Niagara region over the last few months has been particularly challenging. Niagara Health System reports that while *C. difficile* presents a real challenge for all hospitals, what is unusual is the increasing number of people who are already carrying the bacteria when they arrive to a hospital [1]. Public Health Ontario (PHO) molecular specialist and University of Toronto Assistant Professor, Dr. George Broukhanski, has been working closely with researchers and infectious disease prevention and control staff at PHO to improve laboratory analysis of *C. difficile* and to enhance our understanding of how outbreaks originate and develop.

Currently used methods for studying the epidemiology of *C. difficile* fail to distinguish individual strains in the hypervirulent NAP1 clusters. A more discriminatory method, Modified Multilocus Variable-Number Tandem-Repeat Analysis (MMLVA), was introduced by Dr. Broukhanski at PHO and has been shown to be a successful tool for the investigation of *C. difficile* outbreaks [2]. Dr Broukhanski explains that, "MMLVA results are transferred to BioNumerics software that evaluates genomic diversity using phylogenetic trees and defines clonal differences between clinical isolates." Although MMLVA is a useful tool for *C. difficile* investigation, the criteria used to define clusters and outbreak strains are still indeterminate.



Left: High resolution image of a *C. difficile* colony. Right: Spores (stained blue) and vegetative cells (stained red) of *C. difficile* at 1000x magnification. Photos by Dr. G. Broukhanski.

Therefore, Dr. Broukhanski is working on determining the cut-off values for specimen differences in order to allow for proper clonal definitions. The C.difficile genome is known to be plastic and overly discriminatory cut-off values may overstate differences between the isolates [4]. Dr. Broukhanski's team is conducting a study on sequential subculturing of *C. difficile* isolates of the hypervirulent NAP1 strain. The study uses MMLVA to analyze variations, if they are present, at each subculture cycle to observe changes in repeat sequences at 5 loci of the NAP1 genome.

There are currently two methods of analyzing MMLVA data for *C. difficile* studies: numerical and categorical coefficients. According to Dr. Broukhanski, Manhattan distance-based clustering is used to define numerical coefficients and has a lower cut-off value that does not consider a 1 repeat difference as a significant difference between isolates. Categorical coefficients, however, have higher cut off values that cause MMLVA analysis to be too discriminatory. Dr. Broukhanki's study shows that gradual increases or decreases in the number of repeats (+1 or -1) support the use of numerical Manhattan distance-based coefficient clustering for defining clonality in clinical isolates. Changes involving multiple repeats at a time support the use of categorical coefficient. The results of Dr. Broukhanki's study will improve the efficacy and accuracy of MMLVA analysis of *C. difficile* outbreaks and will allow hospitals and health centers to better understand the routes of *C. difficile* transmission.

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JULS RESEARCH

The association between comorbid anxiety disorders and pro-inflammatory cytokine levels in individuals with bipolar disorder

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Abstract

Studies have shown that abnormal activation of inflammatory responses play a salient role in the pathogenesis and progression of bipolar disorder (BD). The amount of pro-inflammatory cytokines, mediators of inflammatory responses, has been found to be elevated in individuals with BD. Meanwhile, the prevalence of anxiety disorders has been reported to be higher among individuals with BD, which usually worsen its course. This study aimed to investigate the effects of comorbid anxiety disorders on inflammatory cytokine concentrations of BD patients. Cytokine levels were compared between BD individuals with and without different comorbid anxiety disorders. We found that [1] BD individuals with comorbid social phobia had higher levels of interleukin 8 (IL-8), and [2] BD individuals with comorbid generalized anxiety disorder (GAD) had higher concentrations of IL-5 and IL-8. Taken together, these findings suggest that an association exists between the presence of anxiety disorders and increased levels of pro-inflammatory cytokines, notably IL-5 and IL-8, in individuals with BD.

Introduction

The etiology of BD is complex, having genetic, physiological, and environmental components [1, 2]. In previous years, growing evidence has indicated that alterations in inflammatory responses play a salient role in both the pathogenesis and progression of BD [1, 2, 3, 4]. An altered cytokine profile has been one of the most common observations found in individuals with BD. Elevations in pro-inflammatory cytokines, including IL-6, soluble IL-2 receptor (sIL-2R), and tumor necrosis factor-alpha (TNFα), have been observed during manic and depressive episodes, suggesting that the two episodes are associated with a pro-inflammatory state [1, 5, 6]. Some decreases in the anti-inflammatory cytokines, including IL-4 and IL-10, as compared to a euthymic state, have also been suggested [1]. The pro-inflammatory state of mania and depression is probably resolved during euthymia [7]. However, there are studies reporting that cytokines are also altered in euthymia [5].

The balance between pro-inflammatory and anti-inflammatory cytokines is closely associated with neurotoxic and neuroprotective mechanisms [3, 8, 9]. The process of illness progression in BD patients can be attributed to the shift of the balance towards the pro-inflammatory side during mania and depression [3, 10]. TNFα initiates the neuronal apoptosis cascade and inhibits the insulin-like growth factor 1 (IGF-1) receptor, contributing to neurological impairment in the central nervous system (CNS) [6]. IL-1 mediates stress-induced decreases in brain-derived neurotrophic factor (BDNF) [11]. It also promotes the inflammation process and augments inflammatory brain injuries by stimulating the generation of other factors including phospholipase A2 cyclooxygenase, nitric oxide (NO), and superoxide, all of which mediate excitotoxicity and apoptosis [3, 12]. Neuronal apoptosis can be further triggered via interferon-gamma (IFNγ), which stimulates the production of NO by inducible Nitric Oxide Synthase (iNOS) [13].

Besides their negative impact on neuronal survival, pro-inflammatory cytokines can influence patients' moods by altering monoamine neurotransmitter metabolism [1]. They induce the expression of indoleamine-2,3-dioxygenase (IDO), an enzyme catalyzing the synthesis of kynurenine from tryptophan in immunocompetent cells [11, 13]. Tryptophan is a requisite precursor for the synthesis of several important neurotransmitters, including serotonin and melatonin. Thus, increased IDO activity leads to the degradation of tryptophan, which reduces the synthesis of serotonin in the brain, and hence contributes to the depressive symptoms of BD [11, 13]. Elevated mono-amine oxidase A (MAO-A) induced by cytokines has been considered as another factor which contributes to the monoamine imbalance [14]. Increases in monoamine metabolism lead to a moderate to severe monoamine loss in depressed patients [14].

Another proposed endocrine signaling mechanism links altered cytokine levels to abnormal glucocorticoid receptor (GR) signaling in individuals with BD [1, 2]. The hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis is caused by the reduced sensitivity of GR [15, 16]. Pro-inflammatory cytokines not only decrease the gene expression of GR, but also block the translocation of GR between the nucleus and cytoplasm [15]. Moreover, the expression of GR beta, an inert isoform of GR, is induced by inflammatory cytokines

[15]. However, the elevated level of glucocorticoids cannot elicit a corresponding level of anti-inflammatory effect due to insufficient glucocorticoid signaling [1].

The interactions among the immune system, CNS, and the neuroendocrine system, as described above, play a salient role in the pathophysiology of not only BD and major depressive disorder (MDD), but also in other neuropsychiatric conditions such as anxiety disorders, schizophrenia, and Alzheimer's disease. [17]. In fact, significant percentages of individuals with BD have been diagnosed with these psychiatric comorbidities. Among them, anxiety has been reported as one of the most common comorbidities, and the prevalence of anxiety disorders in the BD population is higher than that in the general population [18]. It has been shown that comorbid anxiety disorders in individuals with BD worsen the course of the disease by increasing both the frequency and duration of depressive episodes [18, 19]. The presence of two or more illness conditions of shared pathophysiology in individuals with BD is suspected to intensify the pathophysiological processes, which can be manifested as increased amounts of mediators and enhanced signaling pathways.

Thus, the focus of this study is to test the potential additive effect of comorbid anxiety disorders on cytokine levels in individuals with BD. More specifically, we hypothesized that the presence of anxiety comorbidities in individuals with BD will be associated with elevations in pro-inflammatory cytokine concentrations.

Methods and Materials

Participants

One hundred and three individuals clinically verified with BD I/II were enrolled in the randomized, double-blind, placebo-controlled trial evaluating the effect of intranasal insulin on neurocognitive function. Forty-five participants who completed the baseline visit and provided a blood sample were included in this post hoc analysis. DSM-IV-TR defined BD I/II was verified with the Mini International Neuropsychiatric Interview (M.I.N.I. Plus 5.0). All subjects were prospectively verified to be euthymic, which was defined as a score of 3 or less on the Hamilton Depression Rating Scale (HAMD-7) and 7 or less on the Young Mania Rating Scale (YMRS). Eligible participants were outpatients of the Mood Disorders Psychopharmacology Unit (MDPU), University Health Network (UHN).

Procedures

Blood samples were obtained at the baseline visit after a 12 hour overnight fast. EDTA tubes (6ml) were used to collect the blood samples for cytokine analysis. Samples were centrifuged at room temperature at 3000 rpm for 10 minutes, and the plasma was stored at -80^{0}C until analysis. Plasma cytokine levels (GM-CSF, IL-1β, IL-2, IL-5, IL-6, IL-8, IFNγ, and TNFα) were measured with the Ultrasensitive Human Cytokine 10-plex bead-based Panel for Luminex (Invitrogen, Cat. No. LHC6004). Assays were performed in duplicate based on the manufacturer's protocol.

Statistical analysis

Data analysis was conducted using IBM SPSS Statistics 15.0 (SPSS Inc., Chicago, IL). Data was first screened for normality. The raw data of cytokine concentrations was positively skewed and it was log transformed in order to achieve normality. A chi-square test was used to detect differences in categorical measures, and an independent-samples t-test was used to detect differences between two groups on continuous measures. A multiple linear regression analysis was employed to determine if the effect of anxiety disorders on cytokine concentrations persists after taking age and gender into account. All p values were two-tailed and the significance level was set at $p<0.05$.

Results

There were seven different anxiety disorders evaluated, namely current and lifetime panic disorder (PD), current and lifetime agoraphobia, current social phobia, current specific phobia, current obsessive compulsive disorder (OCD), current post-traumatic stress disorder (PTSD), and current generalized anxiety disorder (GAD). The socio-demographic characteristics and cytokine levels of individuals with BD were compared between individuals with and without one specific subtype of anxiety disorders. Only lifetime PD, lifetime agoraphobia, current social phobia, and current GAD were selected as independent variables. Among 45 participants, 44 had diagnostic information on current social phobia and 37 had diagnostic information on lifetime agoraphobia. The sample sizes of individuals with current PD, current agoraphobia, specific phobia, OCD and PTSD were too small to do independent-samples t-tests with sufficient validity and reliability.

Socio-demographic characteristics

Six socio-demographic characteristics had been taken into account, which were age, sex, ethnic origin, marital status, education, and employment status (Table 1). Among 45 participants, 18 did not provide information with regard to their marital status and employment status. Twenty three males (51.1%) and twenty two females (48.9%) were included in this study. The mean age of all participants was 41.0±9.9. The mean ages of males and females were 40.2±9.8 and 41.8±10.2, respectively ($p>0.05$). The majority of participants enrolled were Caucasians (93.3%). None of the six socio-demographic parameters were significantly different between individuals with or without a diagnosis of lifetime PD, lifetime agoraphobia or current social phobia. In the comparison between individuals with and without current GAD, there was a significant difference in the composition of employment status ($\chi^2=12.27$, df=5, $p=0.03$). There were more individuals who were unemployed and disabled among individuals with current GAD.

Current social phobia

Statistically significant differences were found in the plasma IL-5 ($t=2.52$, df=40, $p=0.016$) and IL-8 levels ($t=2.55$, df=42, $p=0.014$) (Table 2). IL-5 and IL-8 levels in individuals with social phobia versus no social phobia were 1.72±0.91 vs. 1.18±0.48 and 2.56±0.48 vs. 2.12±0.52, respectively. The multiple linear regression analysis, controlling for age and sex, showed that social phobia significantly contributed to the higher levels of IL-8 ($R^2=0.230$, $p=0.014$) observed in individuals with social phobia. The difference in IL-5 was not significant in the multiple regression analysis ($R^2=0.149$, $p=0.102$).

Current GAD

Statistically significant differences were found in the plasma concentration of IL-5 ($t=3.05$, df=41, $p=0.004$), IL-8 ($t=2.40$, df=43, $p=0.021$), and IFNγ ($t=2.24$, df=43, $p=0.030$) (Table 3). IL-5, IL-8, and IFNγ concentrations in individuals with GAD versus no GAD were 1.83±0.83 vs. 1.18±0.52, 2.57±0.52 vs. 2.15±0.51, and 2.86±1.24 vs. 2.00±1.07, respectively. The multiple linear regression analysis showed that GAD predicted increased levels

Table 1: Sociodemographic characteristics of bipolar patients grouped based on types of anxiety disorders

Parameters	With social phobia	Without social phobia			With GAD	Without GAD		
	Mean(SD)	Mean(SD)	t (df)	p value	Mean (SD)	Mean (SD)	t (df)	p value
Age (years)	n=12 44.7(9.7)	n=32 39.3(9.6)	1.66(42)	0.11	n=11 45.9 (9.3)	n=34 39.4 (9.7)	1.95(43)	0.06
Education (years)	n=11 16.4 (3.1)	n=30 16.3 (2.8)	0.11 (39)	0.92	n=11 16.0 (3.0)	n=31 16.5 (2.8)	0.50(40)	0.62
	n (%)	n (%)	χ^2 (df)	p value	n (%)	n (%)	χ^2df)	p value
Sex	n=12	n=32	0.03 (1)	1.00	n=11	n=34	0.19 (1)	0.67
Male	6(50.0)	17 (53.1)			5 (45.5)	18 (52.9)		
Female	6(50.0)	15 (46.9)			6 (54.5)	16 (47.1)		
Ethnic origin	n=12	n=32	3.42 (2)	0.18	n=11	n=34	3.75 (2)	0.15
Caucasian	11 (91.7)	30 (93.8)			10 (94.1)	32 (90.9)		
Black	-	-			-	-		
Asian	0 (0)	2 (6.3)			2 (5.9)	0 (0)		
Other	1 (8.3)	0 (0)			0 (0)	2 (9.1)		
Marital status	n=9	n=18	3.12 (3)	0.37	n=10	n=17	2.62 (3)	0.45
Never married	3 (33.3)	10 (55.6)			4 (40.0)	9 (52.9)		
Married	6 (66.7)	6 (33.3)			5 (50.0)	7 (41.2)		
Divorced	0 (0)	1 (5.6)			0 (0)	1 (5.9)		
Widowed	0 (0)	1 (5.6)			1 (10.0)	0 (0)		
Employment status	n=9	n=18	3.40 (5)	0.64	n=10	n=17	12.27 (5)	0.03
Employed	5 (55.6)	8 (44.4)			4 (40.0)	9 (52.9)		
Disability	1 (11.1)	2 (11.1)			3 (30.0)	0 (0)		
Student	0 (0)	1 (5.6)			0 (0)	1 (5.9)		
Unemployed	2 (22.2)	1 (5.6)			2 (20.0)	1 (5.9)		
Homemaker	-	-			-	-		
Retired	0 (0)	1 (5.6)			1 (10.0)	0 (0)		
Other	1 (11.3)	5 (27.8)			0 (0)	6 (35.3)		

Table 1: Sociodemographic characteristics of bipolar patients grouped based on types of anxiety disorders (continued)

Parameters	With	Without			With	Without		
	Mean(SD)	Mean(SD)	t (df)	p value	Mean (SD)	Mean (SD)	t (df)	p value
Age (years)	n=10 41.0(9.4)	n=35 41.0(10.2)	0.00(43)	1.00	n=10 38.8(11.1)	n=27 42.7 (7.4)	1.25(35)	0.22
Education (years)	n=9 16.8 (2.9)	n=33 16.2 (2.8)	0.61 (40)	0.55	n=9 16.0 (2.9)	n=25 16.7 (3.1)	0.61 (32)	0.55
	n (%)	n (%)	χ^2 (df)	p value	n (%)	n (%)	χ^2 (df)	p value
Sex	n=10	n=35	0.64 (1)	0.43	n=10	n=27	1.09 (1)	0.30
Male	4 (40.0)	19 (54.3)			4 (40.0)	16 (59.3)		
Female	6 (60.0)	16 (45.7)			6 (60.0)	11 (40.7)		
Ethnic origin	n=10	n=35	4.64 (2)	0.10	n=10	n=27	5.71 (2)	0.06
Caucasian	8 (80.0)	34 (97.1)			8 (80.0)	27 (100)		
Black	-	-			-	-		
Asian	1 (10.0)	1 (2.9)			1 (10.0)	0 (0)		
Other	1 (10.0)	0 (0)			1 (10.0)	0 (0)		
Marital status	n=6	n=21	1.78 (3)	0.62	n=5	n=17	0.73 (2)	0.70
Never married	2 (33.3)	11 (52.4)			3 (60.0)	7 (41.2)		
Married	4 (66.7)	8 (38.1)			2 (40.0)	9 (52.9)		
Divorced	0 (0)	1 (4.8)			0 (0)	1 (5.9)		
Widowed	0 (0)	1 (4.8)			-	-		
Employment status	n= 6	n=21	5.93 (5)	0.31	n=5	n=17	8.47 (4)	0.08
Employed	3 (50.0)	10 (47.6)			1 (20.0)	10 (58.8)		
Disability	2 (33.3)	1 (4.8)			0 (0)	2 (11.8)		
Student	0 (0)	1 (4.8)			1 (20.0)	0 (0)		
Unemployed	1 (16.7)	2 (9.5)			2 (40.0)	1 (5.9)		
Homemaker	-	-			-	-		
Retired	0 (0)	1 (4.8)			-	-		
Other	0 (0)	6 (28.6			1 (20.0)	4 (23.5)		

Table 2: Comparisons of pro-inflammatory cytokine levels between BD patients with and without current social phobia and between BD patients with and without GAD.

Cytokine concentrations (Log transformed)	With social phobia Mean(SD)	Without social phobia Mean(SD)	t(df)	p value	With GAD Mean (SD)	Without GAD Mean (SD)	t(df)	p value
GM-CSF	n=12 3.15 (1.54)	n=30 2.79 (1.28)	0.78(40)	0.439	n=10 3.27 (1.39)	n=33 2.79 (1.31)	1.01 (41)	0.319
IL-1β	n=11 0.00 (0.34)	n=32 0.06 (0.41)	0.39(41)	0.696	n=11 -0.06 (0.22)	n=33 0.06 (0.43)	0.88 (42)	0.386
IL-2	n=11 1.76 (0.99)	n=30 1.71 (1.05)	0.12(39)	0.902	n=11 1.79 (0.89)	n=31 1.74 (1.09)	0.11 (40)	0.911
IL-5	n=12 1.72 (0.91)	n=30 1.18 (0.48)	2.52(40)	0.016	n=11 1.83 (0.83)	n=32 1.18 (0.52)	3.05 (41)	0.004
IL-6	n=12 1.95 (0.73)	n=31 1.85 (0.89)	0.35(41)	0.731	n=10 1.94 (0.65)	n=34 1.88 (0.89)	0.21 (42)	0.838
IL-8	n=12 2.56 (0.48)	n=32 2.12 (0.52)	2.55(42)	0.014	n=11 2.57 (0.52)	n=34 2.15 (0.51)	2.40 (43)	0.021
IFNγ	n=12 2.53 (1.41)	n=32 2.07 (1.06)	1.17(42)	0.248	n=11 2.86 (1.24)	n=34 2.00 (1.07)	2.24 (43)	0.030
TNFα	n=10 2.75 (0.71)	n=32 3.06 (0.91)	0.97(40)	0.338	n=11 2.61 (0.66)	n=32 3.14 (0.90)	1.77 (41)	0.085

of IL-5 (R^2=0.197, p=0.034) and IL-8 (R^2=0.214, p=0.018). IFNγ however, was not predicted by GAD after the controlling of age and sex (R^2=0.140, p=0.100).

Lifetime PD and agoraphobia

The cytokine levels of BD individuals with lifetime PD and agoraphobia were not different from individuals without lifetime PD and agoraphobia, respectively.

Discussion

The main findings of this post hoc analysis are 1) current social phobia in individuals with BD is associated with increased concentration of IL-8 as compared to individuals without social phobia, and 2) current GAD in individuals with BD is associated with increased concentrations of IL-5 and IL-8 as compared with individuals without GAD.

IL-5 and IL-8 levels in patients with social phobia/GAD

Although increased pro-inflammatory cytokine concentrations in individuals with BD have been reported in many studies, few studies have compared cytokine levels between BD individuals with and without anxiety comorbidity. In the study herein, increased IL-8 concentration was strongly associated with the presence of social phobia and GAD in euthymic individuals with BD. The concentrations of IL-5 were also found to be higher in individuals with comorbid GAD compared to individuals without GAD. IL-5 and IL-8 are pro-inflammatory cytokines that are able to stimulate inflammation under certain circumstances. IL-5 is an interleukin produced by mast cells and T helper-2 cells, and its main function is to stimulate the growth and differentiation of eosinophils and B cells [20]. Few studies have related IL-5 to mood disorders or any other psychiatric disorders. Most of the time, elevations in IL-5 concentration have been associated with asthma and some allergic responses. Increases in IL-8 during both manic and depressive states have been reported in previous studies [21]. IL-8 is produced and secreted by macrophages and several other cell types including epithelial cells. It acts as a chemo-attractant that directs the movement of immune cells through chemotaxis [13]. Elevations in concentrations of these two pro-inflammatory cytokine in individuals with comorbid social phobia or GAD support our hypothesis that anxiety comorbidities are associated with increased pro-inflammatory cytokines in individuals with BD.

Other cytokine levels in patients with social phobia/GAD

There were no significant differences in other pro-inflammatory cytokine including IL-1β, IL-2, IL-6, and TNFα between individuals with and without comorbid anxiety disorders. In fact, increases in IL-1β, IL-6, and TNFα have been reported mostly in studies that compared BD to healthy controls. It was unclear why IL-5 and IL-8 levels increased while others did not. We speculated that the euthymic state of individuals with BD might be one of the factors that contributed to the non-significant results. Most of the elevations in IL-1β, IL-6, and TNFα were found during manic and depressive episodes. It has been suggested that the pro-inflammatory state of mania and depression resolves during euthymia [5, 7]. Therefore, pro-inflammatory mediators including IL-1β, IL-6, and TNFα might not show differences during the euthymic state, which is not considered to be a pro-inflammatory state. On the other hand, IL-5 and IL-8 which are mainly involved in initiating rather than mediating inflammation responses might be present in higher concentrations during euthymic states.

Cytokine levels in patient with lifetime anxiety disorders

BD individuals with lifetime but not current anxiety disorders, including PD and agoraphobia, did not show any difference in cytokine concentrations as compared to patients without anxiety disorders. This finding suggests that the association between cytokine levels and anxiety disorders might be temporary. Increased cytokine levels were more likely to be associated with the current state of anxiety disorders than with lifetime anxiety disorders.

Limitations and future directions

The major limitation of this study is the relatively small sample size. It prevented us from evaluating BD-I and BD-II separately. It

is possible that anxiety disorders affect the cytokine levels of individuals with BD-I and BD-II differently. Also, we were restricted to studies of only two current anxiety disorder diagnoses, social phobia and GAD, both in which significant effects on cytokine levels were identified. We were not able to study the effects of PD, agoraphobia, specific phobia, OCD, and PTSD due to the small sample size. Another limitation is that cytokine levels can be affected by many variables including body mass index (BMI), smoking habits, and drinking habits, which were not controlled, as otherwise the resulting sample size would be too small to study. The use of other medications can also affect the cytokine levels in individuals with BD. This is also the reason why there are many inconsistent findings regarding cytokine concentrations in BD patients. More studies, with larger sample sizes and more complete patient profiles, are needed to further detect and evaluate the effects of anxiety disorder comorbidities on cytokine concentrations in individuals with BD.

Conclusion

The major findings of this study are the increased concentrations of IL-5 and IL-8 in BD individuals with comorbid social phobia or GAD. They support our hypothesis that anxiety disorder comorbidities are associated with elevations in pro-inflammatory cytokine levels. Also, comorbid anxiety disorders might enhance cytokine levels selectively. BD individuals with lifetime, but not current anxiety disorders, showed no difference compared to patients without anxiety disorders, suggesting that such an association might require the current state of anxiety disorder.

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Neuroprotective Effects of Radix Rehmanniae and Arisaema Cum Bile on Amyloid-Beta-Induced Toxicity in PC12 Cells

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Abstract

Amyloid-β peptide (Aβ) induced toxicity has long been regarded as the primary cause of the neuronal deaths observed in Alzheimer's disease. In this experiment, the toxic effects of Aβ on an Aβ-sensitive neuronal cell line were used as a screening tool to find a potential anti-Alzheimer's drug. Eleven Chinese herbal drugs were chosen (*Caulis spatholobi, Rhizoma cyperi, Cortex Cinnamomi, Radix Astragali, Coptis chinensis Franch, Rhizoma atractylodis macrocephala, Radix Aconiti Lateralis Preparata, Radix Scrophulariae, Radix Rehmanniae, Poria cum Radix Pini, Arisaema Cum Bile*) for examination of their ability to protect PC12 cells from Aβ-induced reactive oxygen species (ROS) formation, caspase-3 activity, and DNA fragmentation. Compared to the control condition in the absence of any tested compounds, Radix Rehmanniae and Arisaema Cum Bile significantly increased cell viability. While exposure of the control to 20μM $A\beta_{1\text{-}40}$ for 24 hours resulted in a significant amount of ROS formation, Radix Rehmanniae and Arisaema Cum Bile were able to suppress oxidative stress in a dose-dependent manner. Similarly, Radix Rehmanniae and Arisaema Cum Bile also decreased caspase-3 activity. Since previous studies have illustrated that capase-3 induces DNA fragmentation, the measured decrease in DNA fragmentation reflects the amount of intracellular capase-3 activity. All things considered, the study demonstrates that Radix Rehmanniae and Arisaema Cum Bile play a protective role in the presence of $A\beta_{1\text{-}40}$-induced cell death and should be considered as a potential therapeutic drug for Alzheimer's disease.

Introduction

Alzheimer's disease (AD) is a debilitating disease that affects an increasing number of people worldwide. It is characterized by neuronal degeneration, extracellular formation of amyloid plaques, consisting of aggregates of amyloid-β (Aβ) peptides, and intraneuronal neurofibrillary tangles (NFT) composed of aggregates of the tau protein [1, 2]. Aβ is a 39-43 amino acid peptide fragment derived from the proteolytic cleavage of amyloid precursor protein (APP). Aβ aggregates into amyloid fibrils, which have been reported to be neurotoxic in vitro and in vivo [3, 4]. Thus, the modulation of Aβ toxicity has been widely utilized as a therapeutic method in finding potential anti-dementia agents. In this regard, Aβ-induced toxicity in a cultured neuronal cell line can be used as a screening tool for identifying potential agents for the treatment of AD.

Chinese herbal medicine has a long history of treating dementia and neurological symptoms [5]. An example of such a treatment is the Korean Palmul-Chongmyeong-Tang's ability to protect against cerebral ischemia-induced neuronal and cognitive impairments [6]. However, in depth analyses of such herbs and their potential neuroprotective effects against AD have been largely lacking.

In this experiment, eleven potential Chinese herbs were selected for assessment in their ability to protect rat adrenal medulla pheochromocytoma 12 (PC12) cells from the Aβ-induced cytotoxic effects. The main neuroprotective activities assessed include the ability of these herbs to reduce reactive oxygen species (ROS), caspase-3 activity, and DNA fragmentation.

Methods and Materials

Chemicals and Reagents

Amyloid-β peptides ($A\beta_{1\text{-}40}$), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Dulbecco's modified Eagle medium (DMEM) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2',7'-Dichlorofluorescin diacetate (DCFH-DA) was obtained from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco (Grand Island, NY, USA). All other reagents and chemicals used in the study were of analytical grade.

Plant Materials and Extraction

All the herbs were purchased from Zhixin Pharmaceutical Co. (Guangzhou, China), and authenticated by one of the authors (ZXL, an herbalist and pharmacognosist) by comparison with authenticated specimens stored in the Herbarium of the School of Chinese Medicine, Faculty of Science, The Chinese University of Hong Kong. Voucher specimens have been deposited in the Herbarium of the School, with reference no. AD 01-11.

To prepare the extracts, all herbal substances were ground to a powder using an electrical blender. 50g of the powdered medicinal substances were then macerated in 2000 mL of 80% aqueous ethanol for 1 hour at

Table 1: The yield of extracts in herbal drugs preparation.

Name of Herb drugs	Yield (%)
Caulis spatholobi	12.75
Rhizoma cyperi	5.64
Cortex Cinnamomi	11.30
Radix Astragali	20.80
Coptis chinensis Franch	19.85
Rhizoma atractylodis macrocephala	7.14
Radix Aconiti Lateralis Preparata	1.72
Radix Scrophulariae	23.49
Radix Rehmanniae	34.74
Poria cum Radix Pini	2.18
Arisaema Cum Bile	12.56

room temperature, and then refluxed for 2 hours. The extraction was repeated twice. All three filtrates were combined and then concentrated in a rotary evaporator under reduced pressure, followed by freeze drying. The yields of the extracts are shown in Table 1. The resultant extracts were stored at -20°C for subsequent bioassay testing.

The extracts were reconstituted in dimethyl sulfoxide (DMSO) to produce respective stock solutions and then diluted with culture medium to various concentrations. The final DMSO concentration in each sample was less than 0.1%, and this concentration did not affect cell growth or cause cell death.

Preparation of Aggregated $A\beta_{25-35}$

$A\beta_{1-40}$ was dissolved in deionized distilled water at a concentration of 1 mM and incubated at 37°C for 4 days to induce aggregation (Li et al. 2008). After aggregation, the solution was stored at -20 °C until use.

Cell Culture and Drug Treatments

PC12 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 unit/mL penicillin, 100 µL/mL streptomycin, 6% fetal bovine serum, and 6% horse serum at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The PC12 cells were split at a density of 2 x 10^4 cells/well, plated into poly-D-lysine-coated 96-well plates (Corning Incorporated, USA) and allowed to adhere for 48 h at 37°C with complete DMEM. The cells were seeded onto 96-well culture plate at a density of 2 x 10^4 cells/well, unless otherwise specified. The cells were first stabilized at 37°C for 48 h and subsequently cultured in serum-free medium followed by incubation with different concentrations of the herbal extracts for 2 hours. $A\beta_{1-40}$ at a final concentration of 10 µM was added to the culture for an additional 24 hours.

Cell Viability Assay

Cell viability was measured by quantitative colorimetric assay with the methylthiazol tetrazolium MTT method as described previously by Xian, Y.F [3]. 24 hours after drug treatment, the cells were treated with 20 µL/well of MTT solution (final concentration: 1mg/mL) for 4 hours. The supernatants were aspirated off and the dark blue formazan crystals formed in the intact cells were dissolved in 150 µL DMSO. The optical density of each well was measured with FLUOstar OPTIMA microplate reader (BMG Labtech, Offenbury, Germany) at 570 nm. Cell viability was expressed as a percentage of non-treated control.

Measurement of Reactive Oxygen Species (ROS)

Intracellular ROS levels were measured using the 2',7'-dichlorofluorescin diacetate (H_2DCF-DA) method [3]. H_2DCF-DA is a non-fluorescent molecule that diffuses passively into cells where acetates are cleaved by intracellular esterases to form H_2DCF. In the presence of intracellular ROS, H_2DCF is rapidly oxidized to the highly fluorescent DCF. After the end of drug treatment, the cells were washed with D-Hanks solution and incubated with H_2DCF-DA at a final concentration of 10 µM for 30 min in the dark at 37 °C. The cells were then washed twice more with D-Hanks solution to remove the extracellular H_2DCF-DA. The fluorescence intensity of DCF was measured with a microplate reader at an excitation wavelength of 485 nm and emission wavelength of 538 nm. The level of intracellular ROS was expressed as percentage of non-treated control.

Measurement of Caspase-3 Activity

The activity of caspase-3 was measured using a colorimetric assay kit (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's protocol. In brief, PC12 cells were seeded onto a 6-well culture plate at a concentration of 2 x 10^6 cells/well. After drug treatment, the cells were washed with D-Hanks solution and incubated with 500 µL lysis buffer on ice for 30 min. The cells were then transferred to a centrifuge tube and centrifuged at 16,000xg for 10 min at 4°C. An aliquot of the supernatant was incubated with the substrate (acetyl-Asp-Glu-Val-Asp-p-nitroanilide) at 37°C for 90 min. The activity of caspase-3 was measured spectrophotometrically using a microplate reader at 405 nm, and data were expressed as a percentage of non-treated control.

Measurement of DNA Fragmentation

Quantification of DNA fragmentation was determined by Cell Death Detection ELISA^{Plus} kit (Roche Applied Sciences, Basel, Switzerland) according to the manufacturer's protocol. After herbal drug treatment, the cells were washed with D-Hanks solution. The cells were incubated with 200 µL lysis buffer for 30 min at room temperature while the plate was centrifuged at 200xg for 10 min at 4°C. 20 µL of the supernatant from each well was transferred to a streptavidin-coated microplate and incubated with a mixture of anti-histone-biotin and anti-DNA-peroxidase. The apoptotic nucleosomes were captured via their histone component by the anti-histone-biotin antibody, which was bound to the streptavidin-coated microplate. Simultaneously, DNA-peroxidase was also bound to the DNA component of the nucleosomes. After removing the unbound antibodies, the amount of peroxidase retained in the immunocomplex was quantified by adding 2,2'-azinobis (3-ethylbenzthiazoline-6-sulphonic acid; ABTS) as the substrate. The reaction mixture absorbance was measured at 405 nm using a microplate reader. The absorbance is directly proportional to the number of apoptotic nucleosomes. The extent of DNA fragmentation was expressed as percentage of non-treated control.

Statistical Analysis

Data are expressed as mean ± SEM. Multiple group comparisons were performed using one-way analysis of variance (ANOVA) followed by Dunnett's test to detect inter-group differences, using the non-treated group as the control. Statistical analysis was performed using the GraphPad Prism software (Version 4.0; GraphPad Software, Inc., San Diego, CA). Differences were deemed statistically significant if $p < 0.05$.

Results

Effect of Herbal Drugs on $A\beta_{1-40}$-induced Cytotoxicity in PC12 Cells

The cytotoxic effect of $A\beta_{1-40}$ peptide on PC12 cells and the protective effects of compounds on cell viability were assessed by MTT assay. As shown in Figure 1, treatment with $A\beta_{1-40}$ for 24 hours reduced cell viability to 58% of the control value (100%).

Among the 11 medicinal substances tested, only Radix Rehmanniae (100 and 200 µg/mL) and Arisaema cum Bile (50, 100 and 200 µg/mL) significantly increased cell viability (Figure 1). The remaining extracts failed to significantly increase cell viability, indicating that only Radix Rehmanniae and Arisaema Cum Bile conferred protection against $A\beta_{1-40}$-induced cytotoxicity to PC12 cells.

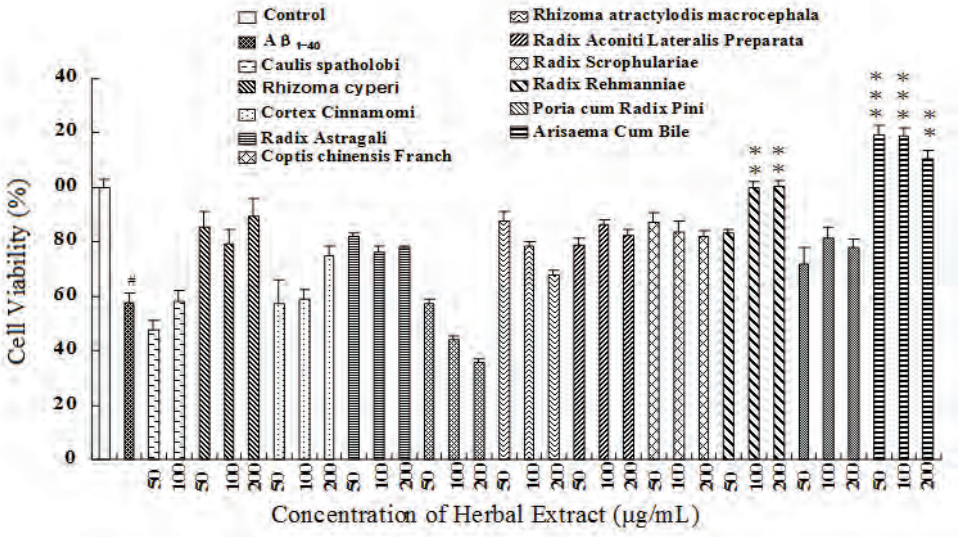

Figure 1: Effects of eleven herbal extracts on $A\beta_{1-40}$-induced cytotoxicity in PC12 cells based on concentration of herbal extracts (µg/mL) versus cell viability (%). Values given are the mean ± SEM (n = 6). #p<0.001 compared with the control group; **p<0.01, ***p<0.001compared with the Aβ1-40-treated control group.

Effect of Radix Rehmanniae and Arisaema Cum Bile on $A\beta_{1-40}$-induced Oxidative Stress in PC 12 Cells

As shown in Figure 2, exposure of PC12 cells to 10 µM $A\beta_{1-40}$ for 24 hours significantly increased the level of intercellular ROS to 267% of the control value (100%), which suggests that $A\beta_{1-40}$ provoked oxidative stress. Treatment with Radix Rehmanniae (50, 100 and 200 µg/mL) and Arisaema cum Bile (25, 50, 100 and 200 µg/mL) significantly decreased the amount of intracellular ROS in a dose-dependent manner (Figure 2).

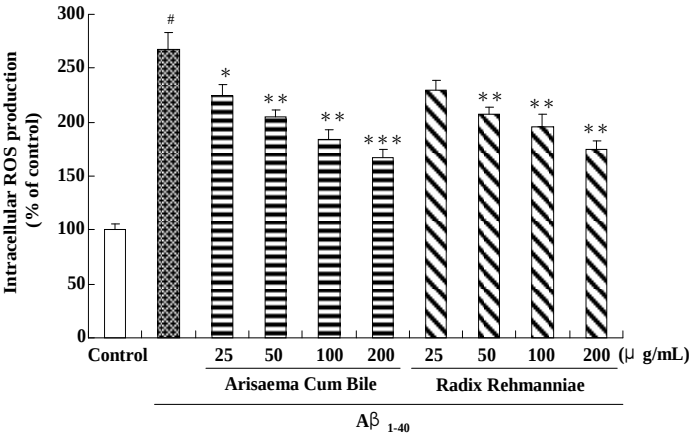

Figure 2. Effects of Radix Rehmanniae and Arisaema cum Bile on the intracellular ROS production in Aβ1-40-treated PC12 cells. Values given are the mean ± SEM (n = 6). #p<0.001 compared with the control group; *p<0.05, **p<0.01 and ***p<0.01 compared with the Aβ1-40-treated control group.

Effect of Radix Rehmanniae and Arisaema Cum Bile $A\beta_{1-40}$-induced Caspase-3 Activity in PC12 Cells

Incubation of PC12 cells in the presence of $A\beta_{1-40}$ for 24 hours significantly augmented caspase-3 activity to 259% of the control value (100%). Caspase-3 activity decreased significantly in a concentration-dependent manner when treated with Radix Rehmanniae (50, 100 and 200 µg/mL) and Arisaema cum Bile (25, 50, 100, and 200 µg/mL) (Figure 3).

Effect of Radix Rehmanniae and Arisaema Cum Bile on Aβ1-40-induced DNA Fragmentation in PC12 Cells

In this experiment, DNA fragmentation was quantified by the presence and amount of peroxidase retained in the immunocomplex, which suggests the peroxidase's unattached state to the DNA. As shown in Figure 4, exposure to $A\beta_{1-40}$ in the absence of any drugs significantly increased the DNA fragmentation to 256% of the control value. Treatment with Radix Rehmanniae (50, 100, and 200 µg/mL) and Arisaema cum Bile (50, 100, and 200 µg/mL) significantly attenuated the extent of DNA fragmentation in a dose-dependent manner.

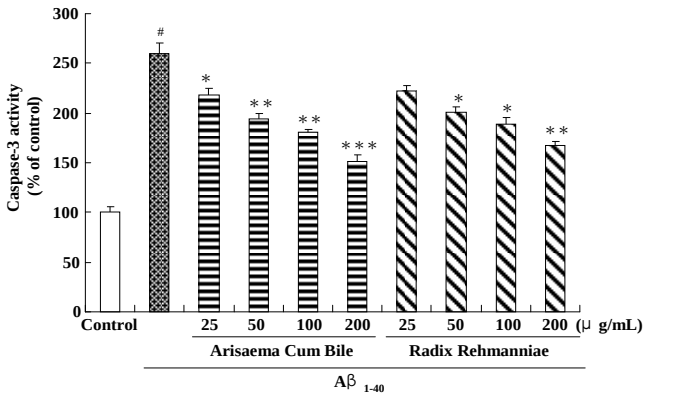

Figure 3: Effects of *Radix Rehmanniae* and *Arisaema cum Bile* on caspase-3 activity in $A\beta_{1-40}$-treated PC12 cells. Values given are the mean ± SEM (n = 6). #p<0.001 compared with the control group; *p<0.05, **p<0.01 and ***p<0.01 compared with the $A\beta_{1-40}$-treated control group.

Discussion

Aβ has been vastly classified as the main cause of Alzheimer's disease. Although the mechanism is still unclear, it has been noted that Aβ induces oxidative stress through the production of free radicals such as H_2O_2, as well as resulting in membrane injury by forming calcium-permeable channels in the cell membrane [6, 1]. Since a cure has not been found, our study focuses on the search for potential anti-dementia drugs that may prevent the development of Alzheimer's disease.

Eleven Chinese herbal compounds were selected to be screened for neuroprotective activities in PC12 cells: Caulis spatholobi, Rhizoma cyperi, Cortex Cinnamomi, Radix Astragali, Coptis chi-

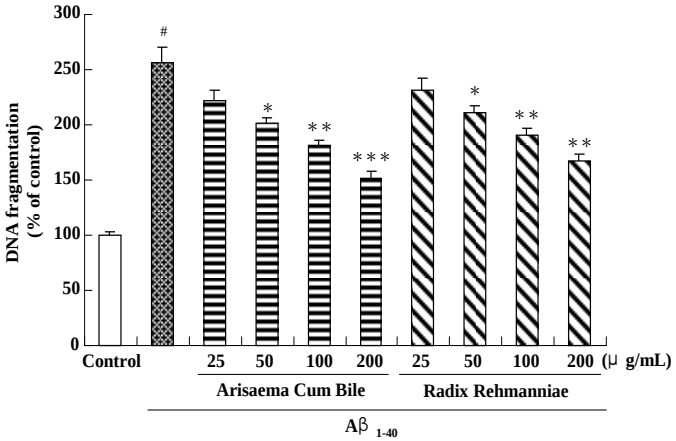

Figure 4. Effects of Radix Rehmanniae and Arisaema cum Bile on DNA fragmentation in Aβ1-40-treated PC12 cells. Values given are the mean ± SEM (n = 6). #p<0.001 compared with the control group; *p<0.05, **p<0.01 and ***p<0.01 compared with the Aβ1-40-treated control group.

nensis Franch, Rhizoma atractylodis macrocephala, Radix Aconiti Lateralis Preparata, Radix Scrophulariae, Radix Rehmanniae, Poria cum Radix Pini, and Arisaema Cum Bile. Amongst these compounds, Radix Astragali, Radix Rehmanniae, and Arisaema Cum Bile demonstrated the ability to protect neurons against 20 μM $A\beta_{1\text{-}40}$, with Radix Rehmanniae and Arisaema Cum Bile exhibiting the most significant effects. To date, Radix Rehmanniae has been noted for its neuroprotective effects against ischemia-induced cholinergic neuron deaths but no in-depth studies have been done on Arisaema Cum Bile in the field of neuroscience [6]. Similarly, neither compound has been probed with regards to its neuroprotective capability against the cytotoxicity of Aβ. Thus, the results of this study provide a preliminary rationale for the use of Radix Rehmannae and Arisaema Cum Bile in the treatment of AD.

Previous studies have provided evidence that Aβ induces the productions of intracellular reactive oxygen species (ROS), lipid peroxidation, and protein oxidation, all of which contribute to the increased oxidative stress observed in AD patients [8]. Likewise, studies have also suggested that antioxidants such as vitamin E can block Aβ toxicity in this respect [9]. In this study, Radix Rehmanniae and Arisaema Cum Bile reduced the production of ROS induced by $A\beta_{1\text{-}40}$ in a dose-dependent manner, which suggests that the two compounds provide neuroprotective effects in part by their antioxidant mechanisms.

Current evidence also implies that Aβ toxicity causes neuronal cells to enter apoptosis through the activation of a family of aspartic acid-specific cysteine proteases referred to as caspases [1]. Of the dozens of caspases, caspase-3 activity has been proposed as the main source of Aβ-induced apoptosis because it cleaves the death causing substrate poly-(ADP-ribose)-polymerase (PARP) to a specific 85kDa form [10]. Thus, the decrease in caspase-3 activity at a dose-dependent manner when exposed to Radix Rehmanniae and Arisaema Cum Bile supports evidence from previous studies that implicates the activation of caspase-3 during apoptosis.

Previous studies have also identified a protein designated as the DNA Fragmentation Factor (DFF). Caspase-3 cleaves the 45kDa DFF subunit at two sites to generate an active factor responsible for DNA fragmentation [11]. Thus, the ability of Radix Rehmanniae and Arisaema Cum Bile to decrease DNA fragmentation in a dose-dependent manner also lends support to previous findings that caspase-3 activity is the source of Aβ-induced apoptosis. The decreased DNA fragmentation reflects the decreased caspase-3 activity as expressed in Figure 3.

All things considered, the present study demonstrates the protective role of Radix Rehmanniae and Arisaema Cum Bile in the presence of $A\beta_{1\text{-}40}$-induced neuronal cell death. The neuroprotective effects of these drugs significantly decrease reactive oxygen species (ROS) production, caspase-3 activity, and DNA fragmentation. However, it must be noted that the $A\beta_{1\text{-}40}$ fragment, rather than the more potently toxic and disease-related $A\beta_{1\text{-}42}$ fragment was used in this experiment [12]. Furthermore, a wide body of evidence suggests that non-fibrillar aggregates are far more toxic than amyloid fibrils, and that fibrils may not be the most relevant models for Alzheimer's disease progression [13]. Thus, more research is required to confirm the therapeutic potential of Radix Rehmanniae and Arisaema Cum Bile in treating AD. Regardless, the use of Radix Rehmanniae and Arisaema Cum Bile should be further investigated as potential therapeutic agents for AD.

Acknowledgements

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JULS RESEARCH

Precise Mapping of Genomic Loci Involved in Complex Yeast Genetic Trait

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Abstract

It is currently estimated that only about 20% of total genes in eukaryotic genomes are essential for cell viability. For instance, approximately 1,000 out of 6,000 *Saccharomyces cerevisiae* genes are required for survival, and the remaining 5,000 genes are dispensable. Interestingly, it has recently been shown that only 5% of the essential genes are truly necessary for viability, and this is strain-dependent ('conditional essentiality'). The buffer system in the yeast genome which could explain conditional essentiality is largely unknown. For example, two different yeast strains, S288c and Σ1258b, show a contrasting phenotype when a conditionally essential gene, such as *RET2*, is deleted. *RET2* is dispensable for Σ1258b survival, but it is essential for S288c viability. Through mating and sequencing, preliminary results indicated that two genomic regions, each 75 Kb in size, contribute to Σ1258b survival in the absence of *RET2*. The scope of this project was to find a method to narrow down these regions in order to identify specific genomic features that enable Σ1258b to survive without *RET2*. Two independent approaches were examined: a backcross of the F1 *ret2Δ* progeny with the parental S288c and a high throughput F1 selection using Synthetic Genetic Array (SGA) markers. The F1 SGA selection method demonstrated the capacity to narrow these regions down to 10 Kb, which is an impressive 7.5-fold increase in resolution. In conclusion, a recombinant progeny selection using SGA markers proved to be an effective approach in mapping a conditional essential network in *S. cerevisiae*.

Introduction

Understanding the genotype-phenotype relationship remains a major challenge facing modern genetics in all organisms [1]. Complex gene interactions at multiple loci lead to various phenotypic traits, such as heritable human diseases [2]. Identification of such modifier genes allows us to understand the factors that cause disease variability and to distinguish new targets for therapy [3]. However, there is little knowledge of modifier-loci interactions. Here, we tried to delineate this genetic interaction network using *Saccharomyces cerevisiae* as a model organism.

S. cerevisiae is a highly tractable organism that is actively studied. Indeed, most of our current knowledge of molecular biology in eukaryotes has been derived from this budding yeast. First, it is a simple unicellular organism with a small genome (12 Mb) that can be grown rapidly [4]. Second, approximately 30% of all yeast genes share significant homology with those of mammals [5]. For example, *PGL*, a human gene mutated in the hereditary cancer paraganglioma is homologous to the yeast gene *SDH5* [1], which is a subunit of an enzyme complex involved in succinate oxidation and reduction [6]. As a result of a substantial homology between yeast and mammalian genes, functional characterization of evolutionarily conserved yeast genes provides valuable knowledge about other eukaryotes. Third, *S. cerevisiae* has been widely used to study complex genetic networks because, in addition to being the first eukaryotic organism to have been completely sequenced [7], a single gene deletion collection for its 6,000 genes is available [8]. From this collection it was found that about 20% of all *S. cerevisiae* genes are required for life ('essential') [9]. This single gene deletion mutant collection has been used in a recent study to examine nearly 5.4 million gene pair deletions, enabling the exploration of multiple gene interactions often encountered in human diseases [10].

More recently, another deletion mutant collection was constructed for the *S. cerevisiae* strain: Σ1258b (hereafter referred to as Sigma). Similar to previous findings, it was found that approximately 19% of Sigma genes are essential for cell viability [11]. However, the overlap of the essential gene sets in the two yeast strains, S288c (also known as BY) and Sigma, differs by approximately 5% [11]. This, in turn, gave rise to a new class of genes named 'conditional essential' (essential in a defined strain genome). For example *RET2*, which codes for an endoplasmic reticulum protein [12], is conditionally essential in BY but non-essential in Sigma. To date, 13 genes have been classified as necessary in BY but dispensable in Sigma and 44 genes have been identified in the opposite case using each other as a reference strain [13].

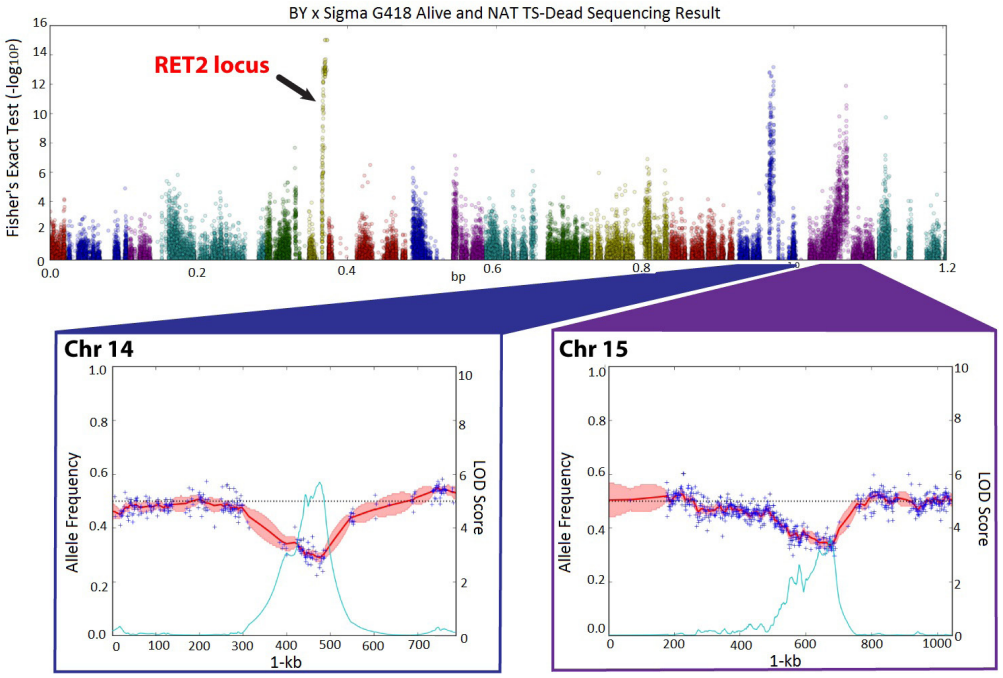

Figure 1: Preliminary Sequencing Result. (Upper panel) Sequencing result of the F1 BY and Sigma G418-Resistant and NAT-Resistant TS-dead progeny (about 500 spores) focused on the Sigma background. The y-axis shows statistical enrichment of Sigma SNPs inheritance (individual dots) and the x-axis represent their physical locations along each of the 16 yeast chromosomes (colour coded 1 to 16). (Lower panels) Magnified view of chromosomes 14 and 15, each containing one statistically enriched region associated with RET2 deletion survival. Blue crosses represent a SNP position along the chromosome (x-axis). The y-axis represents the deviation from the probability of acquiring SNPs from each parent (expected to be 0.5). Red lines indicate their deviation average. Based on statistical analyses, all points above the dotted threshold lines were considered critical, forming approximately a 75 Kb region in each graph.

Conditional essentiality can be viewed as a synthetic lethal interaction between several loci in one strain genome and a central gene. Briefly, a genetic interaction refers to a deviation from the expected phenotypic outcome deduced from single mutants [14]. As an example, consider a case where a wild type is assumed to have a fitness of 100% and two single mutants each with 50% fitness. If the two mutants were to mate and create a double mutant, the expected fitness would be 25 % (0.5 x 0.5 = 0.25). If this double mutant has a greater fitness than this predicted value, this is defined as positive epistasis or interaction, whereas a fitness level lower than the expected value is described as negative interaction. Furthermore, negative interaction can be termed "synthetic lethal" if viable single mutants lead to death in a double mutant [13]. This example illustrates the simplest form of synthetic lethal interaction involving only two loci. In reality, genetic interactions exist between more than two loci; current estimates range from 2 to more than 20 loci to account for a simple trait, such as heat tolerance or better drug resistance [15]. Human genome-wide association studies for complex traits such as diabetes, schizophrenia, obesity and cancer, show that hundreds of loci could be implicated [16].

The overall goal of this study was to elucidate the synthetic lethal environment created by a conditionally essential gene, *RET2*, which is homologous to the human ARCN1, which is involved in the coatomer protein complex [17]. As a preliminary observation, BY and Sigma were crossed to produce an F1 population from which approximately 500 G418-Resistant and NAT-Resistant TS-Dead (drug described later) spores were selected for sequencing (Figure 1). Preliminary sequencing results (Dr. Gerald Fink group, unpublished) outlined two precise, but fairly large enrichment regions found in the Sigma background that contribute to its viability without *RET2*. This project specifically examined methods to refine these genomic regions through two independent methods: backcrossing and the Random Spore Analysis (RSA). By reducing the preliminary genomic regions and examining sources of genome variation, other genes that may not be involved in the *RET2* conditional essential network can be eliminated.

Methods and Materials

Strains

We worked with two *S. cerevisiae* strains, S288c (BY) and Σ1258b (Sigma), from the Boone lab collection [10]. As the first eukaryotic genome to be sequenced [18], BY is used widely as a reference strain in the yeast community. As a similar strain to BY, the Sigma genome has also been fully sequenced and its own deletion collection is available [11]. Both BY and Sigma strains carried drug resistant cassettes in association with the *RET2* locus. These cassettes had unique antibiotic resistance phenotypes that did not affect the growth of either strain [19]. In Sigma, *RET2* was replaced by the geneticin (G418) resistant marker KAN (*ret2*Δ::KAN). In BY, a temperature-sensitive mutant version of *RET2* was linked to the nourseotricin resistance gene (*RET2*-TS NAT). The temperature sensitivity mutant (TS) of BY permitted colony growth at 24°C to 30°C, but yielded lethality or sickness at 38.5°C. The use of the TS-mutant was needed in order to account for all modifier loci present in the BY-Sigma genome. It was hypothesized that the G418-Resistant spores should contain functional versions of modifier loci, whereas NAT-Resistant TS-Dead should possess deleterious copies.

Media Preparation

The standard rich medium used for *S. cerevisiae* was YPED. One liter of YPED medium required the following: 10 g yeast extract, 120 mg adenine, 20 g peptone, 20 g agar, 900 ml deionized water, and 50 ml 40 % glucose after autoclave [20]. MSG medium used for the Random Spore Analysis was made in two separate bottles due to a solidification problem. One bottle was prepared with 1.7 g yeast nitrogen base without amino acids and ammonium sulphate, 1 g L-glutamic acid sodium hydrate, 2 g amino acid drop out – HIS/ARG/LYS (for mating type a) or – LEU (for mating type α) and 100 ml deionized water [20]. In another bottle, 20 g agar and 850 ml deionized water were combined [20]. The two mixtures were combined after autoclaving and 50 ml 40 % glucose, 0.5 ml canavanine and 0.5 ml thialysine were added [20]. Media were supplemented with kanamycin and nourseotricin [21] at a final concentration of 200 µg/ml [20]. Enriched sporulation medium was prepared by technicians and contained the following: 10 g potassium acetate, 1 g yeast extract, 0.5 g glucose, 0.1 g amino acid supplement powdered mixture for sporulation and 1 L of water [10].

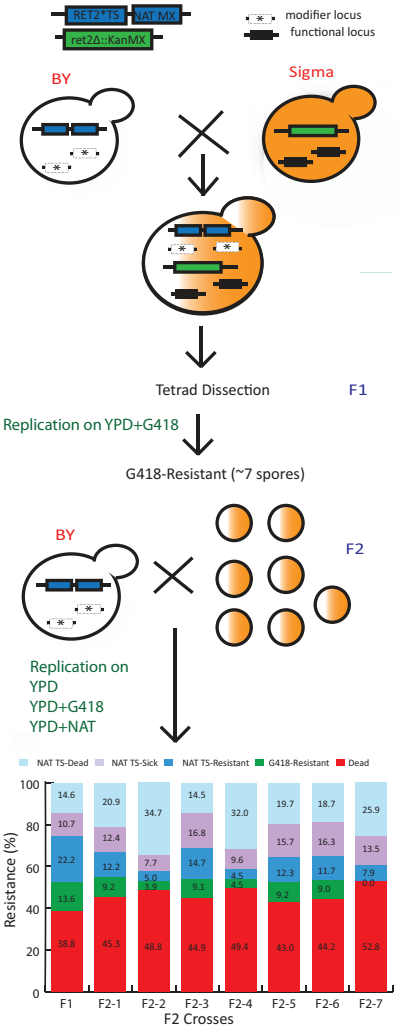

Figure 2: Schematic Outline of the Backcross Approach. Modifier loci can be speculated to exist in two states: deleterious and functional. Hence, when a modifier loci network around a specific gene is only composed of non-functional copies, the central gene is necessary for viability in a particular *S. cerevisiae* strain. Two strains, BY (S288c) and Sigma (Σ1258b), mated to form a hybrid diploid strain that was resistant to both G418 and NAT drugs. This strain was forced through sporulation to create haploid spores, which were germinated. Seven G418-Resistant F1 colonies (MAT**a**, *ret2*Δ::KAN) with various growing fitness were selected randomly. Each one of the seven colonies was backcrossed with the parental BY (MATα, *RET2*-TS NAT) to give rise to an F2 population with a genome predicted to be 75 % BY and 25 % Sigma. The hybrid diploids were then sporulated and about 100 asci (400 spores) were analyzed per cross. The marker segregation patterns among the four spores were examined by replica plating the F2 spores on YPD supplemented with G418 and NAT drugs (Bar Graph). The x-axis represents F2 crosses and the y-axis shows the resistance percentage whose colours are defined in the legend. A F1 segregation pattern bar graph on the far left is included as a reference from the first cross.

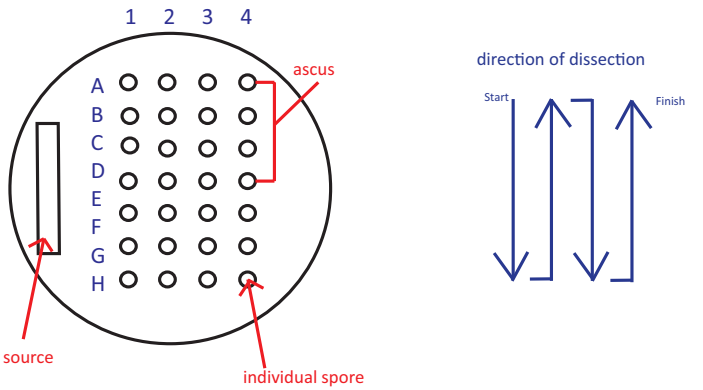

Figure 3: Schematic Representation of Dissection Pattern. A petri dish is represented by the large circle. Sporulation culture treated with zymolase is spread on the source site. Using a mechanically dissecting microscope, asci can be broken and their spores can be rearranged as shown. A set of four spores (i.e. A to D or E to H) constitutes four spores from one ascus. Coordinates are registered by the dissector machine to facilitate spore arrangement. Dissection starts at A1 and ends at A4 following the direction as illustrated by the arrows on the right.

Culture Condition

Yeast colonies were routinely incubated in 30°C or 38.5°C incubators while liquid sporulation cultures were incubated at room temperature (24°C).

Tetrad Dissection

Liquid sporulation cultures grown at room temperature (24°C) were monitored daily for asci formation after 48 hours. Once asci were visible under light microscope, cultures were treated with zymolase (0.5U/µl) and spread on YPED plates. Treated cultures were then mechanically dissected using a microscopic dissector (Singer Tetrad Dissector) (Figure 3).

Genetic Analysis

Genomic DNA extraction protocols from the Dr. Fink group and Qiagen (DNeasy Blood Tissue Culture) were modified by Dr. Pierre Côte. HiSeq 2000 Illumina technique was used for sequencing. Data analyses involved simple genome mapping against a reference genome and enrichment region calculations, which were conducted externally at the MIT (Dr. Fink group). A statistical model that combined information across nearby markers (SNPs) accounted for varying sequence depth. The model calculated the probability of a non-random association and compared it to the probability of no association. These probabilities were used to calculate LOD scores (-log base 10 of the odds of linkage), which were plotted across the genome.

Results

Method 1: Backcross (F1 x BY)

Initially, two haploid parental strains BY (MAT**a**, *RET2*-TS NAT) and Sigma (MATα *ret2*Δ::KAN) of opposite mating type were mated to create a hybrid diploid strain containing genome composed of 50 % of each parental strain. This hybrid also harboured two dominant drug resistance markers that conferred resistance to G418 and NAT. The diploid was subjected to meiosis to create sets of four recombinant haploid cells called spores. A set of four spores encapsulated in an ascus could be released via a mild treatment of the lytic enzyme zymolase (0.5 U/µl for 20 min at 37°C). The weakened asci were spread on YPED plates and mechanically dissected. Their four spores were re-arrayed using a mechanical dissecting microscope (Singer Tetrad Dissector). (Each recombinant haploid spore will be referred to as an F1 population from hereafter). The F1 colonies were replicated on YPED supplemented with either the NAT or G418 drug. Subsequently, the genomes of all G418- and NAT-Resistant Temperature Sensitive mutant colonies were selectively transferred and re-organized as separated groups. From over 500 tetrads we isolated 291 TS-dead and 272 G418-resistant individual F1 recombinant spores. These two populations were then sequenced; the results show a clear enrichment for two 75 Kb regions, one each on chromosomes 14 and 15 (Figure 1).

Building on these initial results, seven G418-Resistant F1 colonies (MAT**a**, *ret2*Δ::KAN) with varying fitness (i.e. robust, normal, medium and weak) were mated with their parental BY haploid (MATα, *RET2*-TS NAT). This backcross gave rise to an F2 population with a predicted genome composed of 75 % BY and 25 % Sigma (Figure 2). One hundred asci were dissected from each cross, with each individual ascus giving rise to four F2 spores. These spores were then grown on YPED plates and then replicated after 3 days on YPED+G418 and YPED+NAT plates followed by incubation at 30 °C for 3 days (Figure 2). In order to measure the temperature sensitivity (TS-Resistant, Sick or Dead) of the spores, the YPED+NAT plates were further replicated onto two fresh YPED+NAT and incubated at different temperatures: 30 °C and 38.5 °C (Figure 2). Following replication on the selective media

and incubation, the segregation pattern for each cross was scored (Figure 2 bar graph). For example, the F2-1 cross showed the following phenotypic outcomes: NAT TS-Dead (20.9 %), NAT TS-Sick (12.4 %), NAT TS-Resistant (12.2 %), G418-Resistant (9.2 %) and Dead (45.3 %) (Figure 2 bar graph). For each ascus, a segregation patterns indicating a 2 to 2 ratio was expected due to meiosis. A schematic representation of the tetrad dissection approach and an image of the final tetrad dissection plates are provided (Figures 3, 4). Finally, the genomic DNA of 192 G418-Resistant F2 colonies was extracted and sent for sequencing (results pending).

Method 2: Random Spore Analysis

Both the preliminary and the first method relied on a human intervention to manually separate each recombinant spore. Manual dissection enables an accurate retention of spore segregation patterns thus allowing one to determine parental genotype. However, the microscopic dissection is an extremely time consuming step, because it takes about 10 minutes on average to dissect 10 asci. In order to obtain a better resolution by increasing the number of G418-Resistant F1 spores by 10-fold, 10 times more asci than the preliminary results would have to be dissected. In the case of the *RET2* conditional essential network, approximately 4,000 asci would take 66 hours of disection without a definite guarantee that a larger sample size would render enough resolution. Such a labour-intensive method proves to be inefficient in investigating complex conditional essential gene networks involving several loci. To resolve this problem, we have coupled the genetic-based selection approach of the Synthetic Genetic Array (SGA) with rapid and large-scale spore production used in the Random Spore Analysis (RSA) to examine 4,000 recombinant F1 G418-Resistant spores.

Briefly, the SGA technology is based on the double-negative selection using two drugs, canavanine and thialysine, that mimic the amino acids arginine and lysine, respectively [10]. The incorporation of these two drugs in the cell leads to lethality. Canavanine and thialysine uptake is prevented by the respective deletion of *CAN1* and *LYP1*, both of which are non-essential genes [10]. Diploid cells harbouring heterozygous deletions (+/-) or haploid cells with only one of the two deletions are killed. Furthermore, the SGA markers have been engineered for auxotrophic mating-type driven selection: the *CAN1* locus is replaced by a MAT**a**-dependent His5 expression cassette and *LYP1* locus by a MATα-dependent Leu2 expression [10]. As a result, a sporulation culture grown on SD-his supplemented with canavanine and thialysine would select for MAT**a** haploid cells, whereas SD-leu medium supplemented with the two drugs would select for MATα haploid cells. Ever since its debut about a decade ago, the SGA technology has pioneered an extremely robust way to select specific recombinant cells after mating [10].

Before implementing the SGA approach in a large scale population, the accuracy of its mapping resolution was assessed by applying the SGA selection after the BY-Sigma mating. Mating of haploid parents, diploid selection, and sporulation steps were followed as described previously. After the integration of the SGA markers, the MAT**a** Sigma parental strain harboured the *can1*Δ::Ste2pr_Sphis5 *lyp1*Δ::Ste3pr_LEU2 genotype, whereas the MATα BY parent carried the wild-type version of both genes (*CAN1* and *LYP1*). This genetic difference allowed the sporulation culture to be spread directly on SD-his medium supplemented with canavanine and thialysine to recover selectively recombinant MAT**a** spores. The net result was the isolation of approximately 4,000 F1 G418-Resistant MAT**a** spores carrying canavanine and thialysine resistance deletions. Subsequently, each colony was cherry-picked and individually re-grown in 125 µl YPD media in 41 plates of 96 wells. After 2 days of incubation, a fraction of each culture (25 µl) was pooled together and the genomic DNA was extracted. These 4,000 spores were sequenced and the three loci of interest (MAT**a**, *CAN1*, and *LYP1*) were each mapped with a 10 Kb resolution (Figure 5).

Discussion

Two genetically distinct *S. cerevisiae* parental strains, S288c (BY) and Σ1258b (Sigma) were used to study the conditionally essential gene, *RET2*. As part of the preliminary experiment, a hybrid of BY and Sigma was created and approximately 500 G418-Resistant and NAT-Resistant TS-Dead spores were selected for sequencing. This preliminary sequencing result outlined two genomic regions (each 75 Kb in size) in the Sigma background that contributed to the progeny viability in the absence of *RET2* (Figure 1). Based on the pilot result, the goal of this project was to develop and test two new methods, the backcross and the Random Spore Assay, to allow for a better resolution of parental-specific genome contribution to a progeny phenotype.

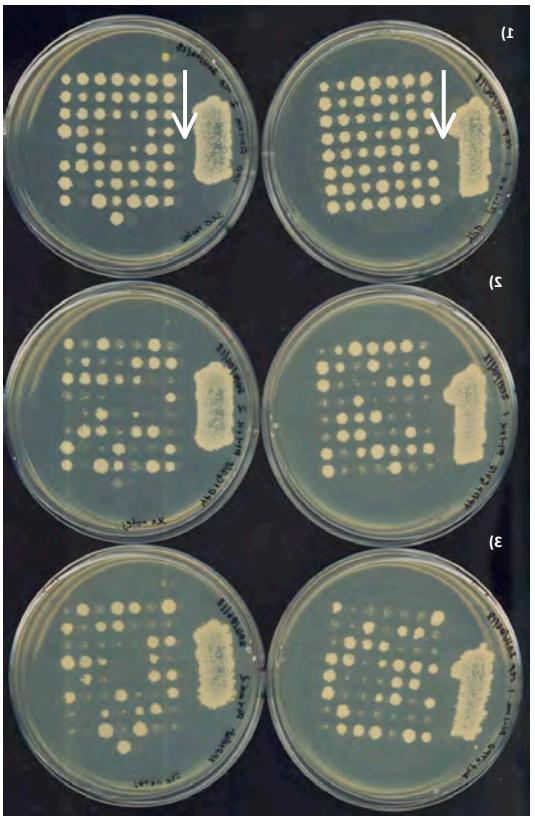

Figure 4: Tetrad Dissection and Replication on Selective Media. 1) Original tetrad dissection plate on YPD media. Patches on the left show source position from where asci were collected. Spores are counted from top to bottom as indicated by the arrows in groups of four that originate from a single ascus. **2)** Replication of YPD plate on YPD+G418 media. **3)** Replication of YPD plate on YPD+NAT media. In **2)** and **3)**, segregation pattern of 2:2 is observed. Natural spore death can account for the empty spaces on the YPD plates.

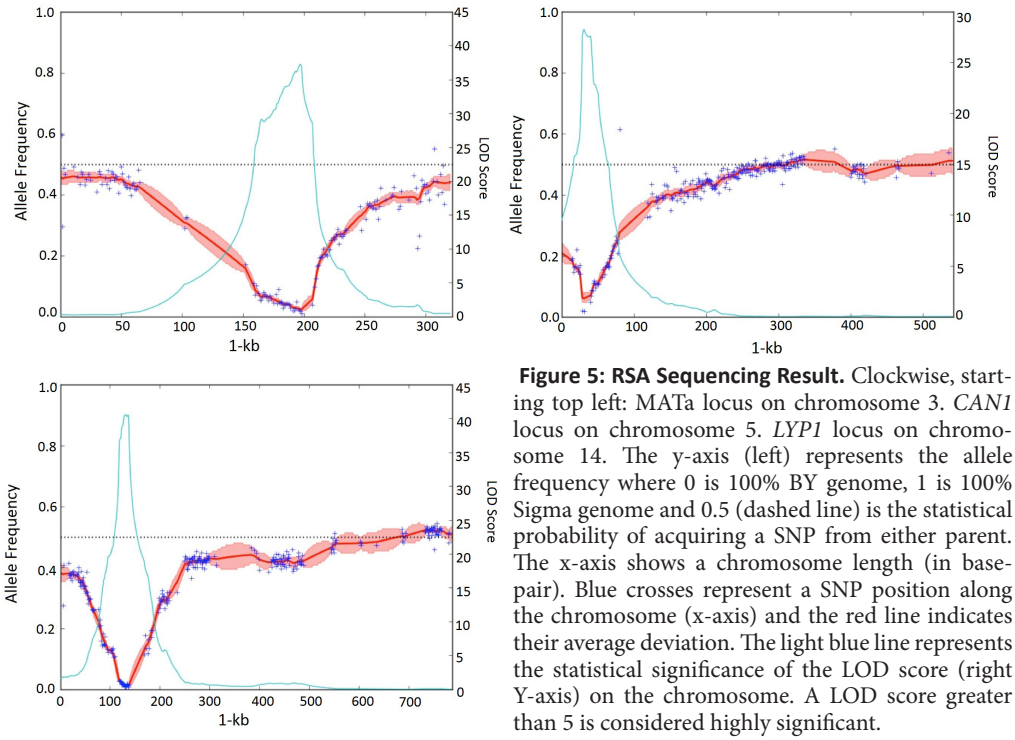

Figure 5: RSA Sequencing Result. Clockwise, starting top left: MATa locus on chromosome 3. *CAN1* locus on chromosome 5. *LYP1* locus on chromosome 14. The y-axis (left) represents the allele frequency where 0 is 100% BY genome, 1 is 100% Sigma genome and 0.5 (dashed line) is the statistical probability of acquiring a SNP from either parent. The x-axis shows a chromosome length (in base-pair). Blue crosses represent a SNP position along the chromosome (x-axis) and the red line indicates their average deviation. The light blue line represents the statistical significance of the LOD score (right Y-axis) on the chromosome. A LOD score greater than 5 is considered highly significant.

To benchmark this approach, we mated SGA marker-compatible BY and Sigma strains and looked for the resolution at which we could map MAT and SGA marker-associated loci. In this mapping we sequenced approximately 4,000 F1 G418-Resistant colonies, a number exceeding our target of a 10-fold increase. Through this approach we were able to very precisely (<10Kb) and significantly (LOD score >28) locate loss of heterozygocity at MAT**a**, *CAN1* and *LYP1* loci in the F1 progenies. Interestingly, although we have increased the number of sequenced F1 spores by over 10-fold, the resultion has improved only by 7.5-fold. This suggests that a plateau in resolution-gain may have been reached and that a further increase in the number of sequenced spores would not necessarily yield a better resolution.

Backcross

The purpose of the backcross was to assess whether multiple chromosomal recombination in a small sample size would be enough to gain better resolution in modifier loci mapping. Despite the fact that the sequencing results are still pending, the segregation pattern of F2 could be analysed. The F2 G418-Resistant population represent a reduced fraction (largely 4.5% or 9%) (Figure 2) of the overall population when compared to the F1 G148-Resistant population (13.6%, Figure 2 left most bar). Although modest alterations of the segregation ratio could be expected in backcrossing assays, the reduction observed here is stronger than anticipated. Theoretically, the Sigma parental strain and the F1 G418-Resistant should have the same number (set) of modifier loci to survive in the absence of *RET2*. Therefore, we would expect the cross between BY and F1 to give rise to the same ratio of G418-Resistant as the BY-Sigma cross. Because we observe a marked reduction, we speculate that the backcrossing tends to reduce or dilute the effect of Sigma modifier loci. After many back crossings, a backcrossed progeny segregation pattern will ultimately mirror the one of BY, showing no modifier loci and a *RET2* essential phenotype.

Random Spore Analysis

We explored the possibility of increasing the number of test progeny after a single F1 generation. Although this approach is quite simple, and mainly involves the use of a mechanical dissector, the procedure is rather time-consuming. Gathering approximately 300 F1 G418-Resistant colonies require a screening of approximately 2,000 spores, itself representing about 1 to 2 days of work. Based on mathematical calculations, to refine the genome mapping by an order of magnitude, the number of colonies screened should be incerased 10-fold. As a result, we resorted to the Random Spore Analysis using the SGA markers. This allowed for a rapid selection of haploid cells with desired resistance phenotype at the cost of segregation linkage patterns.

Conclusion

The purpose of this study was to identify suitable methods for high throughput strain-specific loci mapping. This was examined through two methods: the analysis of F2 recombinant spores resulting from backcrosses between seven F1 G418-Resistant spores and BY and the RSA-SGA of 4,000 recombinant F1s using the SGA selection marker as benchmark. High-througput sequencing using the RSA-SGA approach proved to be more effective than the backcross. Multiple backcrosses of small sample sizes led to a diluted effect of Sigma modifier loci as shown in the diminished proportion of G418-Resistant spores in the F2 population in comparison to the one from the F1 population. In contrast, the RSA-SGA combined method led to a very precise detection of heterozygoity loss around MAT**a**, *CAN1* and *LYP1* in the F1 recombinant population. Building on the success of the RSA-SGA pilot assay, the *RET2* network is currently being sequenced. Using the same method, we will then systematically explore all known conditional essential genes to date [11] and decipher their strain specific modifer loci network.

In the wake of tremendous advances in genotyping and sequencing technologies, genetic variation has become the focus of recent biomedical science. Recent studies outline that genome-wide association investigations can only account for a small fraction (5-10 %) of trait heritability, underlining the importance of a more global understanding of complex phenotypes; the paradigm of single locus effect is insufficient because most phenotypes are likely determined by the interplay of many different variants. Ultimately, translating genotype into phenotype remains one of the major challenges in biology. Results from our project will go a long way towards understanding the genetic causes of complex diseases.

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JULS RESEARCH

A novel combination of plumbagin and paclitaxel for the treatment of renal cell carcinoma

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Abstract

Paclitaxel is a chemotherapeutic known to up-regulate Nuclear Factor kappa B (NF-κB) transcription, promoting chemoresistance. This report focuses on whether plumbagin, a quinonoid, can sufficiently down-regulate NF-κB activation and sensitize Renal Cell Carcinoma (RCC) cells to paclitaxel. Through MTT assay and Sub-G1 analyses methods, it was found that plumbagin possesses chemotherapeutical properties. Furthermore, down-regulation of the NF-κB pathway modulated gene products were identified by Western Blot. A co-treatment of both plumbagin and paclitaxel was analyzed by various methods and the results suggested a strong synergy between the two drugs. Live-Dead assay showed that the co-treatment of the drugs was much more effective in induction of apoptosis in RCC cells compared to those of the individual drugs. Sub-G1 analyses verified the results from the Live-Dead assay as there were many more cells in Sub-G1 phase of the cell cycle when treated with the combination of the drugs. The DNA binding assay data made it clear that plumbagin down regulated the NF-κB pathway that was upregulated by paclitaxel. Overall, a novel combination of plumbagin and paclitaxel appears to have great potential for the treatment of renal cell carcinoma.

Introduction

Kidney cancer is among the top ten most common cancers world-wide, with 90% of cases being of the subtype Renal Cell Carcinoma (RCC). Males are twice as likely as females to be affected by the disease with a mortality rate two times higher [1]. Nephrectomy is the best available treatment option of kidney cancer, but it is often not sufficient. Due to the asymptomatic nature of all RCC subtypes, the tumour is usually malignant by the time the cancer is detected. Symptoms include hematuria, unexpected weight loss, edema, and prolonged fever, none of which are specific to kidney cancer [2].

The first line of defence against RCC includes targeted therapeutics such as sorafenib [3], bevacizumab [4, 5], sunitib [6] and pazopanib [7]. Paclitaxel, also a first line of defence, is a chemotherapeutic agent that exerts its effects by stabilizing microtubules, preventing extension and thus causing mitotic arrest [8]. However, it also up-regulates the NF-κB pathway, which plays an important role in the innate humoral immune response in normal cells. NF-κB has been found to be constitutively active in various solid tumours, as well as haematological malignancies including RCC. Paclitaxel regulates over 400 genes including those involved in proliferation, survival, invasion, angiogenesis and metastasis [9]. Evidently, it plays a major role in chemoresistance and radioresistance observed during cancer therapy.

Plumbagin is a potential chemotherapeutic that downregulates the NF-κB pathway. It is extracted from plants of the *Plumbaginaceae*, *Droseraceae*, *Ancestrocladaceae*, and *Dioncophyllaceae* families. It is currently used as a superoxide generator, an antibiotic, and an anti-neoplastic agent. Plumbagin works mainly by down-regulating or inhibiting NF-κB and gene products related to it. It has been shown to have anti-cancer effects *in vitro* as well as *in vivo* in studies using lung cancer, prostate cancer and leukemia models [10].

This study examined whether plumbagin could potentiate the apoptotic effects of paclitaxel in RCC.

Methods and Materials

Reagents

MTT, Tris, SDS, PBS, glycine, NaCl, BSA, plumbagin and paclitaxel were purchased from Sigma–Aldrich (St. Louis, MO). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), 0.4% trypan blue vital stain, and antibiotic–antimycotic mixture were obtained from Invitrogen. Mouse monoclonal antibodies against Bcl-2, Bcl-xL, cyclin D1, survivin, Mcl-1, VEGF and Goat anti- mouse horseradish peroxidase were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).The RCC cell line RCC786.0 was purchased from ATCC (Virginia, USA).

MTT Assay:3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

The effect of plumbagin treatment on the viability of the RCC cell line was measured by the MTT dye uptake method based on the ability of live and active cells to cleave the MTT (tetrazolium salt) to purple formazan that is absorbed at 570 nm. 5.0 x 10^3 cells/well with a final volume of 200

µl/well were seeded in 96-well plates for 24 h and the cells were further incubated with different concentrations of plumbagin. After 24, 48 and 72 hrs, 20 µl MTT reagent (5 mg/ml in PBS, stored at 4°C in the dark) was added to each well, and the cells were further incubated at 37°C for 2 hours. 100 µl of MTT lysis buffer (20% W/V SDS in 50% of each DMF and dH2O) was then added to each well and the cells were incubated for 4 more hours, followed by reading on a scanning multi-well spectrophotometer (TECAN) at the optical density of 570 nm. Untreated cells were used as controls. Experiments were performed with 3 repeats per concentration.

Western blotting

For detection of Bcl-2, Bcl-xL, cyclin D1, survivin, Mcl-1, and VEGF, plumbagin-treated whole-cell extracts (2 x 10^6/ml) were lysed in lysis buffer (20 mM Tris (pH 7.4), 250 mM NaCl, 2 mM EDTA (pH 8.0), 0.1% Triton X-100, 0.01 mg/ml aprotinin, 0.005 mg/ml leupeptin, 0.4 mM PMSF, and 4 mM NaVO4). Lysates were then spun at 13,000 rpm for 5 min to remove insoluble material and resolved on a 10% SDS gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane, blocked with 5% non-fat milk, and probed with a primary antibody (1:1000) overnight at 4°C. The blot was washed, exposed to anti-mouse secondary antibodies for 1h, and examined by chemiluminescence (ECL; Amersham Pharmacia Biotech). The respective nitrocellulose membranes were stripped with stripping buffer (Thermo Scientific), blocked with blocking buffer, and probed with antibodies against β-actin overnight at 4°C. The protein bands on the membrane were detected by enhanced chemiluminescence (ECL; Amersham) the following day. β-actin was the loading control.

Live-Dead assay

Viability of cells was also determined by Live/Dead assay (Molecular Probes, Eugene, OR, USA). This technique operates by measuring intracellular esterase activity and is dependent on the plasma membrane integrity. Calcein, a polyanionic dye, produces an intense yet uniform green fluorescence because live cells retain the dye. EthD-1 is another dye, but unlike calcein, it only enters cells that have a damaged cell membrane and produces a bright red fluorescence in the nucleus of the dead cells. Briefly, 1 x 10^6 cells were treated with 1 mM plumbagin and 2 nM paclitaxel either alone or in combination for 48 hours at 37°C. After washing with PBS, the cells were stained with the Live/Dead reagent (5 µM ethidium homodimer, 5 µM calcein-AM) and then incubated at 37°C in the dark for 30 min. Working solution was discarded and processed cells were analyzed under a fluorescence microscope (Olympus, Japan).

Flow cytometer: Sub-G1 analysis

To determine the effect of plumbagin and paclitaxel on the cell cycle, 2 x 10^6 RCC 786.0 cells were incubated overnight in the absence of serum. They were then treated with 1 µM plumbagin or 2 nM paclitaxel alone or in combination for 48 h at 37°C. After, cells were washed and fixed with 70% ethanol. Cells were then washed again, re-suspended, and stained in PBS containing 25 µg/ml propidium iodide (PI) for 30 min at room temperature. Cell distribution across the cell cycle was analyzed with a CyAn ADP flow cytometer (Dako Cytomation).

DNA Binding Assay

To confirm the synergy between plumbagin and paclitaxel on the NF-κB pathway, a DNA binding assay was conducted. RCC 786.0 cells (2 x 10^6 /mL) were seeded and then treated with 5 µM plumbagin for 0, 2, 4, and 6 hours. The plumbagin-treated cells were then stimulated with paclitaxel (50 µM) for an additional 12 hours. Nuclear extracts were prepared, and 20 µg of the nuclear extract protein was used for ELISA-based DNA binding assay.

Results

We investigated the effects of plumbagin on a RCC cell line with and without the influence of paclitaxel. The structures of both plumbagin and paclitaxel are shown in Figure 1a and 1b.

Plumbagin suppresses proliferation of RCC cells in a time dependent manner

The suboptimal dose at which plumbagin can exert its effects in RCC cells was investigated by means of MTT assay. RCC 786.0 cells were plated, treated with different concentrations of plumbagin and then subjected to MTT assay. As seen in Figure 2, proliferation decreases as the time interval of the treatment increases. This is not as clearly evident in the treatment with 10µM plumbagin, since there is no proliferation at all. As the concentration of plumbagin increases, the RCC proliferation decreases.

Plumbagin induces apoptosis in RCC cells

Flow cytometry was used to assess whether plumbagin can exert its chemotherapeutic effects via inducing apoptosis. RCC 786.0 cells were incubated, treated with 5µM of plumbagin, washed, fixed, stained with PI, and analyzed for DNA content by flow cytometry. There is a significant amount of cell accumulation in the Sub-G1 phase as seen in Figure 3. Plumbagin induces apoptosis in a time dependent manner as the amount of cells in other phases of cell cycle have decreased periodically.

Plumbagin suppresses NF-κB-regulated gene products

Western Blotting was used to investigate whether plumbagin can downregulate NF-κB in RCC cells. RCC 786.0 cells were treated with

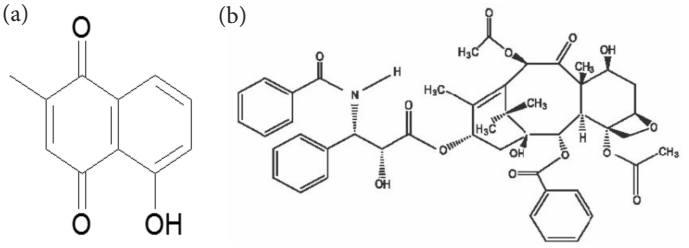

Figure 1. (a) The structure of plumbagin. **(b)** The structure of paclitaxel.

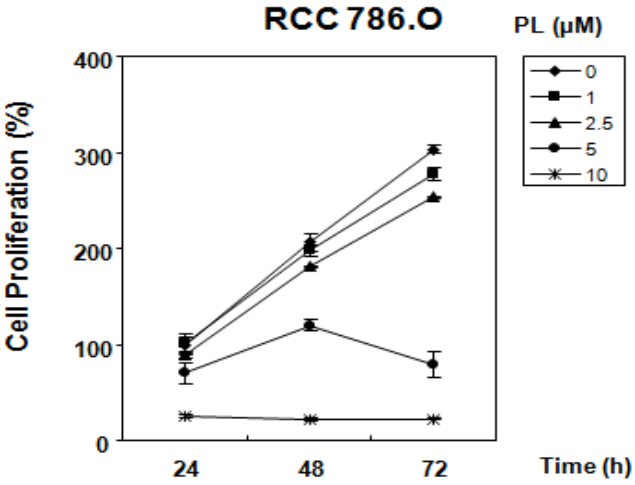

Figure 2: Decrease in proliferation was dose-dependent and time-dependent. RCC 786.O cells (5 x 10^6 /mL) were plated in triplicate, treated with indicated concentrations of plumbagin, and then subjected to MTT assay after 24, 48 and 72 hours to analyze proliferation of cells. Standard deviations between the triplicates are indicated.

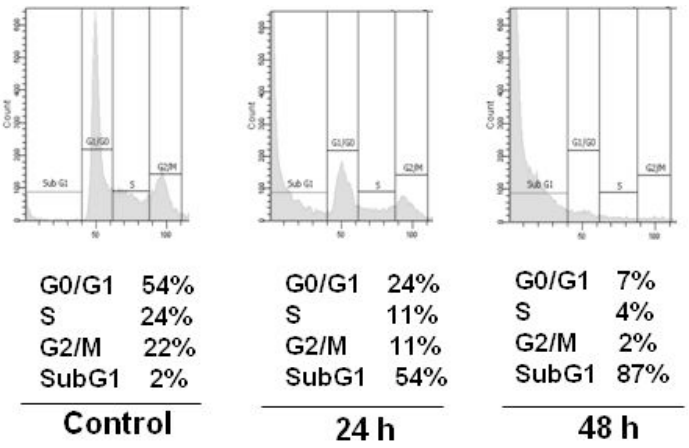

Figure 3: Plumbagin possesses chemotherapeutic properties. RCC 786.O cells (2 x 10^6 /mL) were incubated overnight in the absence of serum and then treated with 5 μM of plumbagin for the indicated time, after which the cells were washed, fixed, stained with PI, and analyzed for DNA content by flow cytometry.

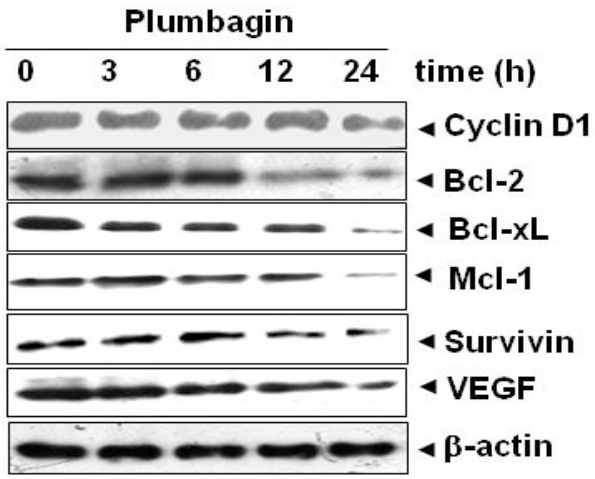

Figure 4: Plumbagin down-regulates gene products of NF-κB. RCC 786.O cells (2 x 10^6 /ml) were treated with 2.5 μM plumbagin for the indicated time intervals, after which whole-cell extracts were prepared and 30 μg portions of those extracts were resolved on 10% SDS-PAGE, membrane sliced according to molecular weight and probed against cyclin D1, Bcl-2, Bcl-xL, Mcl-1, Survivin and VEGF antibodies. The same blots were stripped and re-probed with β-actin antibody to verify equal protein loading.

plumbagin, extracts were prepared and resolved on 10% SDS-PAGE, the membrane was then sliced and probed to be analysed. There is a gradual, time dependent decrease in the amount of proteins present as seen in Figure 4. The proteins tested for are the gene products of NF-κB involved in proliferation, survival, invasion, angiogenesis and metastasis of a cell. This excludes β-actin which was used as a control, and shows constant amounts throughout the time gradient.

Plumbagin potentiates apoptotic effects of paclitaxel

The treatment of RCC cells with sub-optimal doses of plumbagin or paclitaxel alone or in combination with each other were investigated to determine possible synergistic effects via live/dead assay as well as Sub-G1 analysis. RCC cells were treated with plumbagin or paclitaxel either alone or in combination followed by washing, staining and further incubation before analysis. There is an evident synergy between plumbagin and paclitaxel as seen in Figure 5a. The amount of cells fluorescing green (alive) is reduced when treated with each of the drugs individually but the effect is

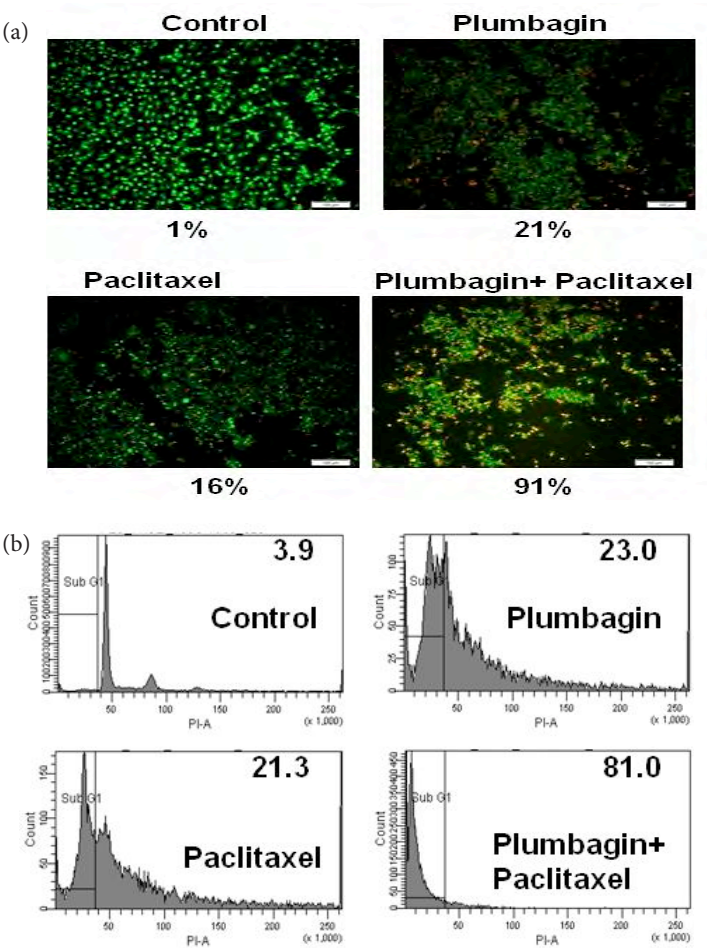

Figure 5. (a) Plumbagin induced apoptosis was measured by Live and Dead assay and Sub-G1 cell cycle analysis. RCC 786.O cells (1 x 10^6 /ml) were treated with 1 μM plumbagin or 2 nM paclitaxel either alone or in combination for 30 min and then analysed under a fluorescence microscope. **(b)** Sub-G1 cell cycle analysis. RCC 786.O cells (1 x 10^6 /ml) were treated with 1 μM plumbagin or 2 nM paclitaxel alone or in combination for 48 h at 37°C after which the cells were washed, fixed, stained with PI, and analysed for DNA content by flow cytometry.

much more pronounced when treated with the combination of the two drugs. The number of cells fluorescing red (dead) increased in the treatment with the combination of plumbagin and paclitaxel.

Plumbagin suppresses NF-κB-regulated gene products

A DNA-binding assay was used to determine whether the treatment of RCC cells with paclitaxel up-regulates the NF-κB pathway and whether plumbagin can down-regulate the pathway. Cells were treated with paclitaxel and plumbagin as seen in Figure 6 and after each time interval, 20 μg protein extracts were used for an Elisa-based DNA binding assay. The treatment with paclitaxel alone has high levels of NF-κB bound to the DNA. Addition of plumbagin results in a decreased amount of NF-κB bound to DNA in a time dependent manner. At the 6 h interval, the NF-κB levels are close to those in cells with no drug treatment at all.

Discussion

This study was conducted to investigate whether plumbagin can sensitize RCC cells to paclitaxel, a chemotherapeutic that causes chemoresistance via stimulation of the NF-κB pathway. There is evidence

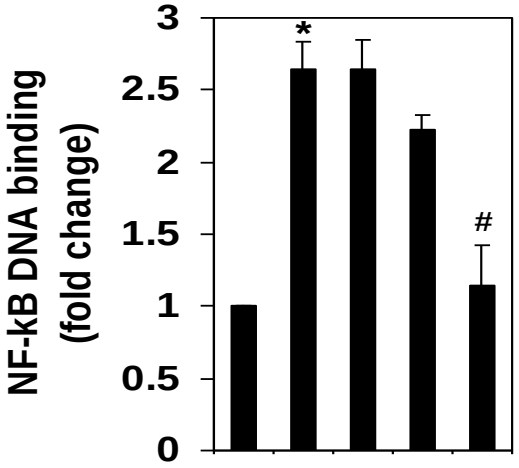

Figure 6. RCC 786.O cells (2 x 10^6 /mL) were treated with 5 μM plumbagin for 0, 2, 4, and 6 h and then stimulated with paclitaxel (50 μM) for an additional 12 h; nuclear extracts were prepared, and 20 mg of the nuclear extract protein was used for ELISA-based DNA binding assay.

that plumbagin inhibits the NF-κB pathway [11] and suppresses metastasis. It has been shown that plumbagin is a potent chemotherapeutic through in vitro and in vivo experiments using several cancer models [12]. The MTT experiment done in this study confirms that plumbagin suppresses proliferation in RCC cells. Moreover, the results have identified the range of sub-optimal doses of plumbagin to be used in further experiments. However, the MTT assay only quantifies cell death and does not expose the mechanism of cell death.

Sub-G1 analysis is a powerful tool that allows cells to be categorised and quantified based on the mitotic stage they are going through. The results show suppression of proliferation and accumulation in Sub-G1 phase of RCC cells suggesting that cells are in an apoptotic condition, away from proliferative phases of mitosis. The results also show that cell death observed in MTT caused by plumbagin occured via apoptosis. Some studies have successfully demonstrated that plumbagin suppresses NF-κB activation and consequently down-regulates the anti-apoptotic gene products associated with it [13].

The western blot results showed decreased levels of cyclin D1, Bcl-2, Bcl-xL, Mcl-1, Survivin and VEGF. These are involved in proliferation, survival, invasion, angiogenesis and metastasis of a cancerous cell. Using a 2.5μM concentration of plumbagin results in an observable, time-dependent decrease in NF-κB protein product. The NF-κB pathway was successfully down-regulated. Further experiments were performed to investigate whether suppression continued with co-administration of paclitaxel. All of the results showed that co-administration of paclitaxel produced an even greater suppression effect.

Live/dead assay was performed to determine the effects of the sub-optimal doses of plumbagin (1μM), paclitaxel (2nM) and a combination of the two. A synergy was observed confirming that the ability of plumbagin to down-regulate NF-κB is able to produce significant results when used in combination with paclitaxel. Studies have shown that paclitaxel is a chemotherapeutic that up-regulates NF-κB leading to chemoresistance [14, 15]. A Sub-G1 analysis was conducted with identical concentrations of plumbagin and paclitaxel. The results were parallel to those of live/dead assay which provided further proof of the synergy between paclitaxel and plumbagin. The accumulation of cells observed in the Sub-G1 phase proved that the cell deaths were indeed apoptotic.

Finally, a DNA binding assay was performed to investigate whether plumbagin down-regulated NF-κB activation-. The results showed that paclitaxel up-regulates NF-κB compared to the control. When treated with plumbagin, the NF-κB concentration decreased to normal levels in a time-dependent manner.

Conclusion

From the MTT experiment with plumbagin on RCC 786.0, it is evident that a 10μM dose of plumbagin is toxic, whereas 1μM - 5μM concentrations can be a range of suboptimal doses for further experiments. The Sub-G1 analysis with plumbagin treatment confirms that plumbagin indeed behaves as a chemotherapeutic and causes cell death via apoptosis. Western Blotting shows that plumbagin down-regulates the NF-κB pathway as evident by the time-dependent decrease in the protein products of the pathway. The live/dead assay as well as Sub-G1 assay with plumbagin or paclitaxel or a combination of both produced results that show a synergy between the two drugs caused by apoptotic cell death. The DNA-binding assay further proves the synergy, as well as suggesting a mechanism as it is clear that plumbagin down-regulates the NF-κB pathway, which is upregulated by paclitaxel. These results suggest that plumbagin may potentially be used to sensitize RCC cells to the chemotherapeutic paclitaxel, as it successfully suppresses NF-κB pathway and its gene products that cause chemoresistance.

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JULS RESEARCH

Effects of *Dan Shen* on Mice Myocyte Ionic Channels and Its Potential Use in the Treatment of Brugada Syndrome

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Abstract

Brugada Syndrome (BrS), a type of arrhythmia, is a genetic disorder characterized by sudden cardiac failure caused by abnormalities in myocytic ion channels. A recent study has shown the potential of *Dan Shen* (dimethyl lithospermate), a traditional Chinese medicine, in the treatment of BrS. This report analyzes single ion channel conductance via the patch clamp technique in order to examine the effects of *Dan Shen*. Sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}) ion channels were studied in *Dan Shen*-treated myocytes from healthy mice. The results showed that *Dan Shen* had no significant effects on regulating either Ca^{2+} or Na^+ currents, but had a negative influence on K^+ channels ($p<0.05$). Given that it has been observed that Dan Shen inhibits K^+ channel activity by inducing a declined K^+ current, it may be hypothesized that *Dan Shen* reduces K^+ efflux, serving as a K^+ channel blocker. Consequently, *Dan Shen* may balance the depolarization and repolarization period disturbed by BrS without increasing the activity of Ca^{2+} and Na^+ channels, thus correcting the arrhythmia in vitro. However, further research is required to examine the clinical benefits of *Dan Shen* on BrS in vivo.

Introduction

Today, almost one third of the North American population suffers from chronic cardiovascular (CV) disease. The most common CV disease is well known as cardiac dysrhythmia (or arrhythmia) [2]. It is commonly caused by abnormal electrical activity in the heart, which results in an irregular heartbeat [3]. Cardiac dysrhythmia can be further divided into two subtypes: ion channelopathy (ICP) or ionic channel disease (ICD), which may have a genetic basis [2]. In particular, Brugada Syndrome (BrS) is a genetic ICD caused by an autosomal dominant mutation on SCN5A [6]. SCN5A is a crucial gene coding for voltage-gated Na^+ channels. Mutations in SCN5A may result in non-functional channel proteins, preventing the entry of Na^+, thereby causing an abnormally fast rhythm resulting in ventricular tachycardia [6].

The three dominant ionic channels in cardiac myocytes are voltage-gated Ca^{2+}, Na^+, and K^+ channels. When the membrane potential reaches the threshold for generating an action potential, Na^+ channels open quickly and promote Na^+ influx and a subsequent depolarization. Meanwhile, Ca^{2+} channels open slowly, releasing Ca^{2+} from the sarcoplasmic reticulum (SR), generating a prolonged depolarization. Changes in concentrations of both Na^+ and Ca^{2+} result in an action potential and subsequently in muscle contraction [3]. Compared to the Na^+ channel, K^+ channels open more slowly, leading to repolarization once the Na^+ channels are closed [3]. Cardiac rhythm is formed from coordination between these three ionic phases. In BrS patients, mutations in SCN5A result in aberrant Na^+ channels, which cause reduced Na^+ influx. This delays depolarization in the cardiac myocyte, which interferes with subsequent repolarization, consequently leading to arrhythmia.

Currently, the best way to study BrS is by examining the conductivity of cardiomyocytic channels under known electronic pressure to determine the factors involved in any abnormality [2]. The patch clamp facilitates the study of various ion channels in cardiomyocytes and is widely used in cardio-pathological experiments. The patch clamp uses an electrode that induces an electrical pressure in cells and controls the activity of a single ion channel [4]. Ion channel activity is observed by the change in membrane potential and analyzed by a computer program called Clampex. With the same concentration of extracellular and intracellular fluids in isolated cells, the voltage clamp is inserted into the fluid and is placed close to a single ion channel. The three stages involved are attaching, sealing, and pulling. A short circuit is formed in a single channel by interrupting the current, and resistance can be measured to study the activity of the ion channel [1]. The patch clamp measures normal cardiac ion channel activity and produces negative Na^+ current (I_{Na}), negative Ca^{2+} current (I_{Ca}), and a positive K^+ current (I_{to}) as described in previous studies [4].

Traditional Chinese medicines have been widely used in the treatment of cardiovascular disease. Particularly, *Dan Shen* (dimethyl lithospermate B, or *Salvia Miltiorrhiza*) has stood out for its minimal side effects and great potency on cardiac control [8]. Previous studies indicate that *Dan Shen* is involved in lowering blood pressure by causing coronary artery dilation, increasing the heart's ejection fraction, preventing clotting in the bloodstream

by reducing blood cholesterol, and relieving severe pain [8]. Since *Dan Shen* has served as a remedy for CV disease, cardiologists have focused on its potential as beta-blocker to control Na^+ channel activity in order to treat BrS [9]. *Dan Shen* can also block I_{Ca} and balance the arrhythmic effects in BrS patients [9]. However, there are fewer studies related to its effect on K^+ channels. This report investigates the hypothesis that the efficacy of *Dan Shen* in BrS treatment involves modifying K^+ channel behavior [2]. By comparing the K^+ channel activity between the control and treatment groups, the effect of *Dan Shen* on I_{to} behavior can be examined.

Materials and Methods

In this study, eight large white mice were used. They were divided into two groups: 1) control with normal daily feeding and 2) treatment with *Dan Shen* induced for one month (2g/day). The treatment group was fed twice daily with *Dan Shen* mixed into regular crops. Mice were anesthetized by a 15 min treatment with 4L of Nembutal. Afterwards, their hearts were isolated, washed with distilled water and desktop liquid, and the aortal branch was removed. The isolated hearts were then hung over the wash apparatus placed in the temperature controlled cup and digested by an enzyme mixture of proteases, pancreases and collegenase II at 37°C. The digestive solution ran from the right atrium to the right ventricle then to left ventricle [2]. The speed of digestion was controlled by a speed control machine. At various time points (from 10 to 30 min), pieces of cell tissue were cut and placed into cell dishes with KB solution (simulated cellular environment) and desktop liquid (Na^+ rich solution). Individual cardiomyocytes were isolated and then selected under a microscope based on their quantities and qualities, and stored in a Ca^{2+} rich solution at room temperature for 30 min, and at 4°C for another 30 min [6].

Patch clamp tips were prepared from glass micropipettes by a micropipette puller and polished by the microscope forge. A metal wire was inserted into a glass micropipette with the solution simulating the intracellular fluid. Another solution was used to mimic the extracellular environment and contained the myocytes selected. To test each ion channel, the intracellular and extracellular fluid containing corresponding ions were required [2].

Whole-cell patch clamp was conducted under a fluorescence microscope. Before the tips were attached to the cell membrane, pipette resistance was kept at 2.0 Ω. While continuously pressing the clamp down, the tip was gently attached to the cell. This allowed the current to pass through a single ion channel without disturbing other ion channels inside the cell. When it was completely sealed to the membrane, the seal resistance reached the maximum and was held over 1GΩ [4]. After giving slight pressure, the cell membrane was broken, and leaked the internal contents out; a large drop in resistance could be observed [5]. During the whole process, voltage was kept constant. Clampex 14.0 was used to measure each change in resistance and to calculate the current (I = U/R) at each time log and recorded on the graph. A two tailed t-test was performed to analyze the statistical significance of the difference between control and drug-induced groups.

Results

Voltage-Gated Ca^{2+} Channel (VGCC)

According to Figures 1 and 2, normal Ca^{2+} current (I_{Ca}) formed a downward peak. I_{Ca} was decreased when seal resistance increased, and dropped when resistance exceeded 1GΩ. In fact, I_{Ca} was dropped to -1000 pA (negative sign indicates influx) before *Dan Shen* induction and -1500 pA after induction. As the experiment progressed, I_{Ca} recovered to -500 pA before induction and -555 pA in the *Dan Shen* treatment group, and it was continuously increased in each time period. Before the membrane was broken, I_{Ca} remained close to zero. However, at the point of membrane breaking, it suddenly recovered to zero. The resistance fluctuated around 100 – 200 Ω after membrane breaking [1].

Voltage-Gated Na^+ Channel (VGNC)

Normal Na^+ current (I_{Na}) formed a large downward peak [1]. I_{Na} declined when seal resistance increased based on the formula I = U/R, and I_{Na} current dropped when resistance exceeded 1GΩ. I_{Na} was kept around zero before the membrane was broken [1]. Since highly concentrated Na^+-containing fluid was used, the results at the peak were could not be read; therefore, the result for first time log was used. At the first time log, I_{Na} was dropped to -3500 pA in the control group and to -3000 pA in the *Dan Shen* treatment group. It then recovered to zero at the same rate as with the control. When it was broken, the intracellular contents leaked out of the cell and uniform resistance was formed between the cell and pipette. After breaking the membrane, the resistance ranged from 100 – 200 Ω [1].

Voltage-Gated K^+ Channel (VGKC)

In the control, the K^+ current (I_{to}) formed a large upward peak. I_{to} declined when seal resistance increased [1]. However, unlike other ionic channels discussed, I_{to} rose above 2000 pA when resistance exceeded 1GΩ and declined again after the membrane was broken. According to Figure 5 and 6, I_{to} reached 2500 pA in

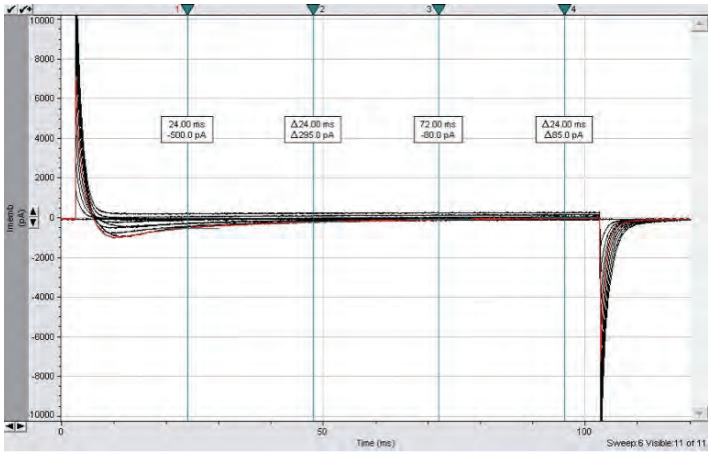

Figure 1: Ca^{2+} current before drug induction, analyzed by Clampex.

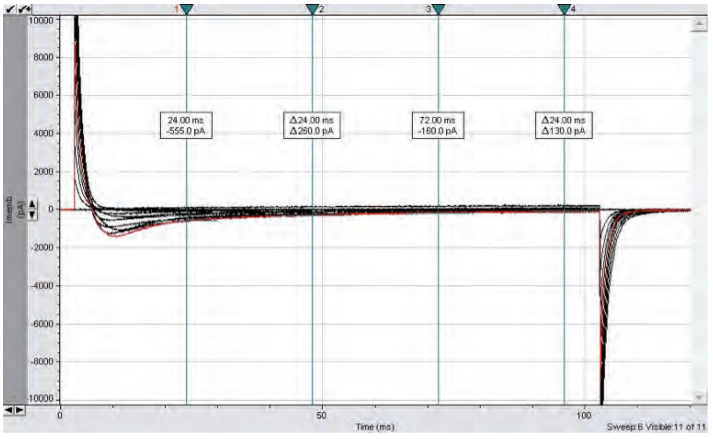

Figure 2: Ca^{2+} current after *Dan Shen* induction, analyzed by Clampex.

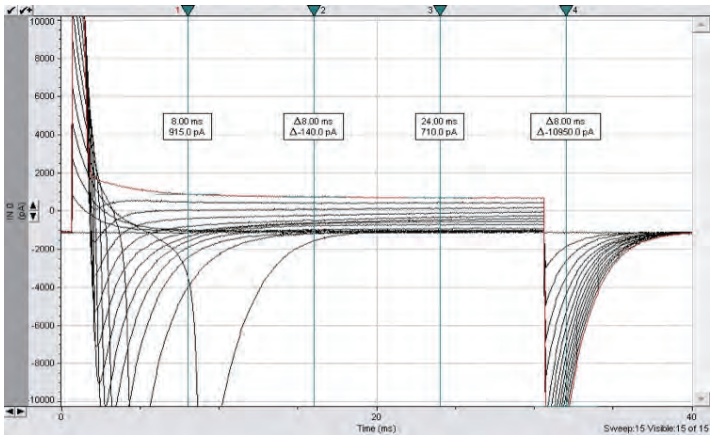

Figure 3: Na+ current before drug induction, analyzed by Clampex.

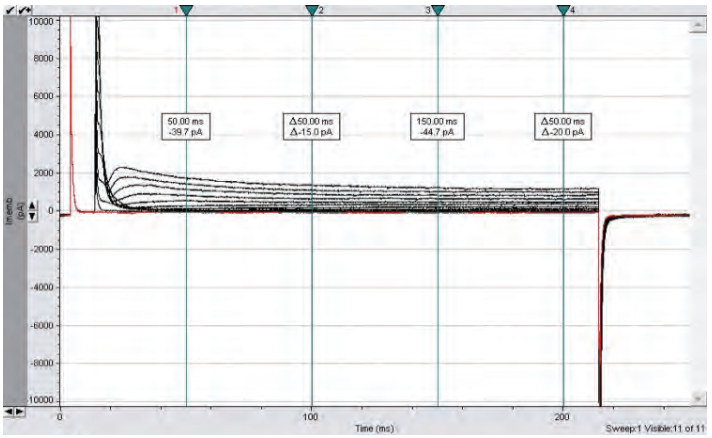

Figure 5: K^+ current before drug induction, analyzed by Clampex.

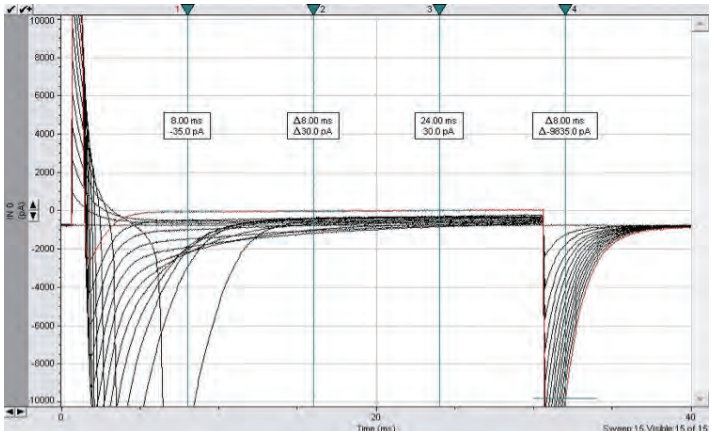

Figure 4: Na^+ current after *Dan Shen* induction, analyzed by Clampex.

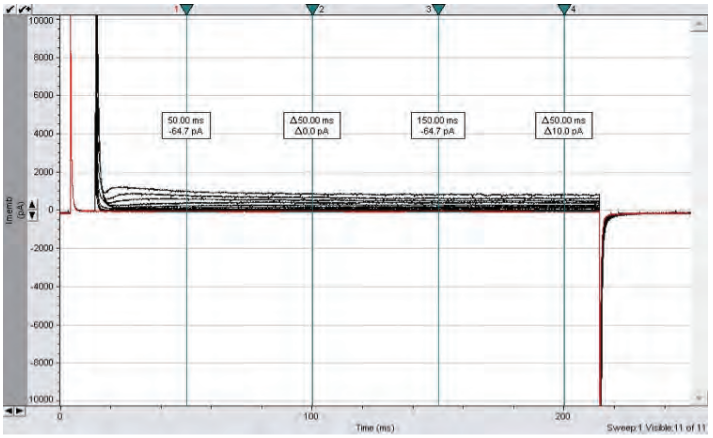

Figure 6: K^+ current after *Dan Shen* induction, analyzed by Clampex.

the control group, but only rose to 1200 pA in *Dan Shen*-treated group. Both measurements declined after the complete sealing, but compared to the control group (260 pA/s), the drug-induced group recovered at lower rate (70 pA/s). Unlike I_{Ca} and I_{Na}, I_{to} did not recover to zero and remained positive. Resistance ranged from 100 – 200 Ω after the breakage on the cell membrane [1].

Discussion

The patch clamp examines ion channel performance under normal cellular conditions. According to this study, normal cardiomyocytes are expected to have a downward Ca^{2+}peak and an even larger downward Na^+ peak, but form an upward K^+ peak [1]. This is because in cellular membranes, there is an influx of Ca^{2+} and Na^+, but an efflux of K^+ in membrane potential activation and transmission [3]. In the process, sealing induces the greatest resistance because it blocks the channel, forming complete isolation in a particular cell region. Immediately after giving external pressure, the membrane breaks and the current remains at zero pA. This can be attributed to the intracellular contents of the myocyte rushing into the pipette and equalizing the overall resistance [4]. After breakage, the patch clamp absorbs some membrane contents, resulting in a final resistance of 100 – 200 Ω.

Statistical analysis was performed in order to evaluate the significance of Clampex results. The null hypothesis is that there is no difference in three ion current measurements in control and drug-injected myocytes.

Table 1. Summary of Results in Three Ion Currents in Control and Drug-Induced Groups, Calculated by Clampex 14.0

	Ca^{2+} current (pA)	Na^+ current (pA)	K^+ current (pA)
Control - No *Dan Shen* Treatment	-1000	-3500	2500
	-500	-1000	1750
	-100	-750	1500
	-50	-500	1250
	0	0	1200
Dan Shen Treatment	-1500	-3000	1200
	-555	-1000	1000
	-100	-750	900
	-50	-500	900
	0	0	850

Table 2. Summary of Statistical Analysis by Excel (t-test)

	Ca^{2+} current (pA)	Na^+ current (pA)	K^+ current (pA)
p value	0.3199	0.3739	0.01867
α value	0.05	0.05	0.05
Degrees of freedom	4	4	4

According to Table 2, both p-values of Ca^{2+} and Na^{+} current are greater than 0.05. This confirms that there was no significant difference in I_{Ca} and I_{Na} between control and drug-induced individuals. In fact, *Dan Shen*'s benefit in arrhythmias appeared to be unrelated to the Ca^{2+} channels; rather, it may be implicated in regulating blood circulation, increasing blood flow, and reducing the chest pain resulting from arrhythmia [10]. In addition, the results of this study did not support that *Dan Shen* can balance abnormal I_{Na} in BrS patients in which the early depolarization and irregular heartbeat are observed [11].

K^{+} channels were the most difficult channels to measure in this experiment because K^{+} caused the repolarization of the membrane potential, leading to the relaxation of myocytes [5]. In K^{+} rich solution, cells may die or become inactivated due to irregular repolarization [1]. This study showed that I_{to} formed a positive current which was above zero and stayed in the upper region of the graph. This was due to the efflux of K^{+} when the K^{+} channel opened, which left a greater negative charge inside the cell [4]. While measuring the difference between extracellular and intercellular potential, I_{to} generated a positive value. Statistical analysis rejected the null hypothesis, ($p=0.01867 < 0.05$); therefore, a significant reduction in K^{+} channel activity was observed after drug induction. This may suggest that, after the treatment mice were fed with drug-containing food, their K^{+} channels were blocked, thus preventing K^{+} efflux. Accordingly, it is possible that a greater positive charge was present inside the cell, which facilitated depolarization. Consequently, this may suggest that *Dan Shen* has negative effects on K^{+} channel activity, which may contribute as a solution to arrhythmia caused by abnormal cardiac muscle contraction [11].

There are several possible concerns which could influence the results of this experiment. First, the time integral was essential for this experiment because if the cardiac cells were not digested for long enough, myocytes would not be completely separable and clusters would form [2]. However, if they were digested for too long, cells might be killed [2]. Second, pressure of the digestion liquid injected into the isolated heart may also contribute to the success of this experiment. If the heart was hung too high, then the pressure of injected solution would be high, breaking the circulation inside of the heart. However, if too low, the heart tissue may not be fully digested and myocytes would not be separable [2]. Moreover, precise concentrations of intracellular and extracellular solutions were required. However, in this experiment, a concern was raised with regards to the concentration of the intracellular solution added to the dish. As shown in Figures 3 and 4, part of the data is out of scope. This is because the solution used as the Na^{+} rich extracellular fluid was not prepared following regular concentrations [11]. In fact, the solution used was more concentrated than regular extracellular fluid. Therefore, while measuring the current, it dropped out of the range which the Clampex could measure [10]. This concern should be addressed in future experiments and the concentration should be conserved.

Conclusion

Brugada Syndrome has been studied widely with regards to its cause and treatment. Before, the most common treatment relied mainly on increasing Na^{+} concentrations in order to re-boost depolarization levels. While not observed in this experiment, some research has suggested that *Dan Shen* can act as a Na^{+} channel agonist [7]. *Dan shen* could extend the time required for Na^{+} channel inactivation, leaving it in its active state and increasing the action potential duration so as to prolong myocyte contraction [7]. More recently, another approach was identified, arguing that BrS can be controlled by Ca^{2+} boosting agents to replenish the cations required for depolarization [8]. Nevertheless, both approaches have potential risks in hyper-rhythmic conditions since it is difficult to control the concentration of both cations [2].

Recently, scientists have moved their attention to K^{+} channels. It has been suggested that if the K^{+} channels could be blocked, overall cardiac ionic activity may be restored in BrS patients, possibly contributing to curing BrS and other arrhythmias. *Dan Shen* (dimethyl lithospermate B) serves as a class III anti-arrhythmic agent that predominantly blocks the K^{+} channels [8]. In this experiment, results indicated that *Dan Shen* (2g/day) represses K^{+} channel activity and maintains an inactivated state for a longer period of time [8]. It is possible that the prolonged repolarization provided more time for the cell to recruit Na^{+} and Ca^{2+}, complimenting the lack of Na^{+} in BrS. This experiment also indicated that *Dan Shen* affects K^{+} channels without affecting Na^{+} and Ca^{2+} channels, suggesting a lower risk treatment for hyper-arrhythmia. However, further research is required to better understand potential therapeutic benefits and side effects of *Dan Shen*.

The limitations of this research include mice conditions, solution preparation, and the major challenges include paralysis technique and lack of in vivo testing. First, mice with different weight were paralyzed with equal amount of Nembutal. This could become a problem because 4L of Nembutal could kill myocytes in smaller mice because of their weaker circulation systems. Another challenge is that this experiment was conducted only in vitro. Results may be complicated in in vivo studies since there would be more interactions between individual ion channels or between ions. Hence, in vivo tests need to be carefully designed and more research is necessarily to reach further conclusions.

Even with the aforementioned limitations, this study appears to show a possible mechanism of how *Dan Shen* may be beneficial in treating arrhythmias and BrS. If further research and clinical trials demonstrate the benefits of *Dan Shen* in the treatment of arrhythmic diseases, *Dan Shen* may provide a promising treatment option for patients with cardiovascular disease.

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The Role of Cortical Cholinergic Inputs in a Selective Attentional Suppression Task

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Abstract

Visual stimuli are in constant competition with one another due to the limited processing capacity of the visual system. As a result, selective attention is necessary to suppress distractor stimuli and enhance perceptual processing of target stimuli. Cholinergic projections from the nucleus basalis magnocellularis (NBM) in the rat basal forebrain to the prefrontal (PFC) and posterior parietal (PPC) cortices play a significant role in the modulation of selective attention. These two cortical areas amplify the signals of the attended stimulus in order to overcome those elicited by the unattended stimulus, thereby increasing the signal-to-noise ratio. In the present study, rats performed a learning-to-ignore task where they learned to respond to a stimulus that they had previously ignored in two different contexts. The cholinergic neurons of the NBM were lesioned with the selective cholinergic immunotoxin 192 IgG-saporin (SAP) to reduce cholinergic cortical afferentation. Selective attention was impaired in the SAP group; this was evidenced by the SAP rats' better performance in the learning-to-ignore task. As hypothesized, the reduction in cortical levels of acetylcholine (ACh) impaired the ability to ignore distractor stimuli and improved performance. The impaired selective attention demonstrated by the SAP group is comparable to that shown in older adults and patients with Alzheimer's disease. The present study confirms the role of ACh in attentional modulation, additionally, it suggests that treatment to improve attentional processing should include therapeutic strategies that increase the level of cholinergic neurotransmission in the central nervous system.

Introduction

Selective attention refers to the ability to focus on a pre-determined target, or behaviourally-relevant stimulus while simultaneously ignoring the distractor or behaviourally-irrelevant stimulus [1]. Selective attention resolves competition between visual stimuli by increasing the firing rate of cells whose receptive fields contain the attended stimulus (i.e. amplifying the signals of the attended stimulus) [2] and suppressing those of the unattended stimulus. This increases the salience of the attended stimulus relative to stimuli that are being ignored [3]. Furthermore, this biased competition model of attention holds true even when the distractor stimulus is naturally more salient than the target stimulus, known as bottom-up bias [3]. As bottom-up processes can interfere with one's ability to process target visual information, top-down mechanisms are necessary to suppress irrelevant stimuli. This is especially true in tasks that are more attentionally demanding as there are more distractor stimuli that would otherwise direct attention away from the target stimulus.

It is hypothesized that selective attention is modulated by acetylcholine (ACh), a neurotransmitter synthesized by cholinergic neurons of the central nervous system. These cholinergic cells originate in the basal forebrain cholinergic complex, more specifically, the nucleus basalis magnocellularis (NBM), as it is known in rats, or in the nucleus basalis of Meynert, as it is known in humans [4,5]. These cholinergic neurons modulate cortical levels of ACh by projecting to the prefrontal (PFC) and parietal (PPC) cortices [4].

As a task becomes more attentionally demanding, cholinergic neurons innervate neurons of the PFC to increase the salience of an attended stimulus [6]. The PFC in turn signals to the PPC, known to be involved in the suppression of irrelevant stimuli [7]. A study conducted by Gills, Sarter and Givens [8] found that during a sustained attention task, cholinergic deafferentation of the rat PFC decreased PFC firing rates associated with the appearance of a visual distractor. This study suggests that a rise in ACh levels in the PFC correlates with neuronal activity associated with higher demands for attentional processing. Furthermore, the PFC has the ability to increase ACh efflux in the PPC when a task is attentionally demanding [9]. Nelson et al [9] found that when carbachol, a cholinergic agonist, was infused into the PFC, ACh efflux in the PPC increased. When carbachol was infused into the PPC however, no change in ACh efflux in the PFC was found. This study demonstrates that the PFC has the unique role of mediating top-down regulation by modulating ACh release in the PPC.

Given the significant role that ACh has on attentional modulation, it is not surprising that the degeneration of cholinergic neuronal cells in the basal forebrain has a huge impact on overall cognitive decline. Reduced cholinergic innervation to the frontal lobe of patients with Alzheimer's disease, for example, was found to be positively correlated with memory impairment [5]. This may be due in part to their reduced ability to inhibit irrelevant stimuli, which in turn compromises their ability to successfully encode relevant stimuli [10].

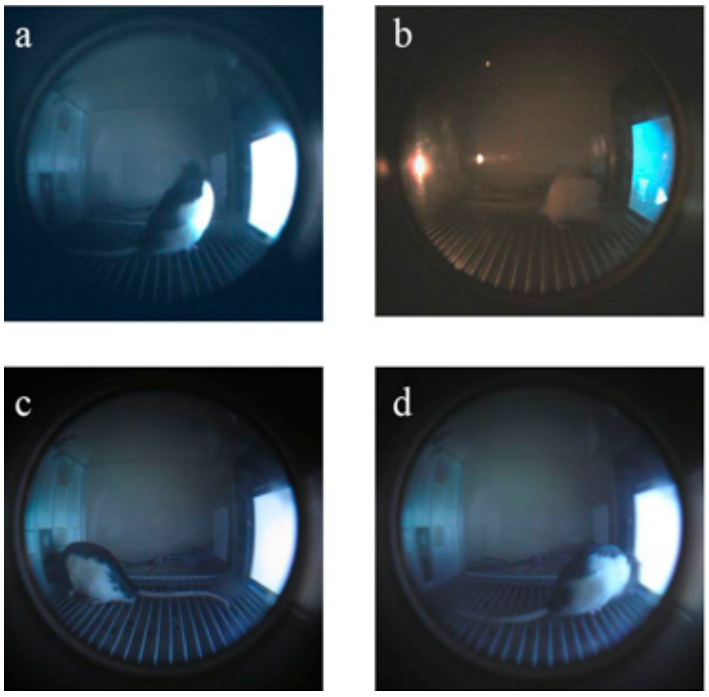

Figure 1: Progress of a rat within the operant chamber during Learning to Learn, Prime 1, Prime 2, and Probe. **(a)** Prior to presentation of the two-second tone and one-second delay, no stimuli were displayed on the touchscreen monitor. **(b)** When the rat touched the target stimulus, the circular light at the rear wall lit up. This signalled to the rat that water had been dispensed into the water-well. **(c)** 0.05 ml of water was dispensed upon correct selection of the target stimulus. **(d)** When the rat touched the distractor stimulus, a white light appeared on the screen indicating to the rat that it had made an error. No water was dispensed into the water-well.

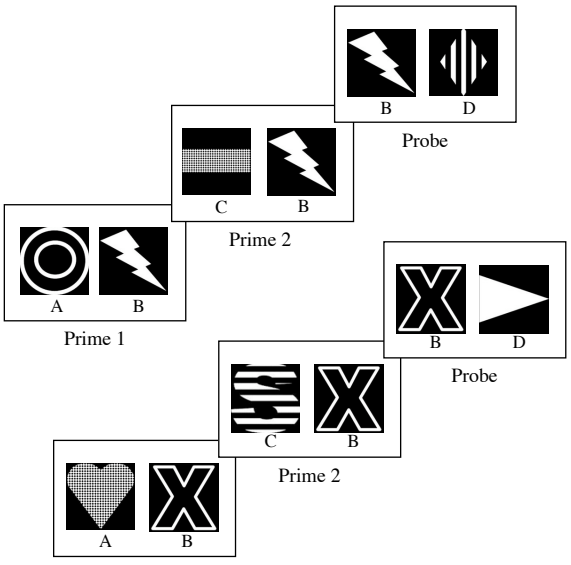

Figure 2. A

Figure 2. B

Figure 2. **(a)** Illustration of the visual stimuli used in each block during pre-surgical training. **(b)** Illustration of the visual stimuli used in each block during post-surgical training.

The following visual operant attentional suppression study demonstrates that the lesioning of cholinergic neurons in the NBM improves the performance of rats in a two contexts learning-to-ignore task [11], i.e., reduces the rats' ability to attentionally suppress task-irrelevant stimuli. In this learning-to-ignore task, rats repeatedly ignored a distractor visual stimulus in two separate stimulus contexts and then responded to the same visual stimulus as a target in a new stimulus context. The cholinergic cells of the NBM were lesioned with the selective cholinergic immunotoxin 192 IgG-saporin (SAP) in one set of rats to determine whether or not they are involved in selective attention. This reduced the number of cholinergic cells in this region and as a result, lowered cholinergic innervation to the PFC and PPC. With a lower cholinergic fiber density in these cortical areas, the SAP group was unable to learn to ignore the distractor stimulus to the same degree as the sham-lesioned control group. As a result, when the previously-ignored distractor stimulus became the target stimulus, the SAP surgical group was expected to perform better compared to the control group.

Methods and Materials

Subjects

The subjects used for this study were twenty male experimentally naïve Long-Evans rats (Charles River, Montreal, Quebec). As one of the rats passed away, the total number of rats used in the study was reduced to nineteen. At the beginning of the experiment, rats were 8 weeks of age and weighed approximately 200 g. Rats were housed individually in 45 cm long x 25 cm wide plastic clear tub cages. Food was restricted to 5 pellets per day and the vivarium was temperature and humidity controlled. Rats were maintained on reversed 12 h light/dark cycle (lights off at 8 A.M) and training was conducted during the dark phase of the light/dark cycle between the hours of 8 A.M and 8 P.M 6 days a week. 2 weeks prior to the start of the experiment, rats were handled 10 minutes per day for 6 days. Water was available ad libitum during this time. During the 24 h period prior to training, rats were water deprived. Following training, rats had access ad libitum to water for 20-30 minutes per day. This study was approved by the University of Toronto's Local Animal Care Committee.

Apparatus

The equipment used for this study is described in Botly and De Rosa (2011) [12].

Visual Stimuli

The visual stimuli were black-and-white computer generated shapes that consisted of one target stimulus and one distractor stimulus. The target and distractor stimuli varied between pre-surgery (Figure 2A) and post-surgery (Figure 2B).

Pre-Surgical Training Procedures

Variable Light Water

Rats underwent a positive reinforcement light-water task where they learned to associate the lighting of a circular light above the watering well with the dispension of 0.05 ml of water. This was done for 30-minute sessions a day.

Touch Light Water

Rats underwent a positive reinforcement touch-light-water task where they were required to learn to associate touching the touchscreen with the lighting of the circular light above the watering well. Rats were required to learn to touch the touchscreen within set boundaries without touching the corners. Rats could touch the screen with either their nose or paws. Moving towards the watering well caused one of the infrared beams to break dispensing 0.05 ml of water. This was done for 72 trials per session or 50-minute sessions, whichever came first, until a pre-determined criterion was met (an average reaction time of 5 seconds for 2 consecutive days).

Single Stimulus

In this task, rats were required to touch a single white square stimulus presented in the middle of the touchscreen. Rats learned to associate touching the stimulus with access to 0.05 ml of water. This was done for 72 trials per session or 50-minute sessions, whichever came first, until a pre-determined criterion was met (an average reaction time of 5 seconds for 2 consecutive days).

Moving Stimulus

This task is essentially the same as the single stimulus task, except that the single white square stimulus was now presented on either the left or right bottom of the screen. This was done for 80 trials per session with no time limit.

Learning to Learn

In this task, rats were required to discriminate between a target stimulus and a distractor stimulus. Touching the target stimulus was positively reinforced with gaining access to 0.05 ml of water. Touching the distractor stimulus was negatively reinforced with the emission of ten seconds of bright light from the touchscreen (Figure 1). This was done for 80 trials per session, without a time limit, until a pre-determined criterion was met (18 correct responses within 20 trials at any point during the session).

Learning-to-ignore – Two contexts (LI)

The conditions consisted of three blocks, namely prime 1, prime 2 and probe (Figure 2A and Figure 2B). Each block was run for ten days. Eighty trials were performed per session with no time limit. In prime 1, rats had to attend to stimulus A and ignore stimulus B. In prime 2, stimulus B was to be ignored while the rats attended to a novel stimulus C. The addition of a novel stimulus to prime 2 created a second context in which stimulus B was to be ignored. This increases attentional suppression of stimulus B, making it more difficult to attend to B in the probe block [11]. In the probe block, rats now attended to stimulus B while ignoring a novel stimulus D.

Surgical Procedure

Rats were divided into two surgical groups, namely sham-lesion (n=8) and NBM-lesion (n=11) (SAP). The two groups were matched based on pre-surgical performance to ensure that any difference in performance between groups observed in the post-surgery probe block was due to the lesion of the NBM and not due to pre-existing individual differences. Surgeries were performed under aseptic conditions. Immediately prior to the surgery, rats received an intraperitoneal injection of atropine (0.05 mg/kg) to prevent fluid buildup in the lungs. Rats were anesthetized with isoflurane (approximate maintenance dose was 2% with 1 L/min of oxygen). Rats in the sham lesion group received 0.2 µL of sterile 0.1M phosphate-buffered saline (PBS). Rats in the SAP group received 0.2 µL of 192 IgG-saporin in sterile 0.1 M PBS (concentration of saporin = 0.3 mg/ml). Injections were given at the following stereotaxic coordinates relative to bregma: anterior NBM: Anterior–Posterior (AP)— 0.8 mm, Medial–Lateral (ML)±2.6 mm, Dorsal–Ventral (DV)— 7.8 mm; posterior NBM: AP—1.3 mm, ML ±3.0 mm, DV—7.3. Four injections were given altogether, two per hemisphere at a rate of 0.1µL/min. After each injection, the needle was left in place for 3 minutes before being removed. 20 minutes prior to the end of the surgery, rats were given subcutaneous injection of analgesic ketoprofen (5 mg/kg) as well as the injection of 3ml of warm, sterile saline. Staples were used to close the wound and EMLA topical analgesic ointment (2.5% lidocaine and 2.5% prilocaine) was applied around the staples. Rats were given a minimum of 10 days to recover with *ad libitum* food and water.

Retrieval of Probe stimuli

After the rats had fully recovered, the probe block of the learning-to-ignore task was run for three days using pre-surgery visual stimuli. The performance of the rats on these three days was compared to that of the last day of pre-surgery probe to ensure that the surgery did not interfere with their ability to perform the task.

Post-Surgical Training

Experimenters were blind to the surgical group of the rats. Each block was run for ten days as in the pre-surgical training. Different stimuli were used for each block in order to avoid interference from pre-surgical training.

Learning-to-ignore – Two contexts (LI)

Rats were once again trained in a learning-to-ignore task using the same three blocks used in pre-surgical training, namely prime 1, prime 2 and probe. Different visual stimuli were used for all blocks in order to avoid interference from pre-surgical training. Each block was run for 10 days. Eighty trials were performed per session with no time limit.

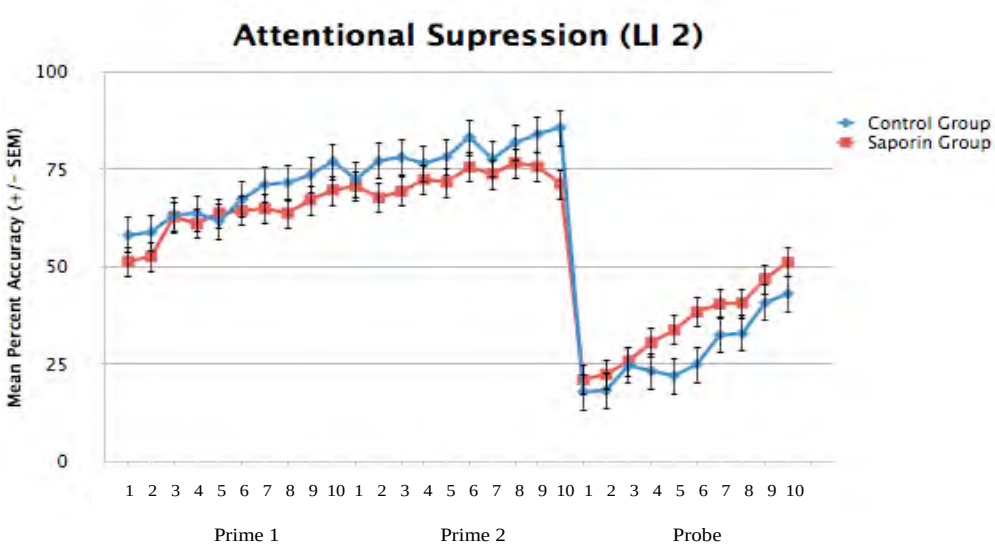

Figure 3. This graph illustrates the performance level of both the control and saporin group in a latent inhibition task two contexts (LI2). The performance of both groups was similar during the Prime 1 and Prime 2 sessions. Mean percent accuracies significantly dropped at the start of the Probe block. The error bars indicate the standard error of the mean (SEM).

Histological Analyses

Isoflurane (3-4%) was used to anesthesize the rats who were subsequently transcardially perfused with 150 mL of saline followed by 150 mL of ice-cold 4% paraformaldehyde. The brains were extracted from the skull and immediately postfixed in 4% paraformaldehyde for 2h at 4°C. Brains were sectioned at a thickness of 60 µm using a cryostat equipped with a freezing-sliding microtome (Leica Microsystems). Choline acetyltransferase (ChAT) immunohistochemistry was performed to determine whether there was a reduction of cholinergic cells in the NBM (Figure 4). To confirm that 192-IgG saporin did not affect other cells in the NBM, namely GABAergic cells, parvalbumin immunohistochemistry was conducted according to the methods described in Baxter et al [13] and De Rosa et al [14] (Figure 5). To

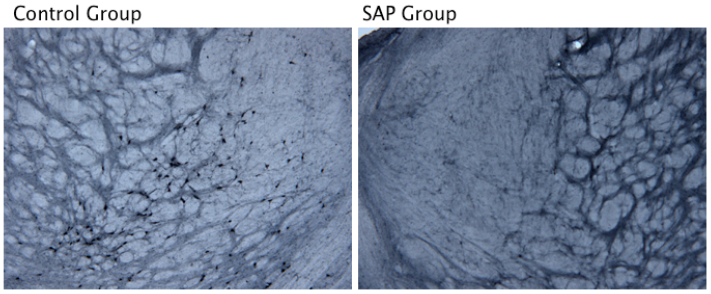

Figure 4: ChAT Immunohistochemistry of the NBM. ChAT immunohistochemical staining shows that there are significantly less cholinergic neurons in the NBM of the SAP group compared to the control group. Darker stained areas depict regions where more cholinergic neurons are present.

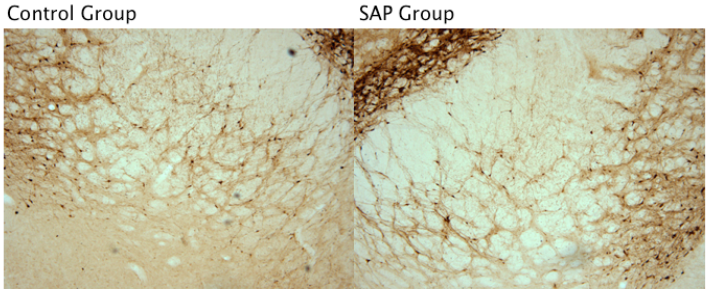

Figure 5: Parvalbumin Immunohistochemistry of the NBM. No significant difference in the number of GABAergic cells can be seen between the control group and SAP group.

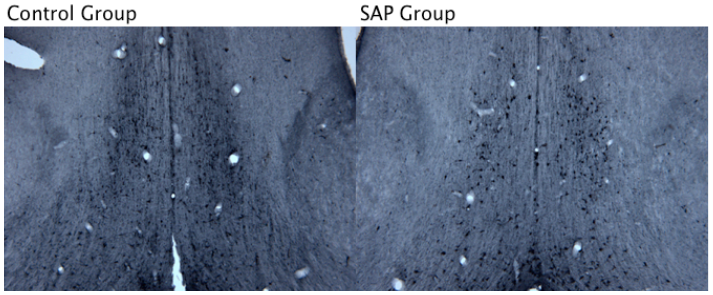

Figure 6: ChAT Immunohistochemistry of the MS/VDB. No significant difference in the number of ChAT-immunoreactive cells can be seen between the control and SAP group.

confirm that there was no reduction of cholinergic cells in other areas of the basal forebrain, namely the medial septum (MS) and vertical limb of the diagonal band of Broca (VDB), ChAT immunohistochemistry was performed (Figure 6). Lastly, acetylcholinesterase (AChE) histochemistry was performed according to the method described by Paxinos and Watson [15]. This was done to determine whether cholinergic fiber loss occurred in the PFC (Figure 7) and PPC (Figure 8). Once the histological assays were completed, brain slices were mounted on slides, dehydrated, and cleared with the use of an ascending ethanol and xylene series. Brain slices were coverslipped with the histological mountant distyrene plasticizer xylene. Slices were then examined under a Leica light microscope (DM4000B).

Histological Quantification

The methods used for cell counting and AChE densitometry are described in Botly and De Rosa [16].

Statistical Analysis

Performance on the visual operant attentional suppression study was measured using percent accuracy. The data from all rotations were compiled to form a complete data set and the average performance accuracy across all 19 rats for each block was calculated. Statistical analyses were

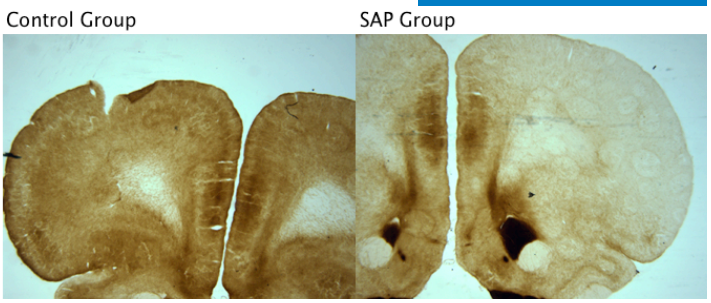

Figure 7: AChE Histochemistry of the PFC. AChE histochemical staining illustrates that less AChE is found in the PFC of rats who have been injected with 192-IgG saporin compared to the control group. Darker stained areas depict cortical regions that have a higher concentration of AChE and therefore a higher cholinergic fiber density.

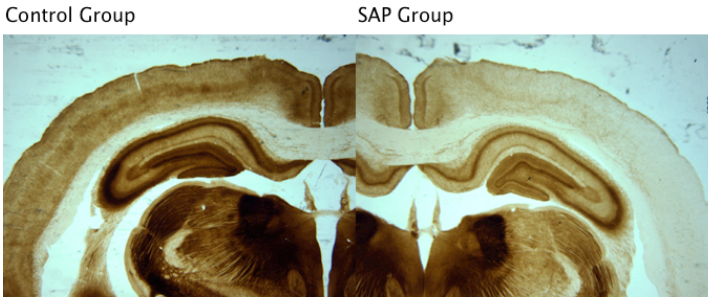

Figure 8: AChE Histochemistry of the PPC. AChE histochemical staining illustrates that less AChE is found in the PPC of rats who have been injected with 192-IgG saporin compared to the control group. Darker stained areas depict cortical regions that have a higher concentration of AChE and therefore a higher cholinergic fiber density.

conducted using SPSS version 17.1 software and a 95% confidence level was used to measure significance.

Results

Retrieval of Probe Stimuli

An independent samples *t*-test comparing the mean accuracy scores of control and lesion rats on the last day of pre-surgery probe revealed no significant difference between the two groups ($t(17)= 0.41$, $p> 0.05$). A between subjects mixed-design repeated measures ANOVA comparing the post-surgery performance of control and SAP rats on the three days of pre-surgery probe yielded no significant group effect [$F(1, 17) = 1.309$, $p> 0.05$].

Performance during the Probe Block

A between subjects mixed-design repeated measures ANOVA was run for each block to compare the performance of the control group to that of the SAP group. For prime 1 and prime 2, no significant difference was found between groups [$F(1,17)=0.856$, $p>0.05$, M_{sham}= 66.5%, M_{NBM}= 62.0%; $F(1,17)=1.939$, $p>0.05$, M_{sham}= 79.4%, M_{NBM}= 72.4%, respectively]. In the probe block however, a significant difference was found [$F(1,17= 6.381$, $p<0.05$, M_{sham}= 27.9%, M_{NBM}= 34.9%]. Two-tailed t-tests were also run for each day of post-surgery probe to see if there was a significant difference in performance between the two groups. A significant difference between groups was found on day four [$t(17)=-2.283$, $p<0.05$], day five [$t(17)= -2.769$, $p<0.05$], day six [$t(17)=-3.045$, $p<0.01$] and day seven [$t(17)= -2.441$, $p<0.05$] of probe (Figure 3).

Histological Analyses

A significant difference was found between the control group and SAP group in the number of cholinergic neurons counted in the

NBM [t(17)= 7.73, p<0.0001], and the cholinergic fiber density of the PFC [t(10)=-9.31, p<0.0001] and PPC [t(11.45)=4.74, p<0.01]. There was no significant difference between groups in the number of cholinergic neurons counted in the MS/VDB [t(15.59]=0.08, p>0.05] or the number of GABAergic neurons counted in the NBM [t(14.69)=-0.26, p>0.05].

Discussion

Performance on Learning-to-ignore Task

As expected, rats in the SAP group performed significantly better relative to the control group in the probe block. In contrast, there were no significant performance differences during the prime blocks. By lesioning cholinergic neurons in the NBM, cholinergic afferentation to the PFC and PPC was reduced. The resulting cholinergic deafferentation in these cortical areas impaired the ability of these rats to ignore distractor stimulus B in both the prime 1 and prime 2 blocks. As a result, the impaired attentional suppression of this stimulus during the prime blocks allowed the SAP group to attend to stimulus B better than the control group when it became the target stimulus during the probe block. The control group demonstrated a comparable learning-to-ignore pattern of behaviour to human participants i.e., learning to ignore stimulus B in two different contexts severely compromised their ability to attend to this stimulus during the previously-unattended probe block [11].

Histological Findings

There was a significant reduction in the number of cholinergic neurons only in the NBM. This confirms that the 192-IgG saporin was indeed injected into the target area and that other areas of the basal forebrain were not affected. The loss of cholinergic fiber density in the PFC and PPC further confirms that there was significantly less cholinergic innervation to these cortical areas, resulting in impaired selective attention.

Clinical Relevance

Our findings demonstrate the importance of ACh in the modulation of selective attention. As loss of cholinergic innervation to the cortex and hippocampus is a marker for Alzheimer's disease, possible avenues for the treatment of this neurodegenerative disease may lie in therapeutic strategies that increase cholinergic neurotransmission and prevent cholinergic cell loss in these brain areas [17]. Such examples of prospective therapeutic agents include neurotropins such as nerve growth factor (NGF) and brain derived growth factor (BDNF). NGF and BDNF are widely accepted as essential signaling factors for cholinergic neuronal differentiation as well as for the maintenance of the cholinergic phenotype [18]. Mouse embryo studies have shown that these growth factors increase messenger RNA expression of cholinergic markers, namely choline acetyltransferase (ChAT), acetylcholinesterase (AChE), choline transporter (CHT) [19, 20] and vesicular acetylcholine transporter (VAChT) [21]. The use of NGF and BDNF in the treatment of Alzheimer's disease is yet to be practiced in a clinical setting.

Conclusion

The present study demonstrates that reducing cholinergic input to the PFC and PPC by lesioning the NBM improves performance of rats in a learning-to-ignore task; this improvement reflects impaired attentional suppression of previously-ignored distractors. Our study is the first of its kind to use touchscreen behaviour in rats to demonstrate that the selective cholinergic lesioning of the NBM is sufficient to impair selective attention. Pharmacological interventions that reduce the loss of cholinergic neurons or increase cholinergic neuronal function may be considered in the treatment of attentional deficits associated with neurodegenerative diseases such as Alzheimer's disease.

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Cellular dynamics involved in the narrowing of the first tarsal segment of the first leg of *Drosophila melanogaster* females used as a control for male sex comb rearrangements

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Abstract

Development of most epithelial tissues involves complex rearrangements as seen in the sex comb rotation of male *Drosophila melanogaster*. The sex comb is a compact row of bristles found on the first tarsal segment of the first leg in male *D. melanogaster*, which undergoes a horizontal to vertical rotation during development. Females do not have sex combs, but despite this sexual dimorphism, the first tarsal segment of the first leg undergoes similar changes during the elongation stage of leg development. This leads to the question of what mechanisms are involved in leg elongation versus the rotation of the sex comb in males, and if female legs can be used as a control to understand the cellular dynamics of male leg elongation. To better understand the development of female *D. melanogaster*'s first tarsal segments, labeled confocal images from both sexes were studied starting from 23 hours after pupariation (AP), after the onset of the larval to pupal transition. It was found that the first tarsal segment of female *D. melanogaster* has fewer cases of cell intercalation and has types of cell rearrangements not previously described in males. Careful analysis of these cell rearrangements suggests that female *D. melanogaster* use a different mechanism for the narrowing of the distal portion of the first tarsal segment as expected due to the absence of the sex comb. We hypothesize that a global rotation of cells in the distal posterior region of the female first tarsal segment has a major role in the elongation process of the leg.

Introduction

Epithelial elongation is a key part of development and has been observed in many systems. In *Drosophila melanogaster*, germ band extension [1, 2], tracheal elongation [3], and egg chamber elongation [4] have all been used to examine the cellular dynamics involved in simple epithelial elongation where the epithelium is uniform and there are homogeneous cell dynamics. However, there remain many more complicated systems of epithelial elongation, for example in epithelia which contain protrusions and where multiple forms of cellular dynamics take place. The processes behind these complex elongations remain poorly understood.

An example of a complex tissue elongation system is the rotation of the *Drosophila melanogaster* sex comb, a row of 9 to 11 bristles located on the first tarsal segment of the first leg in males (Figure 1a, b). Not only do the cell dynamics responsible for sex comb formation occur concurrently with those responsible for leg elongation, but the sex comb and other bristles also serve as greater barriers to cell rearrangements than any epithelial protrusions found in previously studied systems. Atallah's study [5] on the development and rotation of the sex comb found that there was a male-specific cellular behaviour, such as movement of intercalating cells from the distal region to the proximal region. In addition, leg elongation was shown to continue beyond early stages of development of the leg where leg elongation was the principle developmental feat [6, 7]. Atallah's work concluded that leg elongation continues after 12h AP and also that the apical size of cells in the initially distal region increases, while the apical size of cells in the initially proximal region decreases. This change in cell shape, along with cell intercalation in the initial proximal region, generates the final confirmation of the rotated sex comb, yet the actual cellular dynamics responsible for the rotation of the sex comb remain unclear.

Female *D. melanogaster* legs do not develop sex combs (Figure 1c) but still undergo leg elongation. This makes them a useful model for understanding the cellular mechanisms involved in leg elongation but not sex comb rotation.

The current hypothesis suggests that the rotation of cells in the first tarsal segment of the first leg is due to a passive push created by the enlargement of cells in the distal posterior region of the sex comb.

If this is true, then the passive rearrangement of cells in the proximal region should not occur in females as they lack a sex comb. Any cellular dynamics in females should contribute primarily to leg elongation. This means that there will be fewer cases of intercalation in the region between the most distal transverse row and the second most distal transverse rows because there is no sex comb in females. In fact, further analysis of the cell dynamics between these two transverse rows determined a different type of cell rearrangement from cell intercalation in females. There is evidence to suggest that cell intercalation is not the major cause for the elongation of the female's first tarsal segment as predicted by the germ band extension model of elongation. In germ band extension, cells move and interchange neighbours in order to cause the overall tissue to elongate [1, 2]. This form of elongation is not seen in significant amounts in females. The elongation of the female first tarsal segment is now proposed to be due to a simultaneous global rotation of many cells in the distal anterior region.

Methods and Materials

Previously developed methodologies were used to study tissue elongation [1, 8]. Briefly, confocal images, from 23h to 36h AP, of cells from the first tarsal segment of female D. melanogaster pupae that were marked by GFP-tagged cadherin, were analyzed by computer. Horizontal lines of cells were manually colour-coded (Figure 2) using Image J software (software can be found at: http://rsbweb.nih.gov/ij/). These lines of cells were located between the distal end of the first tarsal segment up to the transverse row proximal to the most distal transverse row. A range of cellular dynamics responsible for the rearrangements (see Table 1) was quantified for each of 3 time points: 23h AP, 28h AP and 36h AP.

Using Image J software, lines were drawn connecting common bristles in female D. melanogaster (Figure 4).The resulting polyhedron was used to quantify the locations of cell delamination, as well as other events during leg elongation. This polyhedron in females is the equivalent of the polyhedrons created in similar studies on wild-type males. The number of cells along the distal horizontal length and posterior vertical length of the box were analyzed to look for elongation and narrowing of the female leg. Three angles, A, B and C shown in Figure 4 were also measured to analyze the global tissue rotation of the polyhedron. The movements of the bristles that define the corners of the polyhedron can only occur if neighbouring cells were also involved in the movement.

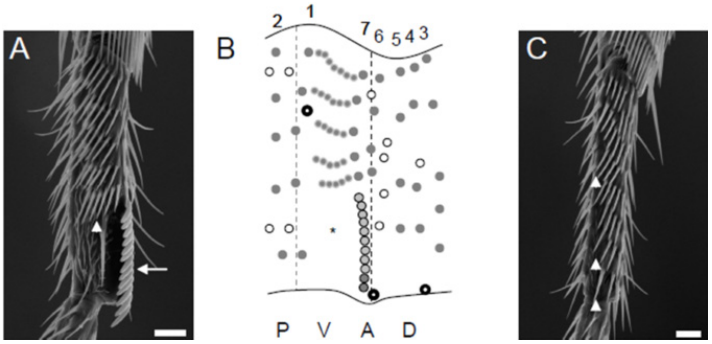

Figure 1: The male *D. melanogaster* first tarsal segment. **(a)** has a sex comb (long arrow) as well as transverse rows of bristles (triangular arrow), while the female **(c)** only has transverse rows (triangular arrows). **(b)** shows a schematic of the male first tarsal segment. The image between the dotted lines is what is visible from the confocal images. The leg has been marked from Posterior (P), Ventral (V), Anterior (A) and Dorsal (D). The completed sex comb is found near the anterior, while the transverse rows are found in the ventral. In females, transverse rows are still found in the ventral. Scale bars for **(a)** and **(c)**: 20um. (Atallah, 2008)

Table 1: Different cell dynamics observed in males that were used as a template for comparison with females.

Cell Dynamic	Description
Cell delamination	A cell delaminates in the line of cells
Intercalcation	One line of cells become two or more lines due to a cell moving up (Figure 4 left)
Intercalcation between lines	Certain cells from one line joins the line above (Figure 4 right)
Clockwise (CW) rotation	Three cells that rotate 15 to 30 degrees clockwise
Counter-clockwise (CCW) rotation	Three cells that rotate 15 to 30 degrees counter-clockwise
Other	Other cell dynamics usually involving a cell moving downwards during elongation
Static	Cell does not move

Using Image J software, lines were drawn connecting common bristles in female *D. melanogaster* (Figure 4).The resulting polyhedron was used to quantify the locations of cell delamination, as well as other events during leg elongation. This polyhedron in females is the equivalent of the polyhedrons created in similar studies on wild-type males. The number of cells along the distal horizontal length and posterior vertical length of the box were analyzed to look for elongation and narrowing of the female leg. Three angles, A, B and C shown in Figure 4 were also measured to analyze the global tissue rotation of the polyhedron. The movements of the bristles that define the corners of the polyhedron can only occur if neighbouring cells were also involved in the movement.

Rotations and intercalations were analyzed as follows: in an elongating tissue, four neighbouring cells in a cluster can create either type 1, type 2 or type 3 patterns (Figure 5). These patterns have previously been associated with tissue elongation, with more type 1 patterns in the non-

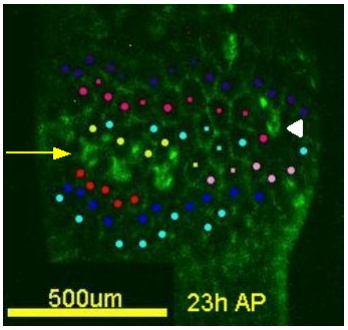

Figure 2: Image of the first tarsal segment in females. Lines of cells are labeled at 23h AP. Note that certain lines do not extend across the entire width of the leg due to the presence of bristles (white triangular arrow) and transverse rows (yellow long arrow) that block them. The visible transverse row is the most distal one.

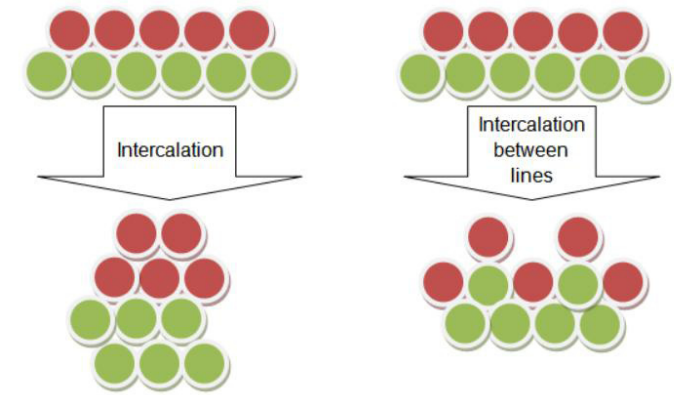

Figure 3: Differences between intercalation within a line (left) and intercalation between lines (right). Red circles represent one line of cells while green circles represent a separate line of cells.

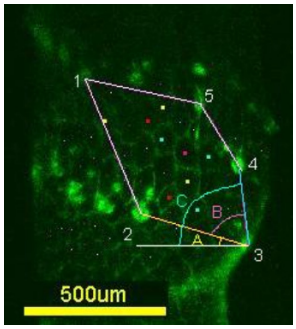

Figure 4: Distal region of the first tarsal segment at 28h AP showing the polyhedron formed by drawing lines to connect the first bristles on the two distal transverse rows (1, 2), the campaniform sensillum (3) and the two chemosensory bristles proximal to the campaniform sensillum (4, 5). The length of the horizontal line (yellow) and vertical line (blue) were measured by counting the number of cells along the lines. Angle A (yellow) measures from the strict horizontal to the yellow line (2,3). Angle B measures between the yellow line (2,3) to the blue line (3,4). Angle C measures from the horizontal to the blue line (3,4).

elongated form of the tissue and more type 3 patterns after elongation [1]. Through the elongation process, it was previously observed that a pattern changed from a type 1 to a type 2, and finally to a type 3, through the process of cell intercalation [1]. Type 2 patterns are unstable due to the fact that four cells share the same meeting point and usually resolve to a type 1 or 3 pattern often due to intercalation [1]. Since the presence of type 2 patterns often indicates an intercalation event is taking place, they were found and labelled within the polyhedron and followed through the times of 23h AP to 36h AP. These patterns were then observed for their method of resolution. Cases in which the patterns resolved due to a rotation rather than intercalation were also taken into account.

Cell delaminations were analyzed as follows: to observe the timing of cell delaminations, delaminating cells were encircled in different colours according to the time at which the delamination occurred. Cells that delaminated inside the box were quantified and compared with cell delaminations in males within the same boxed region. A cell delamination involves any cell that does not return after exiting the visible apical epithelia.

The following data were obtained from analyzing three female first tarsal segments. For all averages and totals in the figures, n=3 movies. All error bars represent the average deviation. In addition to female legs, similar data from three male legs, courtesy of Malagon, were provided for comparison use.

Results

Evidence of differing global cellular processes between males and females

Due to the lack of a sex comb in females, it was predicted that leg development would have different cellular processes. It was found that only 18% to 42% of cells in male first tarsal segments, between the sex comb's finished position and the proximal transverse row, are static. The number of static cells in females in the same region is considerably higher, ranging from 33.6-63.3% (Table 2).

Table 2: The different types of cellular dynamics found in 3 different first tarsal segments of 3 different female *D. melanogaster*.

	Leg 1	Leg 2	Leg 3
Cell Dynamic	63.3%	33.6%	48.6%
Delamination	22.4%	15.0%	18.6%
Intercalcation	2.0%	0%	12.9%
Intercalcation between Lines	0%	13.1%	10.0%
CCW rotation	4.1%	0%	0%
CW rotation	0.0%	30.8%	4.3%
Other	8.2%	7.5%	5.7%

Figure 5: Type 1, 2 and 3 patterns show the type of contact cells have with their neighbours. Type 1 patterns generally show wide epithelia, while type 3 patterns show narrower epithelia. The type 2 pattern is necessary for the change of cell-to-cell contact from a type 1 to type 3, or vice versa. Generally the patterns change from type 1 to type 3 over time to create elongation.

Many of the lines of cells (Figure 6) in females remain fixed and do not break throughout the elongation process. There were also significantly fewer cases of both cell intercalation and intercalation between lines ($p<0.01$). The reduced number of moving cells is likely due to the lack of sex comb rotation in female first tarsal segments. In females, there were also many cell dynamics that were not seen in the males, such as cells moving downwards in the anterior region. This may be due to the formation of the joint, which occurs in that area, being more easily seen than in the case of males where the sex comb rotation may cause one to overlook the joint formation.

The majority of cell movements found in females are due to cell delamination. In males, the majority of cell delaminations occur after 28h AP. The same is true for females, with 80% of cell delaminations occurring after the 28h AP mark. This delamination may be due to an unidentified tension that is present in both sexes after 28h AP. However, the sites of delamination are different in males versus females. In males, cell delamination mostly occurs in a more proximal location than in females. In females, many cells delaminated within the boundaries of the polyhedron (Figure 4). However, in males almost no cells delaminated in that area.

The process of leg elongation

The process of leg elongation was a major determinant for using female leg development as a control for the male leg development. We wanted to see the cellular mechanisms involved in leg elongation without the presence of a sex comb. The vertical length of the polyhedron (depicted by a blue line in Figure 4) increased over time indicating an elongation in the female leg. However, from Malagon's given data, the elongation is more dramatic in the male leg, likely due to the extra cell intercalation caused by the sex comb rotation. Leg elongation in both males and females began after 31h AP.

Aside from leg elongation, female legs also narrow over time as the horizontal line of the polyhedron (depicted by a yellow line in Figure 4) decreases in length. However, the narrowing is less dramatic and starts later than in males (Figure 7).

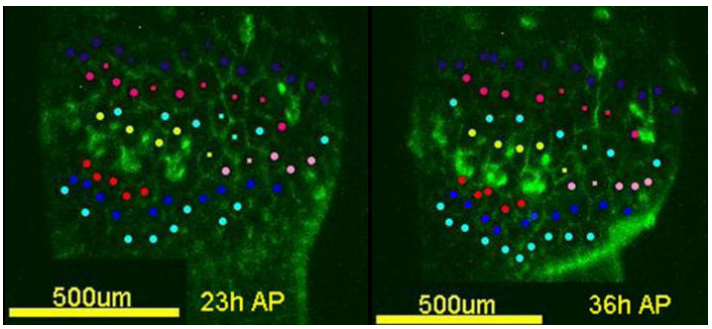

Figure 6: Lines of cells are labelled in different colours at 23h AP (left) and the same lines are seen again at 36h AP (right) with few causes of intercalation between lines. At 36h AP (right) the joint of the leg has been formed.

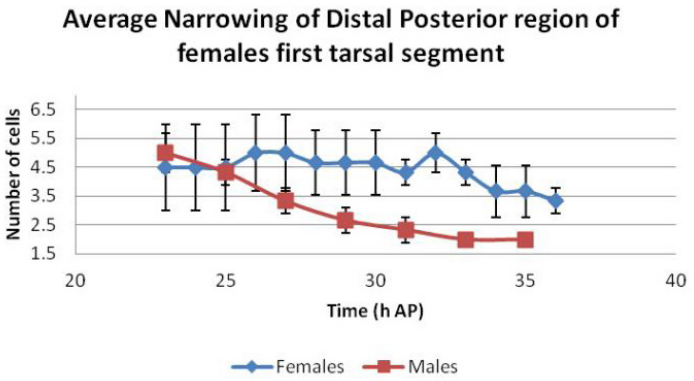

Figure 7: The average narrowing of the tarsal segment in terms of number of cells lying on the vertical line of the polyhedron (on the y-axis). The x-axis shows the time in hours after pupariation. The error bars represent standard deviation. n=3 legs

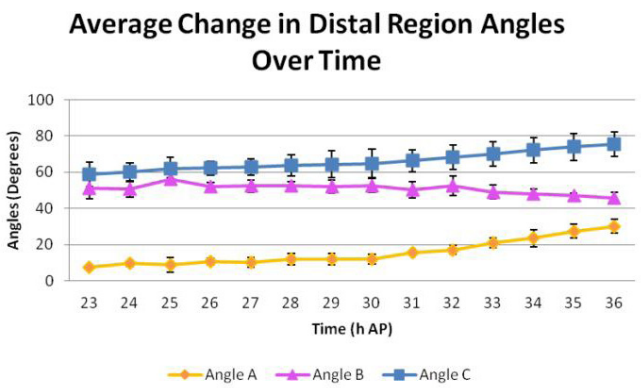

Figure 8 Change in angles of the polyhedron over time. The y-axis shows the angle in degrees, while the x-axis shows the time in hours after pupariation. The error bars represent standard deviation. Angles A and C show a steady increase after 30h AP while angle B stays relatively constant. n= 3 legs

Due to their previous associations with leg elongation, type 1, 2, and 3 patterns were analyzed in the female legs. Unfortunately, GFP fluorescence did not last well throughout the entire duration of the analysis and so cell boundaries were difficult to see in detail for the later time periods. As such, only qualitative observations were made. Between 23h AP and 36h AP, there was an observed overall decrease in type 1 and 2 patterns, leaving only type 3 patterns at 36h AP. This trend of wide and short cell clusters changing to narrow and long cell clusters may be crucial for leg elongation and narrowing in both sexes. However, in females, many patterns resolve due to rotation rather than intercalation. Although the two sexes differ in cell dynamics, the end result is that cell clusters resolve globally to type 3 patterns.

There were also changes in the angles of the polyhedron (Figure 4). Angles A (depicted by yellow line in Figure 4) and C (depicted by blue line in Figure 4) increased while angle B (depicted by pink line in Figure 4) stayed constant (Figure 8). This implies a clockwise rotation in the entire distal anterior region.

Discussion

The lack of a sex comb on female *D. melanogaster* first tarsal segments implied male and female legs undergo differing developmental patterns. The analysis of the epithelia of the female first tarsal segment did show different global changes. Elongation in females is thought to be a good control for analyzing male sex comb development since the mechanism underlying leg elongation can be observed in isolation in females. The female first tarsal segments undergo less cell intercalation, with cell delaminations occurring in the more distal region of the leg than in males. As well, female legs begin to narrow at a later time than males and a global rotation of the distal section of the first tarsal segment is observed. With the differences in development between the legs of the two sexes analyzed, it is possible to use the female leg as a control for analyzing the cellular mechanisms behind the sex comb rotation in the male leg. In particular, certain hypotheses regarding the male sex comb rotation can be made based on the data collected on females.

Epithelial elongation has mainly been described and explained through cell intercalation [1, 2]. While this may be the process in which the male leg elongates, there is not enough intercalation in female legs to cause the same elongation. In place of cell intercalation, female legs likely use cell and tissue rotation for the majority of elongation in the first tarsal segment. Not only do females legs have many cases of type 2 patterns resolving by rotation, but the entire distal anterior region also rotates clockwise.

We have seen that the measured horizontal length of the box narrowed over time, and the vertical length also elongated over time. It should be noted that male first tarsal segments may narrow more than females in the horizontal length due to the method in which lengths were measured. Both horizontal and vertical lengths were measured using number of cells on the line. However, since all cells have different apical sizes, this method may not be accurate. However, measuring distance as seen on the confocal image would not take the curvature of the leg into consideration.

In females, the rotation of the distal anterior region likely causes the leg elongation in the same region. If we take the females to be a control for the males, then the second rotation of the sex comb, involving the anterior section of the sex comb rotating downwards in a clockwise motion (Figure 9), may be a part of the overall rotation of the distal anterior region in males. In terms of evolution, this explanation requires only one small add-on in order to completely change the cellular dynamics for leg elongation in males and females. The sexually dimorphic sex comb rotation would have been a simple addition to a leg which already had a simple elongation method. Since we can note that the overall elongation of the leg as seen in females likely has little effect on the sex comb, it is likely that the sex comb exerts a certain "push" onto the epithelia to cause the variety of cellular processes seen in the males.

Comparison of the first tarsal segment on the second and third legs of males and females can shed more light on how conserved this rotational mechanism is for leg narrowing. If the same pattern is found in the female legs but not the male legs, then the rotation of the tissue may be exclusively a female trait, making the female leg a poor control for studying the male sex comb development. Evidence of similar rotational mechanisms found in both male and female legs will be able to show if the rotation is the mechanism behind the leg narrowing.

However, the rotation of the box did not explain why so many cells delaminate out of the epithelia in the distal region. A likely reason for cell delaminations may be formation of the joint, which narrows the leg further. Cell delaminations in females and males were thought to be caused by the same force since the majority of delamination occurs after 28h AP in both sexes, although in different locations. This force is hypothesized to be a result of the joint formation of the leg, which also occurs around 28h AP. It was thought that

the joint formation creates a narrowing pressure on the leg. In the presence of a sex comb rotation, the location of that pressure might change from the distal posterior region to the more proximal region. In females, the transverse row does not rotate, so cell delamination offers a convenient mechanism for narrowing. Analysis of other female tarsal segments which also have joint formation might help to determine if this pattern of cell delamination is correlated with joint formations in general. How the joint formation creates the force is a mystery due to lack of visible detail around the curvature of the 3D nature of the joint from the confocal images.

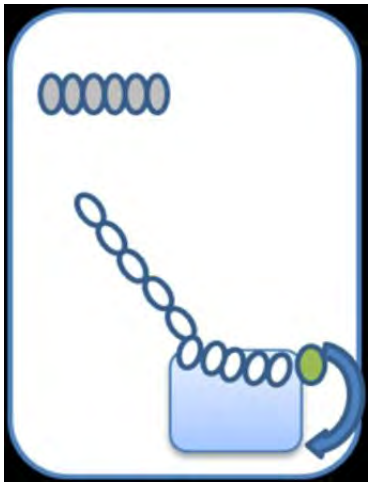

Figure 9: The second process in the sex comb rotation where the more posterior part of the sex comb rotates clockwise downwards. The bottom white circles represent the bristles in the sex comb while the top purple circle represent a transverse row. The green circle represents the campaniform sensillum. The region in the rectangle shows the cells that rotate in the last step of sex comb rotation, with the arrow marking how the rotation will proceed.

Conclusion

The analysis of the first tarsal segment of the first leg of female *D. melanogaster* provided much insight into the elongation of the tarsal segment without the complications created by the rotating sex combs in males. In fact, the complicated nature of the sex comb rotation may simply be explained by the addition of the sex comb, which applies an additional force to change the overall cellular dynamics. The global rotation of the distal posterior region in females suggests that elongation of the first tarsal segment of females after 23h AP is not due to intercalation, but instead due to the rotation of cells. Analysis of the 2nd and 3rd legs can help us determine if the processes described in this study are exclusive to the first leg or are universal. Now that this complex elongation system can be analyzed by using a simpler control, we can attempt to analyze more systems that were previously too difficult to study.

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Dr. Mladen Vranic

Professor Emeritus Mladen Vranic completed his MD and PhD at the University of Zagreb. He completed his post-doctoral training with Dr. Charles Best at the University of Toronto in 1965 and embarked on active research and teaching career at the University of Toronto which has propelled the field of diabetes forward. Dr. Vranic has been inducted into the Canadian Medical Hall of Fame and has received many awards in recognition of his pioneering work, including the Order of Canada (Officer) and Ontario, the Canadian Diabetes Association's Inaugural Life-time Achievement Award, and the Banting and Best Memorial Award, and the Mizuno Lectureship and Award from Japan. From the American Diabetes Association he received the Banting Medal and Lectureship for Distinguished Scientific Achievement and the Albert Renold Award for training scientists (he is the only Canadian to get these two most important world-recognitions in the field of diabetes). Dr. Vranic also received an honourary degree of Medicine from the Karolinska Institute in Sweden, and honourary DSc degrees from the University of Toronto, Saskatchewan, and Zagreb, Croatia. He is a fellow of the Royal Society of Canada, Canadian Academy of Health Sciences, and corresponding member of the Croatian Academy of Arts and Science.

Interview conducted by: Ana Komparic

AK: Could you please introduce yourself and give us a brief overview of your many years of working in the field.

MV: I graduated with a MD in 1955 in Zagreb and then I did my PhD in the field of diabetes in Zagreb. Later, my father developed type II diabetes, so I observed some of his problems first hand. A few years after I started my PhD, I realized that if I wanted to continue, I would need post doctoral training. I had friends in Germany and one of those friends was Creutzfeldt and his father was the co-discoverer of Creutzfeldt-Jacob Disease, also known as mad cow disease. Creutzfeldt had an opportunity to visit the U.S. so he knew a lot about the U.S. and Canada. When I was in Germany, he introduced me to someone from the Joslin Diabetes Center in Boston. We had a meeting of the International Diabetes Federation in Geneva. Today, about 15,000 people attend this event, but at that time, over 50 years ago, a little boat was enough to accommodate us all. He introduced me to Albert Renold, who was one of the leaders in the field of diabetes. Through Renold, I was introduced to scientists from the University of Toronto and eventually came into contact with Charlie Best. After two months, I received an invitation letter to come to Canada. At the time it was very hard to leave from communist Yugoslavia, and it took a guarantee from the Prime Minister of Croatia to allow my family to leave for Canada with me. The Prime Minister worked as a professor at the School of Economics at the University of Zagreb, as did my father, and my mother talked to her to let us all go.

After my arrival to Canada I spent two years in the Department of Physiology as a post doctoral student at the Best Institute. The Best Institute was very different from most places in Toronto because it had many foreign staff and visitors. At that time, Toronto was very restricted to foreigners so Best was criticized for having so many foreigners either as post doctoral students or as scientists. Towards the end of this period, I had to decide if I would go back to Zagreb or if I should find a job in the U.S. or Toronto. I went to the U.S. to search for placements because at the time an offer in the U.S. would make me a competitive candidate in Toronto. A friend of mine was a pilot, so he rented a four-seat Cessna plane and we flew around the U.S. Only later did I find out that he had never before flown outside of Toronto! I got two offers in the U.S. and when I came back to Toronto, I got an offer to stay. When I started my career, my wife was writing her medical exams to get certified to work in Canada, we had our two year old daughter, I had to dedicate half of my time to teaching in laboratories and lectures, and I had to apply for grants from the Medical Research Council. Today you may take two months to write a grant, but they gave me ten days. I had only a quarter of a small laboratory. I had to take a course in both theoretical and practical biochemistry and start my own research. So, it was a very strenuous period. When the Medical Sciences Building was built, we moved here and I got my own lab.

I was very lucky in choosing my students. Several of my students have become presidents of various Diabetes Associations around the world and practically everyone who worked with me is in an academic position either as a leader in basic research or clinical research. So I was either very lucky or I had a good eye for picking out the right people.

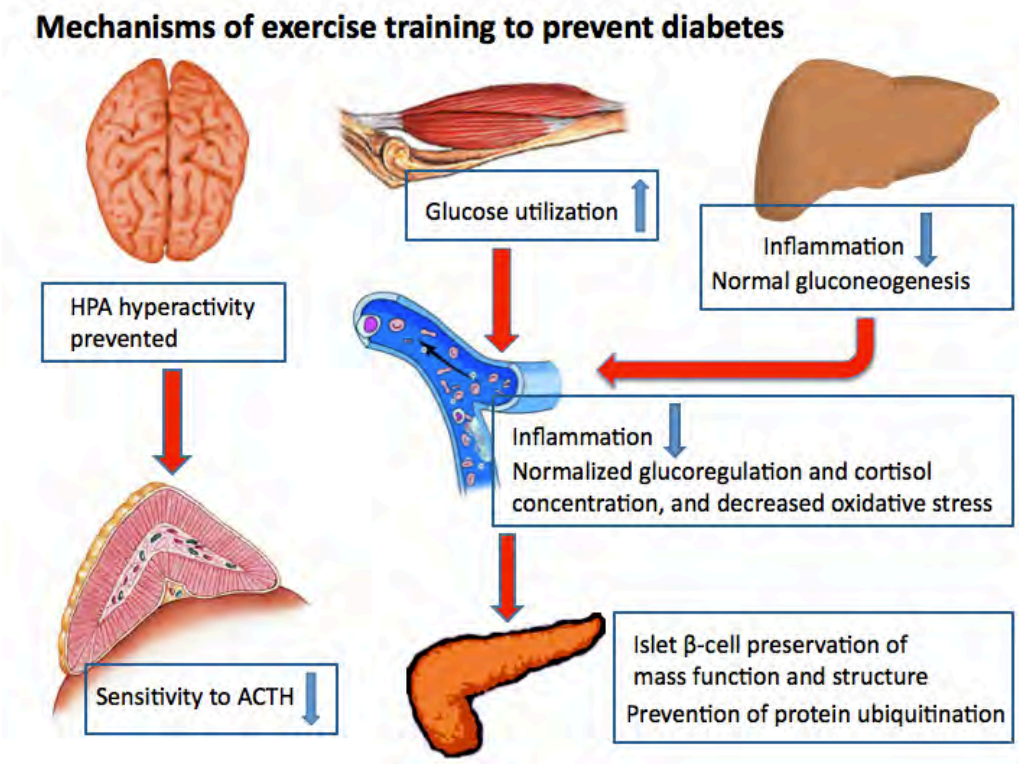
One of the first problems that interested me was that, if you want to learn anything about diabetes you cannot just measure blood sugar levels. You really need to measure how much glucose is produced by the liver and how much glucose is used in the periphery. You also need to measure it quickly when something happens. This can be done by using the tracer method. When I started working, the tracer method was really bad and was not considered as an appropriate method because these methods were never properly validated, so clinicians never used them. Experiments on dogs were done in Toronto and by another group in New York. I was very lucky that one of my students had a strong mathematical background, and later on another one had a background in engineering, so we were able to validate and perfect this method. The moment we validated the tracer method and published a clinical paper with my friend George Steiner it was like a tsunami. Everybody started to do it. I became the president of a group of basic scientists and we started to publish a number of papers showing the validity of these methods. We could quickly accumulate data about how hormones interact and influence blood sugar, what is wrong in diabetes, and this started to clarify how insulin, glucagon, and epinephrine interact. At the time of this experiment, diabetes was considered to be a deficiency of insulin – either in amount or its action – and glucagon. It was clear that these two hormones act on the liver and insulin acts on the liver and the periphery. At that time, however, it was very difficult to measure glucagon. Three laboratories in the world measured glucagon and it was shown that if you take the pancreas out, there is no glucagon in dogs, but you get very severe diabetes. So my question was, how can you have severe diabetes if you remove insulin and glucagon? Today we know that if we remove insulin and glucagon in humans, there is very mild diabetes. So, why was it severe in dogs? And all three laboratories claimed that there was no glucagon outside the pancreas. We were able to show that, actually, the stomach is a very strong source of glucagon in dogs. We did the experiments in Toronto and we proved that the stomach in dogs makes glucagon and that it is the same glucagon as in the pancreas, with the same physiological effects. So, only if you remove the stomach and pancreas in the dog, glucagon secretion is totally eliminated. This suddenly opened the door and we said, yes it is obviously severe diabetes because the dogs can produce a large amount of glucagon. So for four years, this was one of the main topics in international diabetes meetings. On the basis of this work, I got the most important recognitions from the Canadian Diabetes Association (Banting and Best Lectureship and award) and then the most important recognition from the United States, the Banting Medal. I got another award for mentoring students because I had such strong students.

Then I had a very unique opportunity and began working in the field of exercise. When I was Best's student, he delegated me to two preceptors. One preceptor's name was Jerry Wrenshall. He was a type 1 diabetic. He believed, and rightly so, that it was important for him to exercise. So, he would walk after each meal. We started to do experiments in diabetic dogs. When we did not treat them with insulin, their diabetes worsened. We did many experiments and determined that exercise is beneficial if you have an appropriate amount of insulin, and it is not beneficial if your insulin doesn't work properly for some reason, such as when you have the flu, break your leg, or any other state in which insulin does not work properly. I was then invited to host the first symposium on exercise and diabetes in California. These symposia were organized on the ranch that belongs to Mr. Kroc, owner of McDonald's hamburgers. We survived, because we were never fed McDonald's food. Later, epidemiologists came up with firm evidence that exercise can not only improve, but also prevent diabetes. We think the push for these studies came from the first conference. The main problem in type 2 diabetes is insulin resistance. Therefore, they need an increased secretion of insulin. As long as the pancreas can produce enough insulin, the blood-sugar remains normal. However, in a number of obese diabetics, the pancreas is not able to maintain this high production of insulin. With regular exercise, glucose uptake in the muscle is increased. Sub-acute inflammation, which is always present in obesity, decreases. In addition, the function of the hypothalamic-pituitary-adrenal axis is decreased, and the adrenal cortex is less sensitive to ACTH. Thus, less cortisol is produced. All of these factors decrease the oxidative stress of the insulin-producing β-cell, the mass and function of the β-cell is protected, and a number of proteins are protected from abnormal folding and destruction (Figure 1). The recommendation of the surgeon general of the United States suggests that it is sufficient to walk briskly, five times a week for one hour. The same amount of exercise was also necessary for our diabetic rats, and diabetes was eliminated. It was paradoxical when we found that similar repetitive stress exposure also prevented diabetes. We concluded that adaptation to stress is beneficial, but the absence of adaptation is deleterious in many ways and cannot prevent diabetes. One problem that diabetics face when they exercise is that their blood sugar drops and cause hypoglycemia.

It is dangerous if they don't address it right after the exercise. So hypoglycemia is a major problem for diabetics. Why? Because we learnt about twenty years ago that intensive treatment of diabetes greatly decreases the dangers of the big complications of diabetes, such as affecting the eyes, the nerves, the heart, and the kidney. Today diabetes is the main reason for blindness in the U.S and the main reason for kidney failure. But it was shown that if you have intensive insulin treatment, then you can diminish the risk of these complications. The problem with intensive treatment is that you give insulin five times per day, which greatly increases the danger of low blood sugar. So, if I was a diabetic and I am busy, I cannot really risk having hypoglycemia every day, so I would still use intensive treatment, but a little bit less intense. I would risk some of the complications. We have patented a project in which we are preventing low blood sugar in diabetics and so far we have very good results in rats, which have been published. Presently, we are investigating whether there are risks with this approach before clinical investigation can start.

The other luck that I had was that I could collaborate with many younger clinicians, for example, Amira Klip at the Hospital for Sick Children. She is a leader in the field of glucose uptake. With her, we clarified that the muscle actually does not have diabetic complications because there is a built-in mechanism which prevents too much sugar from entering into the muscle. The problem with complications is that many organs are flooded with sugar, which binds with proteins and this is damaging.

Then we had a longer collaboration in Sweden. Using our tracer methods, we did a lot of work on the liver. We also showed that the liver is protected against diabetic complications and we



explained the mechanism both in humans and in dogs. Because of this very intense collaboration in Sweden, I got an honorary degree of Medicine at the Karolinska Institute. The Karolinska Institute is the number two medical school in the world. They have given these honorary degrees over the past hundred years and there are three Canadians who have received them.

And finally, we had a very good collaboration with Gary Lewis, who is now the head of the Diabetes Centre in Toronto, to investigate a very interesting phenomenon. We all know that insulin is a very important hormone which decreases glucose production delivery, which is very important in diabetes because if you produce too much sugar, that is bad. A friend of mine, Richard Bergman, indicated that there is very little evidence that insulin is actually very important in the liver, but rather that it has indirect effects. So, we did experiments in diabetic dogs, and with Gary Lewis we did experiments in humans and we could show that in non-diabetic humans, about half of the effect of insulin is direct, the other half is through the effects of insulin on fatty acids, on glucagon, and other things. And in type II diabetes it seemed that insulin has only indirect effects. With type II diabetes, insulin does not affect the liver, which is important in clinical considerations.

In this department, there are two professors who were my graduate students. One is Pat Brubaker, who has a very interesting career. She had a very productive collaboration with Dan Drucker after she finished her post doctorate with me. Dan Drucker is the previous head of the Diabetes Centre and he is in the Department of Medicine. Dan Drucker worked with GLP, which is a glucagon-like peptide that is produced by the gastro-intestinal tract. Until Dan Drucker started his post doctorate in the U.S., people believed that these peptides have absolutely no function. During his post-doctoral training, he was able to show that these peptides control insulin release by the pancreas. The two newest drugs in diabetes are based on our understanding of GLP— one has the activity of this particular peptide, while another inhibits enzymes which would destroy the natural protein in the body. Pat Brubaker determined many of the physiological aspects of this phenomenon. Additionally, they also discovered a hormone – GLP 2 – and established that it regulates the growth of the gut. This hormone is still being investigated in humans and it could be of importance in many diseases of the gut.

Another one of my students who is now a professor at U of T, Adria Giacca, is very interested in the effect of fatty acids. There are many factors which are good for the pancreas and there are many which are not. So she is investigating the role of fatty acids and she found that when they are elevated for a prolonged period of time, they inhibit the function of the endocrine pancreas.

AK: You mentioned that a lot of your students had varied backgrounds when they came to your laboratory. Do you think there is a value to having a multidisciplinary background when embarking on a research career?

MV: This was absolutely essential. The wrong philosophy of some people is that a student comes with a certain background and the professor wants them to do something else. My approach was: let's have a look at the person and see what their background is and what can they do best. So I had two students, as I mentioned, who had a strong background in mathematics which was very helpful to develop this particular area of tracer methodology. Then I collaborated with Cecil Yip, who was a superb biochemist. So my students collaborated with him when we needed to investigate the biochemical aspects of the extra-pancreatic glucagon. When I was in Geneva, we had to do very complicated experiments by removing part of the gut. Two people in physiology at the University of Geneva were very well-versed surgeons. Another great friend of mine, Philippe Halban, developed a new tracer during his PhD in Oxford. So, we could use this new tracer to investigate certain aspects of exercise in diabetes, which we would not have been able to do otherwise. I was also fortunate here when I collaborated with the present chairman, Steve Matthews, because he is very good at molecular biology. So what do you need today? You need superb surgery, immunology, molecular biology, genetics and many other approaches. It is becoming more and more important to collaborate because no one can control all the areas that are necessary.

AK: What other qualities or personal characteristics do you find are important to have in a career in research?

MV: First: passion. Without passion, it is better not to do anything. You need to be passionate. For example, convincing other scientists of extra-pancreatic glucagon was a big fight for me. My professional life concentrated on this problem for a number of years. In exercise, I was trying to convince people how important it was to study exercise in diabetes.

The other characteristic is survival, because in science you have ups and downs. And so you have to have a similar attitude to a poker player. When I was in Medical School in Zagreb, I played poker. If you win, you try and win as much as possible. If you lose, you try and lose as little as possible. Exactly the same approach applies to science. When you have something good in your hands, do not stop it by any means. You need to follow-through with projects. It is your life goal, you cannot stop.

And then you need to be able to get funding. In other words, you need to know how to write grants. Without that, you cannot do anything. That requires not only good ideas, but also the ability to sell your ideas. If you cannot sell your ideas, the competition is extremely stiff today. And then either you or your collaborators have to have a good knowledge of modern methodology, which changes with the speed of light. I never had the great ability of understanding all the aspects of methodology, but I had collaborators and students. So in other words, obviously you do not need to have all the characteristics yourself.

AK: I noticed you recently wrote an article chronicling a retrospective of your career entitled "Odyssey between Scylla and Charybdis through storms of carbohydrate metabolism and diabetes: a career retrospective". Why did you choose this title in particular?

MV: I discussed this in a convocation speech I gave last year at the University of Toronto. Life can be unfair and tough. It is not easy to get new and original ideas accepted. In that respect, I titled my retrospective "Odyssey between Scylla and Charybdis". You may recall that Scylla and Charybdis were two sea monsters described by Homer. They were located close enough to each other that they posed an inescapable threat to passing sailors, including Odysseus. Avoiding Charybdis meant passing too close to Scylla and vice versa. You need the legendary strength and perseverance of Odysseus to avoid being devoured by sea monsters. The main struggle of a scientist is the struggle for grants and the acceptance of original ideas. So, the key is really originality. When a big discovery is made somewhere, it is very tempting to jump onto the bandwagon. I selected not to do that. I tried to have my own path, just as in Robert Frost's famous poem "The Road Not Taken", where you have to decide between taking two roads: the road that many people walk or the road that is less travelled.

AK: You've worked and taught actively at the University of Toronto for almost 50 years now. How has working at U of T influenced your work? How would you characterize the research environment?

MV: When I came, the research environment in Canada was not very good. When I was canvassing for money I was told that science is done in the United States, and that Canada does not need science. This has totally changed. Today, Canada is a leading country in medical sciences. The best universities in Canada are the University of Toronto and McGill in Montreal. So things changed. Why did things change? Scientists and resources came from Quebec while there was unrest there in the 1970's. In our clinical division of endocrinology, most of the older endocrinologists came from Montreal. And then of course there was an influx of foreigners—students and staff who are very dedicated. And it is a fantastic country. As well, the government started to understand that science is not just fun, but that it creates jobs. The discoveries in medicine made around the world in the last forty years have been incredible. So the governments at least partly understood. The great thing about the University of Toronto is that, if you want to collaborate, you can find anything you want around our campus. And that is very important. Of course one can collaborate through e-mails with anyone in the world, but it is easier if you can walk 5 minutes and drop in on someone and talk with them.

We should just briefly touch upon one last topic since this is the 90th anniversary of the discovery of insulin. For many years, people thought that insulin was discovered by Fredrick Banting and Charles Herbert Best, which is evident if you look around the buildings on campus or the names of various grant agencies. Thirty years ago, Michael Bliss published a book about the discovery of insulin in which he showed that the discovery of insulin would not have been possible if the chairman of the department was not John James Rickard Macleod. Macleod was considered a world-leading metabolic physiologist at the time. He gave lectures, which Best attended. When Banting came, he explained to him how to remove the pancreas, how to ligate the pancreas, how to extract what was later called insulin, so he gave him many suggestions, and also established contacts with the world. And finally, in the last stage, he actually directed research into how insulin acts. They were very simple methods, but some of the main effects of insulin were known very quickly after insulin's discovery. So the Nobel Prize was awarded to Macleod and Banting because insulin had been discovered in dogs, insulin could be given to humans, and the mechanism of insulin was in-part explored. Best finished a pre-med course of four years, so he knew a lot of basic physiology and biochemistry and was an energetic young man. Banting was a little bit bipolar—he could be enthusiastic and depressed. It was very important for Banting to have worked with somebody like Best who was always enthusiastic. And then James B. Collip, a professor of biochemistry, purified insulin. Without insulin purification, insulin could never have been given to humans. There were other people who had extracted the substance and if they injected it into humans it either caused inflammation, so they could not repeat it, or it did not work. So, James Collip's work was also essential. We had a symposium organized by the Gairdner Foundation and there will be a paper published giving the evidence about what was the contribution of each of the discoverers, so that people finally realize that there are more than two discoverers—that all four were absolutely essential.

Best's contribution, in addition to what he did after the discovery of insulin, included leading an institute which had an international setting, which was unique for Toronto at the time. Not as unique for Montreal as McGill was quite open. He helped with funding, with the Canadian Diabetes Association, the American Diabetes Associations, camps for children with diabetes, and organized many conferences. He had a vast importance in the field of diabetes and was highly regarded around the world. Banting died in 1941, in a plane accident, while flying to join the Allied Forces in England. Macleod left because of frictions and was the Dean in Aberdeen and Collip had a fantastic career in endocrinology and is considered the founder of Canadian endocrinology. I want to make it clear that our Department of Physiology is fantastic. The diabetes research scene in Toronto, both in basic and clinical departments, is by far the strongest in Canada.

Dr. Jeffrey M. Friedman

Jeffrey M. Friedman, M.D., Ph.D, of Rockefeller University, is a molecular geneticist whose discovery of the hormone leptin and its role in regulating body weight has changed our understanding of the causes of human obesity. He has received several prestigious awards for this work including the Gairdner Foundation International Award and the Passano Foundation Award.

Prior to his groundbreaking research, little was known about the components of the biologic system that controls weight, leaving many scientists to question the very existence of such a homeostatic system. With Dr. Friedman's discovery of leptin and his subsequent studies, the foundation to understanding an entirely new physiologic system has been established. This knowledge has direct implications for the pathophysiology of human obesity.

Interview conducted by Tina Binesh Marvasti

"I think one of the great challenges we faced was that technology that exists today was embryonic at the time."

TBM: You were pursuing medicine as a career before you got involved in research. What inspired you to change your career path from medicine to research after being trained as a medical doctor?

JF: Well, it was a combination of what I think I saw as my long term interest, but also chance. I had actually done research as a medical student and as a medical resident, but it wasn't particularly distinguished. In fact, I remember the reviews of the first paper I ever wrote came back and both were negative. The first review explained what the reviewer thought were some of the flaws of our paper and the second review simply said that this paper should not be published in the JCI or anywhere else. So, I really didn't think research was necessarily where I was going to end up. However, I think my mentors at the time must have thought that I might enjoy it. I had a gap year between concluding my medical residency and the beginning of what was supposed to be a gastroneurology fellowship. So, I wasn't sure what to do with that year, but one of my professors from the time I did research thought I might like to do more research and introduced me to someone at Rockefeller University who was studying the relationship of specific molecules to addiction. I met Mary Jane Kreek and started working in her lab for about a year and found that I really love research. So instead of going on to my fellowship, I entered the PhD program at Rockefeller and I have been there ever since.

TBM: So it seems like you have had good mentors guiding you throughout the process. In your opinion, how important is it to have a mentor in order to succeed in medicine or research?

JF: I think having a good mentor in research is extremely important. Honestly, I don't feel qualified to speak about that in medicine. And in medicine, there is not just one mentor anyway. I think the evidence seems to indicate that mentors with a proven track record continue to have laboratories that deliver scientists that are more likely to go on to careers of distinction. So, I think a mentor is really important. I should also say that my mentor was a very well known scientist named James Darnell. He studied RNA metabolism and he was one of the early leaders in the field of the molecular biology revolution. By working in his lab, I developed an important level of understanding and a skill set that made it possible to ultimately clone the Ob gene when we set out to do that.

TBM: Intriguing! Most scientists in the field think that your discovery that leptin is a hormone produced by the adipose tissue has resulted in a paradigm shift in the field of endocrinology because

before, we thought that adipose tissues only stored fat, but now we know that they can also be classified as an endocrine organ. So, what were the challenges that you faced from the scientific community while you were trying to convince them of your results?

"Whatever one's specific talent is, the one thing you can't do without is determination."

JF: Yes, we now do realize that the adipose tissue is an endocrine organ. I joke sometimes that it is the largest endocrine organ, but one of my colleagues who studies gut hormones says that about the GI tract. We finally agreed that the GI tract is the longest endocrine organ. So, I think that it is fair to say that part of the discovery of leptin and the notion that there were hormones regulating appetite was disbelieved. The hypothesis was put forward, but I don't think that in the absence of actually identifying the hormone people would have believed it. I don't say that it was an obstacle for us because we were just doing what we were doing, but I don't think most people expected the Ob gene to actually encode a novel hormone. Nor, was it clear at the time that it would come from fat. It could have come from any number of places. For example, you never imagined that insulin would come from a tiny cell-type in the pancreas, but it is true in endocrinology that oftentimes very specialized cells transduce important biological information. We worked for eight years on the identification of leptin and it was a very long time. I think one of the great challenges we faced was that technology that exists today was embryonic at the time, and a project that today would probably take a year took at least eight years back then and it required not only implementing many technologies, but also developing technology. Having worked on something that long, with many people being sceptical whether it was worth it in the first place, it was a rather exhilarating moment to ultimately identify the gene. The initial data with early experiments spoke very clearly to the fact that, not only we had cloned the Ob gene, but that it would encode a hormone. Identifying the gene really went on to prove that. The next step after the gene identification was to try to prove that, in fact, this was a hormone. We needed to show that it circulates in the blood, that its levels increase with obesity and decrease with leanness, and that the protein has bioactivity. This went on to prove that the Ob gene encoded a novel hormone regulating weight.

TBM: Interesting! As a pioneer in the field, I am interested to know your opinion on leptin replacement therapy and its therapeutic potentials.

JF: For any hormone, there are pathologies associated either with hormone deficiency or hormone resistance. There are several conditions associated with leptin deficiency including mutations in the leptin gene, but also forms of diabetes called lipodystrophy. Another condition with leptin deficiency is called hypothalamic amenorrhea, which is often seen in very lean young women who stop menstruating and also start developing immature osteoporosis. Leptin has now been shown to have a very potent efficacy in treating the pathologies associated with leptin deficiency. Obesity, however, is not a leptin deficiency state and it turns out to be a hormone resistant state very much similar to insulin resistance in type II diabetes. Now, typically it is more difficult to treat a hormone resistant state with extra hormone. We now know that leptin is a monotherapy for obesity and it has some efficacy in some set of patients, but the overall effect is not as great as one would have hoped for. However, there is more recent evidence showing that if you combine leptin with other hormones you can get a very robust effect to reduce weight. In one study, a combination of leptin and another peptide called aniline was able to reduce body weight by more than 10% greater than the placebo. Based on that, leptin would have potential as an anti-obesity compound. However, there are certain complications that develop with the treatment – in the form of antibodies to leptin – so, whether or not that combination becomes an anti-obesity therapy depends on how that evolves. But, there is no question that leptin can be a part of a very effective combination to reduce body weight and the hope now would be to replicate that effect with other agents.

TBM: Just as a final question, most of our readers are interested in pursuing careers in medicine, research or a combination of both. What do you think are some personal qualities that they should develop in order to succeed in these fields?

JF: Well, I have done both, although I have spent much more of my career as a research scientist, but I did complete my medical training and a year of medical fellowship. So, in the year between my medical residency and my PhD, I worked in a laboratory, but also attended a GI clinic. There are very good reasons to be a good doctor. It is a very rewarding and satisfying profession and provides opportunities to help other people. I think the reason to be a doctor is if someone wants to take care of patients. The reason to be a scientist is that you like laboratory experimentation and learning new things in the laboratory setting. In research, you learn things that were not previously known, but in medicine you are applying an aggregate body of knowledge to each patient to the best of your ability. These are quite different processes and I certainly think there is a place to do both. A reason to do both, in my opinion, is that someone likes taking care of patients and also wants to work in a laboratory. For someone who likes laboratory research but doesn't want to take care of patients, I am not sure how useful the medical training will turn out to be. In terms of personal qualities, I think everyone has qualities that would be beneficial for any of these fields—like being insightful, confident, working well with other people, and, of course, it is also important to be hard working. However, what I would say is that whatever one's specific talent is, the one thing you can't do without is determination. So, there are many people who are less intuitive than others and become fantastic scientists but there are very few scientists that I can think of who do not bring a tremendous sense of determination and passion to what they do. This is not limited to science, but I think this is the single most indispensable quality.

TBM: It is the key to success it seems.

JF: Yes, in anything.

TBM: It was a pleasure to interview you and on behalf of everyone at JULS I would like to thank you for your time.

JF: My pleasure.

Dr. Aharon Razin

Prof. Aharon Razin was born in 1935 in Tel Aviv, Israel. He received his B.Sc. from the Hebrew University and then went on to earn a M.Sc. and Ph.D. in the laboratory of Prof. Yaakov Mager on the subject of nucleotide metabolism. He conducted his postdoctoral research in the laboratory of Dr. Robert Sinsheimer at Caltech, where commenced his investigations in DNA methylation. Since then, Prof. Razin, along with Dr. Howard Cedar, has pioneered the field of DNA methylation, proposing and demonstrating many groundbreaking concepts in the field. Since 1971 Prof. Razin has been a faculty member Hebrew University where he remains a full professor in Biochemistry. Prof. Razin is an elected member of EMBO and became a member of the Israel Academy of Sciences in 2008. He is the recipient of many prestigious awards including the Israel Prize, the Wolf Prize in Medicine, the Emet Prize in Life Sciences, and the Canada Gairdner International Award.

Interview conducted by David Kleinman

"You start, you study, and then during your studies you decide what is interesting."

DK: Could you tell me a little bit about the path that you took to become the scientist that you are today? How did your education shape this path?

AR: As a matter of fact, the path is like with any student: you start, you study, and then during your studies you decide what is interesting. In my case, I studied mathematics and physics and I found out that only about five students spoke with the same understanding as the lecturer. And although I was very good at mathematics, I realized I could not become a mathematician. So I went into chemistry, as I was also very good at chemistry, and I was happy with the studies. In the last year we had to choose within chemistry— like physics, physical chemistry, biology or something like that. So, I went on to biochemistry and from then on I was a scientist.

DK: You won the 2011 Canada Gairdner International Award for your studies in DNA methylation. How did you get into the study of DNA and DNA methylation?

AR: It was by chance you know. Well, not really by chance. I wanted to study DNA. As a PhD student, I studied nucleotides. I was jealous of those who studied proteins and DNA. For my post doctoral studies I went to Caltech and I studied DNA. One of the papers that I published there was on how proteins bind to DNA. So, that was the first time that I studied methylation and I found that the protein recognizes the DNA by methylation, and from there it's really a story. So, we were pioneers, pioneering the field of methylation that was almost dead; nobody worked on it, it was deserted.

DK: How do you feel that your work and science in general have changed throughout your career?

AR: Our work on methylation was pioneering work. As technology changes, our science changed. Many of the things that you believed you could not do in the early days can be done now with new technology.

DK: What do you think the future holds for epigenetic research?

AR: I think there is a huge future. The future of biology is epigenetics. It is a huge field with branches that touch everything. It

touches development, it is very involved in development. It touches reprogramming, which is a new field of study— reprogramming a cell to become a stem cell.

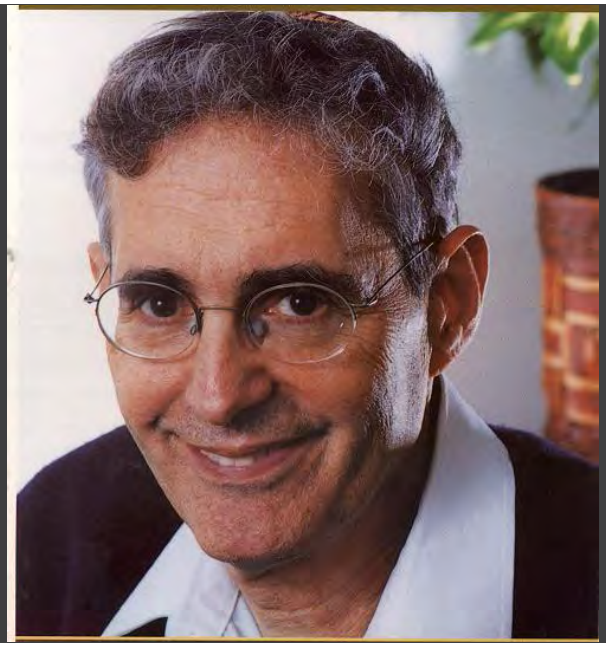
"One of the most important is to never to go for getting awards. They will come by themselves if your work is good."

DK: Like induced pluripotent stem cells (IPS)?

AR: Yes, but IPS is not a very good way of getting pluripotent cells. I don't understand how it works, but it works. When you study methylation, you try to see what it is in the methylation pattern that causes cells to change. And you see that IPS didn't change the cells very well. And it is well understood that you can't use IPS for therapy, because IPS cells are not the same as normal cells.

DK: This journal is primarily for U of T undergraduates, so what advice do you have for aspiring scientists?

AR: I have several pieces of advice. One of the most important is to never to go for getting awards. They will come by themselves if your work is good. So do your work consistently and don't give up if it does not work. To be a good scientist is to be stubborn, and of course you have to be talented, but it goes without saying that students are talented. Use your imagination and don't do things that everyone else does because otherwise you are not special. You have to do things that nobody else has thought about yet. It's not easy, but if you do it, then you have a good career. Also you have to get lucky, although that's not really advice.



Dr. Howard Cedar

Howard Cedar was born in New York. He studied mathematics at M.I.T. and received his M.D. and Ph.D. from NYU. In 1973, Dr. Cedar immigrated to Israel and accepted a position as Professor of Molecular Biology at the Hebrew University in Jerusalem where he remains to this day. Over the past 30 years, his laboratory, along with that of Prof. Aharon Razin, has pioneered the study of DNA methylation to help explain gene regulation, human development and the molecular basis of various diseases. He was elected a member of EMBO in 1983 and became a member of the Israel Academy of Sciences in 2002. Dr. Cedar is the recipient of many prestigious awards including the Israel Prize, the Wolf Prize in Medicine, the Ernet Prize and the Canada Gairdner International Award.

Interview conducted by David Kleinman

"It turns out that there are a lot of basic questions that remain unanswered, that haven't been dealt with."

DK: You studied math at MIT during your undergraduate years. What led you from your studies in math to medicine and then to molecular biology?

HC: You want to hear the story? I went to MIT wanting to study nuclear physics. I got to MIT and at MIT everyone learns all the basic sciences in the first two years. Everybody takes the same math, chemistry, physics— now they have biology as a core subject. Then when I got to third year I took my first course in atomic physics. After my first exam – I did very well on my first exam, I got a 96 – I looked over the exam and noticed that on the main question I had gotten the right answer – I had gotten the right number, the right number was 6 – but I had written the number 35 orders of magnitude off. It was supposed to be and I had written. I looked at this and realized that I'm not really much of a physicist. So, I decided that I was going to change direction. I was very interested in studying medicine, in doing research and medicine. To finish out my degree at MIT I continued in math, which I was very, very good at, and then I went on to medical school.

DK: How do you feel that the medical education that you got at NYU influenced your career as a scientist?

HC: NYU was a wonderful medical school. The teachers were extremely cooperative. There was a very strong basic medical sciences curriculum. There were opportunities for people to work in laboratories. At the clinical level, attending physicians were extremely cooperative; they were always willing to take their own time to work with us. They were always connected to basic sciences, so we learned clinical medicine from the point of view of basic biology. So I thought it was a wonderful experience.

DK: I noticed that you were drafted into the U.S. army, but you didn't have to serve as a soldier; you got to serve at the National Institute of Health as a scientist. How was that experience?

HC: You have to understand—when I finished medical school, the United States was still involved in the Vietnam War. Medical school had gotten me an exemption to study medicine. Everyone who finished had to serve in the army. I was just very, very fortunate and got into the public health service and into a research unit. So it was just luck.

DK: You received the 2011 Canada Gairdner International Award for your work on DNA methylation. What led you to study DNA methylation and how did you put together the link between DNA methylation and gene expression?

HC: All of this research was sort of curiosity-drive. We were interested in development. We were interested in understanding how different cells in the body do different things even though the cells contain the same genetic information. I was fortunate to work with my colleague Aharon Razin who had experience with DNA methylation in bacteria. Together, we came up with the idea that DNA methylation could be a good marking system and we pursued.

"Diversity of knowledge is very important."

DK: What do you research now and how did you get there from DNA methylation?

HC: So, all of my work on DNA methylation is really basic work: basic metabolism of DNA methylation, basic role of DNA methylation, how it works. We've never ventured into live studies. But it turns out that there are a lot of basic questions that remain unanswered, that haven't been dealt with. Those are the things we're working on now—really basic things that no one has ever asked before. And we're doing that now.

DK: Could you give me an example?

HC: Sure, so we know that methylation works like a switch. These switches are set during development. Nobody has really asked what sets the switches and that's what we're doing now— trying to figure how the cell can figure out where to put the methyl groups.

DK: Most of the readers of this journal are undergraduates, many of whom are aspiring scientists. What advice do you have for them?

HC: A couple of things. First of all, diversity of knowledge is very important. If somebody wants to be a scientist in any field, it's important to acquire the basic fundamental sciences: math, physics, chemistry, and biology. It might sound surprising, but later on when you get involved in a specific project or a specific concept all of these things not only come in handy, they're essential.

JULS REVIEWS

Fabry Disease: Gene Therapy as an Emerging Alternative to ERT

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Abstract

The lysosomal storage disorder Fabry disease is commonly treated with enzyme replacement therapy (ERT) using recombinant forms of human α-galactosidase A to supplement reduced levels of this enzyme. Patients who follow a regular regime of ERT experience a reduction in globotriaosylceramide (Gb3) accumulation and a decreased risk of renal, cardiac, and cerebrovascular complications. Despite the clinical benefits, the high cost of ERT and its debatable long-term effectiveness suggest the need for alternative methods of treatment. This review compares ERT with an emerging method of treatment: gene therapy. Gene therapy has been suggested for treating Fabry disease in an attempt to normalize α-galactosidase A expression in targeted cells, thereby resulting in the production of functional protein. Gene therapy may provide patients with a more convenient, less costly, and long-term treatment option. Recent developments in gene therapy pertaining to Fabry disease will be examined along with several issues that remain unresolved including the associated risks of insertional mutagenesis and the need for larger animal models.

Introduction

Fabry disease is a lysosomal storage disorder that is X-linked inherited and characterized by a deficiency in the enzyme α-galactosidase A [1]. This homodimeric protein is a lysosomal glycohydrolase that catalyzes the cleavage of terminal galactose residues from glycosphingolipids [2]. As a consequence of reduced α -galactosidase A activity, Gb3 and similar glycosphingolipids accumulate within lysosomes [3]. The association between the complications of Fabry disease and glycolipid accumulation remains poorly understood. Gb3 further accumulates as the disease progresses, creating a chronic toxicity state [4]. This phenomenon is thought to initiate irreversible renal [5, 6] and cardiac tissue fibrosis [7], oxidative stress [8] and impaired energy metabolism [9, 10].

Symptoms and complications range in severity and are experienced by both hemizygous males and heterozygous females (Figure 1). Classic symptoms arise in childhood and include acroparesthesias (burning, tingling and stiffness in extremities), angiokeratomas (small discoloured skin lesions characterized by thickening of the skin), gastrointestinal complications and debilitating pain [10]. As the individual ages, progressive damage to tissues and organs causes the onset of more severe symptoms including proteinuria (increased amounts of protein in the urine), cardiomyopathy, end-stage renal disease, stroke, and death [11]. Misdiagnosis is common due to the existence of multiple phenotypes of variable onset. The incidence rate of Fabry was previously approximated at 1 in 117,000 live births [12]. However, a significantly higher incidence of mutation in the α-galactosidase A gene (1:3100) was recently suggested by newborn screening surveys [13].

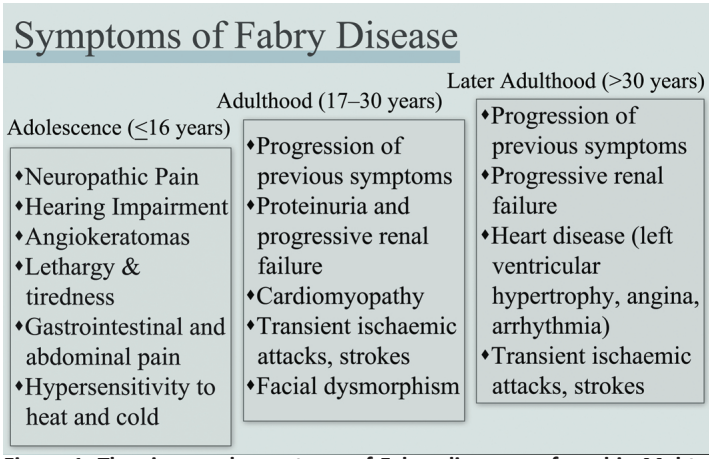

Figure 1: The signs and symptoms of Fabry disease as found in Mehta et al.'s 2010 study. Mehta A, Beck M, Eyskens F, Feliciani C, Kantola I, Ramaswami U, et al. Fabry disease: a review of current management strategies. QJM 2010;103(9):641-659.

The α-galactosidase A gene (GLA) is 12,436 base pairs long [10]; its small size facilitates genotyping and allows for precise analysis of specific GLA mutations. Currently, 431 mutations of the GLA gene have been documented.(The Human Gene Mutation Data Base at the Institute of Medical Genetics in Cardiff.)

Enzyme Replacement Therapy

The method of treatment that is currently used by the majority of Fabry patients is enzyme replacement therapy (ERT). ERT compensates for enzyme deficiency [14] through an intravenous injection of either agalsidase α (Replagal; Shire Human Genetic Therapies Inc, Cambridge, MA) or agalsidase β (Fabrazyme; Genzyme

Corporation, Cambridge, MA). Both of treatments include recombinant forms of the human enzyme, α -galactosidase A. Agalsidase α is isolated from a human cell line that is transduced, while agalsidase β comes from genetically engineered Chinese hamster ovarian cells [13]. Treatment of patients must be continuous and requires reinfusions every two weeks. Agalsidase α is infused over a period of 40 minutes at 0.2 mg/kg while agalsidase β is infused over a period of 4 hours at 1.0 mg/kg [14]. ERT does not cure the disease, though continuous ERT seems to alleviate neuropathic pain, increase Gb3 clearance, and stabilize progression of renal and cardiac symptoms.

The Effects of Enzyme Replacement Therapy

The progressive accumulation of Gb3 causes damage to various renal, cardiac, and cerebrovascular tissues. Patients who receive agalsidase α treatment experience more than 50% of a mean decrease in plasma Gb3 levels, a 30% mean decrease in urine sediments and a 21% mean decrease in kidney Gb3 levels [15]. In a double-blind study, renal-biopsy specimens indicated that Gb3 deposits in renal microvascular endothelial tissue were almost cleared in 69% of patients after 20 weeks of agalsidase β. In addition, patients displayed a decrease in Gb3 deposits in microvascular endothelial tissue of the heart and skin and decreased plasma Gb3 levels [16]. ERT may reduce the accumulation of Gb3 in Fabry patients, delaying the onset of complications in the cardiac, cerebrovascular, and renal systems.

In clinical studies, patients who began ERT with mild or moderate renal symptoms experienced stabilized renal function [17, 18, 19]. Renal function is predicted based on the estimated Glomerular Filtration Rate (eGFR). Fabry patients usually experience a decline in eGFR [20], while those who are treated with agalsidase α continued to show stable eGFR after 1-2 years of treatment [18, 19, 21]. Similarly, the extension of a multicenter phase 3 clinical trial indicated that treatment with agalsidase β stabilized renal function for as long as 30-36 months [22]. These results suggest that ERT significantly delays the onset of more serious renal complications.

However, the progressive deterioration of renal function seems to be related to the severity of glomerulosclerosis and proteinuria before the start of treatment [13, 22, 23]. ERT proved less useful to patients who began treatment with more severe glomerulosclerosis and proteinuria, suggesting that ERT is better used as a preventative measure in the early stages of Fabry. It should also be noted that ERT is not able to reduce proteinuria in Fabry patients [22].

Fabry patients are also commonly affected by cardiomyopathy, especially by progressive concentric left ventricular hypertrophy (LVH). Treatment with agalsidase α resulted in reduction of left ventricular size [18, 24] and mass [25] in patients displaying an enlarged heart at baseline. In addition to the decrease in mass, a decrease in the interventricular septum thickness [13, 26, 27] was observed during treatment with agalsidase β.

The neuropathic pain caused by Fabry can also be treated to some extent using ERT. Fabry patients treated with both agalsidase α and agalsidase β experienced less severe pain and an improved general quality of life [15, 16, 18, 28].

Controversial Aspects of ERT

While many studies show significant benefits of ERT, several concerns such as the costliness and the indeterminate long-term efficiency of ERT have steered researchers towards developing alternative treatments.

Additional therapies are required by some patients to relieve specific symptoms that persist despite treatment with ERT. As ERT is unable to reduce proteinuria [22], many patients require additional anti-proteinuric treatment including angiotensin-converting enzyme inhibitors and angiotensin-receptor blockers to regulate their urine protein excretion and stabilize their eGFR [29]. Patients may also require conventional antihypertensive and antithrombotic therapies for treatment of cardiovascular symptoms [30]. A 24-month follow-up study analyzed several cardiac parameters such as ventricular mass, ECG parameters, and end-systolic volume and concluded that additional therapy is needed because ERT has a minimal effect on cardiac symptoms [30]. Additionally, though ERT seems to reduce neuropathic pain, many patients still require the use of analgesics to reduce the intensity and duration of painful episodes [31].

Another problem associated with ERT is the uncertainty of long-term outcomes due to the use of surrogate endpoints such as proteinuria and left ventricle mass [32]. These surrogate endpoints are used to predict hard endpoints such as the need for organ transplantation, dialysis or a pacemaker. The association between surrogate endpoints and hard endpoints is not absolute, meaning that the predicted results for long-term treatment may not apply in all scenarios. A meta-analysis that examined 41 studies on the treatment of Fabry disease using ERT found that 98% of the studies used surrogate endpoints [32]. Many of these studies also used database registries and historical cohorts which would be subject to different sources of bias [32].

ERT also requires frequent infusions throughout the patient's lifetime, which is not only inconvenient, but also extremely costly. At the recommended dosage, based on ERT costs from the year 2005, Moor et al. calculated that treating a Fabry patient with ERT would cost $175,000 – $350,000 CAD over one year [33]. Accordingly, ERT is inaccessible to Fabry patients in many countries with privatized healthcare. The high cost of ERT has lead healthcare officials to consider the development of other more efficient and less costly options that would make treatment accessible to a wider array of patients.

Gene Therapy: A Possible Alternative

Gene therapy has the potential to provide Fabry patients with a long-term solution that could lead to both the correction of enzymatic function and the reduction of symptoms. Gene therapy involves modifying a patient's genome in order to correct a genetic mutation and treat a disorder [34]. Gene therapy can be separated into two categories: in vivo (genetic material is injected directly or via a vector system into the affected area) and ex vivo (the patient's cells are first harvested, then transduced using a vector system and finally re-introduced into patient) [34]. Fabry disease is a single gene disorder, which makes the mutated gene easier to correct by gene therapy than in many other types of genetic disorders [34]. Furthermore, only 5 – 20 % of regular enzymatic activity levels are required to increase the Gb3 clearance and provide functional results in the various organs [35]. This may be due to "metabolic cooperativity", which occurs when α-galactosidase A secreted by genetically corrected cells is used by nearby deficient cells [36]. Lastly, gene therapy would provide patients with a constant and lasting increase in enzymatic function.

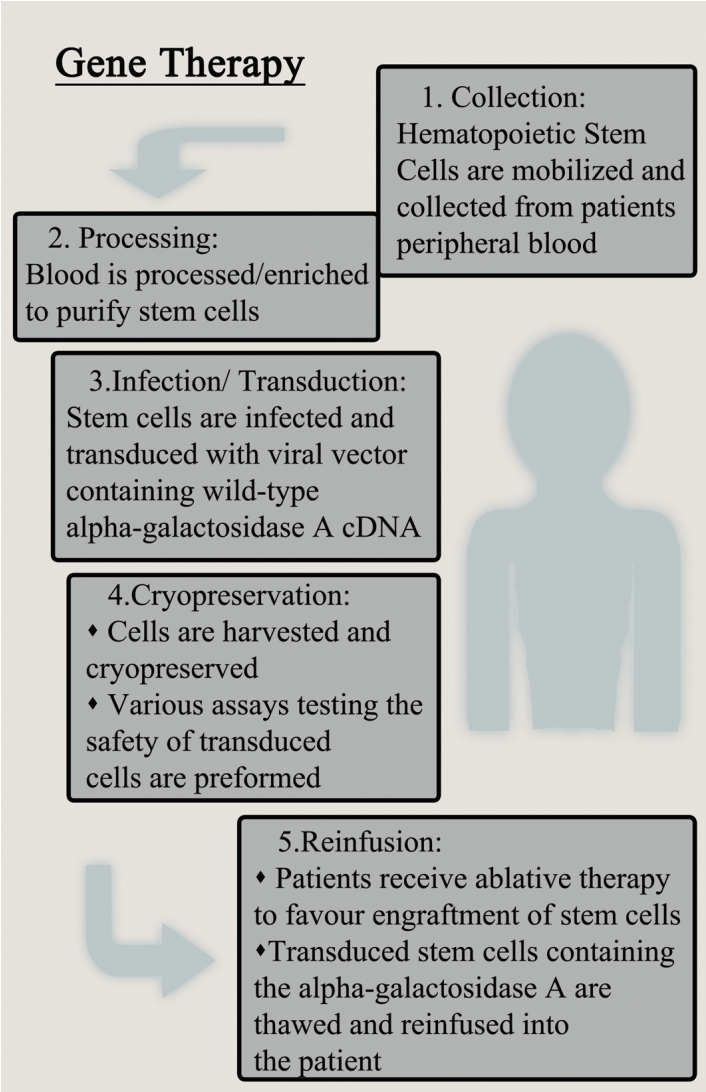

Figure 2: The outline of a proposed Gene Therapy model, modified from Cartier et al.'s (2009) study on gene therapy treatment of X-Ald. Cartier, N., Hacein-Bey-Abina, S., Bartholomae, C., Veres, G., Schmidt, M., Kutschera, I., et al. (2009). Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. Science, 326(5954), 818-823.

In autologous haematopoietic stem cell-mediated gene therapy, the patient's own stem cells are transduced ex vivo using a variety of modified self-inactivating viral vectors in order to correct the genetic mutation (Figure 2) [35]. Transduced stem cells are then transplanted back into the patient [35]. In one study, α-galactosidase A deficient haematopoietic cells were transduced using an oncoretrovirus, encoding for the wild type GLA gene, before being transplanted back into myeloablated Fabry mice [37]. The mice showed increased levels of α-galactosidase A activity in the spleen, heart, lungs, and kidneys and a decrease in Gb3 accumulation in all of those organs except for the kidneys [37].

In another mouse model of Fabry disease, in vivo Lentiviral vector mediated gene therapy was preformed through an intraventricular injection [38]. Seven days after the treatment, the transduced cells expressed functional levels of the transgene and α-galactosidase A increased to 23% of normal levels [38]. There was also a reduction in Gb3 accumulation [38]. When measured at both 3 months and 1 year after treatment, α-galactosidase A activity in the heart of Fabry mice declined to levels present in controls that were not treated [38]. This study shows the possible benefits of in vivo gene therapy localized in the affected area.

Future Areas of Research

The majority of studies are conducted on the murine model of Fabry disease that lacks important phenotypes observed in human patients such as renal failure and stroke [39]. An alternative, larger animal model displaying the disease phenotypes present in humans would provide a better prediction of treatment outcomes in humans, thus speeding-up clinical application. Yoshimitsu et al. found that the coding region of the human and porcine GLA gene were highly homologous and had the same chromosomal location [39]. Furthermore, lentiviral vectors were used to transduce cultured skin fibroblasts of a Fabry Patient with porcine GLA cDNA. The fibroblasts exhibited 4 times the amount of α-galactosidase A activity compared to an uncorrected cell and the decrease in Gb3 was comparable to that of fibroblasts transduced with human α-galactosidase A [39]. The insight provided by this study, suggests the possible use of a porcine model for Fabry disease.

Extensive research performed on animal models supports the possibility of using gene therapy to treat Fabry in humans [36, 37, 38, 39]. However, the safety and efficacy of the therapy in humans must be investigated thoroughly before it can be implemented clinically. One of the primary concerns with gene therapy is that transduced cells do not have a selective growth advantage [40]. Patients must therefore receive myeoablative conditioning (the chemotherapy or radiation therapy regimen given before a bone marrow transplant, which results in the severe depletion of bone marrow cells.) prior to gene therapy for efficient engraftment of transduced haematopoietic stem cells [40]. The effects of myeoablative conditioning may not be well tolerated by patients with moderate to severe Fabry symptoms and may lead to severe complications. Accordingly, the success of gene therapy using reduced intensity conditioning regimes must be further explored.

A recent study applied eight different chemotherapy and radiotherapy reduced intensity (RI) conditioning regimens to Fabry mice. Methods involved low-dose total body irradiation, fludarabine, cyclophosphamide, busulfan, and an innovative approach called local limb irradiation. Adequate reductions in lymphoid and myeloid cells were produced without serious health complications in the mice. In one protocol, cells were transduced and then preselected for functional protein expression and finally transplanted into the mice. Over 182 days, the transduced cells engrafted, while plasma α-galactosidase A activity levels remained elevated and were accompanied by a significant drop in Gb3 levels [40].

In RI conditioning groups, α-galactosidase A activity was also present in the spleen, liver, lung, heart and kidney, though only the high dose of total body irradiation (HD-TBI) group exhibited a significant increase of α-galactosidase A activity in the brain [40]. In addition, the mice did not experience any significant immune responses against α- galactosidase A [40]. Despite producing similar immune and myeloid depletion, the RI regimens varied in their ability to correct α-galactosidase A deficiency in different organs and tissues, indicating that different RI regimens should be considered depending on the desired results [40]. This study established a basis for clinical trials of gene therapy involving RI conditioning regimens to treat Fabry disease.

The risk of insertional mutagenesis is another one of the major concerns of vector-mediated gene therapy [41]. Insertional mutagenesis occurs when vectors are integrated close to or among gene-coding areas causing gene silencing or malignant transformation. This risk was made apparent when a patient developed leukemia due to insertional mutagenesis 30 – 34 months after undergoing treatment with oncoretrovirus-mediated treatment to correct severe combined immunodeficiency [42]. More research is required to determine if the correctional ability of gene therapy outweighs the associated risks.

Conclusion

The development of ERT significantly improved the management of several symptoms in Fabry disease patients. Alternative treatment strategies may be able to offer a more efficient and less costly method of treating the disease. Over the past decade, substantial advances have been made in the development of gene therapy. However, issues including the risk of insertional mutagenesis and the improvement of treatments using reduced intensity conditioning regimes need to be further explored. Future research performed on larger animal models or perhaps clinical studies may give insight into the effectiveness of gene therapy as a permanent solution for curing Fabry disease. These insights may be applied further to treat other lysosomal storage or single gene disorders that are suitable candidates for gene therapy.

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JULS REVIEWS

Treatment Resistant Depression and Current Therapy Options

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Abstract

There are no current first-line therapies for Treatment Resistant Depression (TRD). Pharmacological and neuromodulatory therapies are used to ameliorate depressive symptoms in treatment resistant patients, but no single treatment option has proven both effective and cost efficient. This review will outline current treatment approaches and discuss their relative efficacies. The viability of alternative treatments will be explored with a focus on the potential of aerobic exercise therapy to replace current treatment options. This review is intended to influence the trajectory of research in finding clinically effective treatments for TRD, and to suggest the potential of aerobic exercise in providing a cost effective, low relapse rate therapy for patients.

Treatment Resistant Depression

Treatment Resistant Depression (TRD) is a subset of Major Depressive Disorder (MDD) [3]. Ambiguity regarding the definition of TRD has made it difficult to develop a proper prognosis and course of treatment for this disease [28]. The symptoms of TRD are similar to those of MDD, but often present themselves with a greater duration and severity [19]. The disorder is still largely understudied and under-recognized despite its growing prevalence. Due to a lack of consensus on its definition, the most effective way to perceive TRD is on a spectrum of varying unresponsiveness to treatment. For the purpose of experimental cohesion, the disorder will be defined as patient unresponsiveness to at least two adequate courses of antidepressant therapy from pharmacologically distinct classes [3]. An adequate course of antidepressant therapy consists of a trial of antidepressant medication at a therapeutic dose for an appropriate duration of at least 4-6 weeks [34].

Feelings of regret, low self-esteem, guilt, loss of interest in relationships and life, and a general melancholic disposition are all symptomatic of TRD [19]. People suffering from the disorder exhibit an increased susceptibility to disease and suicide. TRD has been identified as the leading cause of suicide in depressed patients, which itself accounts for 60% of all known suicides [31]. Patients often experience physical symptoms including fatigue, slow movement, sexual dysfunction, insomnia, changes in eating habits, and pain [34].

Effectively treating TRD requires several important preliminary steps. Practitioners must first review the patient's prior treatment before accepting the TRD diagnosis. Often patients will receive inadequate trials of antidepressants and will be misdiagnosed, consequently distorting collective knowledge of the illness [3]. Secondly, clinicians must determine whether a patient is suffering from primary or secondary TRD. Primary TRD is caused by societal distress or biochemical imbalances directly related to the disorder, whereas secondary TRD is caused by distinct illnesses or medications manifesting themselves as depression [3]. Common causes of secondary TRD are thyroid dysfunction, Cushing's syndrome, Parkinson's disease, neurological neoplasms, pancreatic carcinoma, connective tissue disorders, vitamin deficiencies, and daily use of beta-blockers, immunosuppressants, steroids or sedatives [3]. Secondary TRD is also referred to as depression secondary to a general medical condition. If a patient is suffering from secondary TRD, the underlying condition must first be treated before moving on to antidepressant therapy. If the patient has not achieved remission after at least two adequate trials of antidepressant therapy and is suffering from primary depression, then the clinician can re-diagnose the patient with TRD.

Prevalence and Economic Burden

The prevalence of TRD is debated due to varying definitions of the disorder. Recent studies suggest that 60-70% of patients with MDD fail to achieve complete remission [3]. This percentage is based on the fairly lax definition of TRD; some studies place the ratio of MDD patients who fail to achieve remission closer to 30% and 15% [19]. Despite inability to determine exactly how many people suffer from the disorder, there is consensus that the economic burden attributable to the illness is substantial. Liberal estimates place the cost of the disorder upwards of $45 billion [3] and conservative estimates place it around $12 billion [19]. The high prevalence of TRD contributes to morbidity and mortality associated with MDD [31]. TRD highlights the inefficacy of current treatments and demands a re-evaluation of the way we approach depressive illnesses.

Treatment

The broad range of therapy options for TRD demonstrates that the ability to treat the disorder has increased, but that a treatment option viable for the majority of patients has yet to be identified. Outlined below are the therapies used to treat TRD, and how each corresponds with individual and sometimes overlapping theories of pathogenesis. The nature of the disorder requires treatments to be long-term and prevent relapse [3]. Many MDD treatments exhibit antidepressant properties in patients with TRD, but are often unable to achieve complete remission and have high rates of relapse [28]. The staggering cost of newer therapies, and their high relapse rates, indicates that therapy for TRD patients is still inadequate [3].

Antidepressant Medications

Monoamine oxidase inhibitors (MAOIs)

MAOIs were the first class of antidepressants discovered. Their effects were originally observed in the 1940s when they were used to treat patients with tuberculosis [19]. Patients showed no physical improvement, but were reported to feel elated and euphoric. In the 1960s, researchers elucidated that MAOIs inhibit the enzyme MAO, thereby preventing degradation of serotonin and norepinephrine [29].

Tricyclic antidepressants (TCAs)

As amine uptake blocking agents [28], TCAs exert their antidepressant effects by blocking the reuptake of amines in the synaptic cleft, consequently increasing elevations of target monoamines [19]. TCAs are now less commonly prescribed due to their adverse cardiac side effects and potential lethality in overdose.

Selective Serotonin (5HT) Reuptake Inhibitors (SSRIs)

SSRIs were the first generation of TCAs [30]. They work by blocking the reuptake of serotonin in the synaptic cleft, subsequently increasing concentrations of serotonin. However, evidence that norepinephrine (NE) and dopamine (DA) are implicated in the onset of depression has given rise to dual and triple reuptake inhibitors that target biogenic amine transporters for 5HT, NE, and DA simultaneously [10, 15]. By targeting all three transporters, the medication is able to address multiple causes of depression and consequently exert a greater therapeutic effect [19].

Receptor Acting Drugs

The mechanism behind the antidepressant effects of receptor acting drugs is still debated. It is believed that they exert their therapeutic effects by antagonizing the α2-adrenoceptor [19]. Decreasing function of the α2-adrenoceptor would prevent autoinhibition of NE neurons, thereby increasing concentrations of NE in the synaptic cleft [19]. Recently, receptor acting drugs have been refined to target melatonergic and serotonergic receptors as well [29]. Studies have shown that antagonizing serotonergic receptors leads to elevations of dopamine and norepinephrine in the frontal cortex [19].

Augmentation/ Combination of Medications

Many techniques have been explored to adapt existing medications for use in TRD. One approach is optimization. Optimization theory posits that by increasing medication dosage to the highest levels within the safety limit, the efficacy of the medication will increase [34]. For medications that show a linear dose-response relationship, optimization is a viable strategy [19]. However many medications, including SSRIs, do not show a linear relationship and are therefore not more effective at higher levels. In order to prevent dangerously high blood plasma levels, patient drug metabolism must be factored into optimization strategies [19]. Overall, optimization holds some promise in the treatment of TRD, but it often requires costly resources to achieve its full potential and is therefore limited in its range of application [3].

Another common strategy pursued in treating TRD is augmentation. Practitioners augment existing medications by adding a supplementary agent, or another antidepressant in sublevel doses, to achieve remission of depressive symptoms [28]. Augmentation studies have indicated that lithium, olanzapine, triiodothyronine, risperidone, psychostimulants, and tryptophan are among the most effective augmentation agents when combined with SSRIs and non-SSRI antidepressants [35]. Augmentation may increase the risk of weight gain and cause glucose metabolism dysregulation, offsetting efficacy gains [19].

Combination strategies have also been recommended in the treatment of TRD. If a patient shows partial response to one medication, but is still experiencing depressive symptoms, then another medication designed to target the untreated symptoms should be combined with the original antidepressant into a single comprehensive therapy [3, 28]. Combination strategies have also been used to make one medication offset the adverse effects of another. Combination strategies have been shown to increase efficacy in some cases, notably when mirtazapine is combined with an SSRI, but are often accompanied by dangerous and potentially fatal side effects [19]. The lack of methodologically sound studies and the increased risk of side effects limit the efficacy of combination strategies.

Neuromodulation Treatments

The severity of TRD has led clinicians to develop neuromodulatory therapies to treat patients unresponsive to medication. They are accompanied by a variety of adverse effects, and aside from electroconvulse therapy, are still in the investigational phases [3]. The high cost and relapse rates of these treatments present serious drawbacks, but they are constantly being improved and hold promise for future treatment of TRD.

Electroconvulse Therapy (ECT)

ECT is the oldest and most established neuromodulation treatment [1]. Despite widespread public bias, ECT is a relatively safe treatment resulting in fewer than 0.3 deaths per 100,000 patients [19]. One commonly accepted theory explaining ECT's mode of action is that the treatment stimulates a release of dopamine, norepinephrine and serotonin from synaptic vesicles [19]. The resulting sequential modifications in second messengers are believed to be responsible for the alleviation of depressive symptoms. The efficacy of ECT is determined by the duration and intensity of seizures. They should last a minimum of 25 seconds, and be monitored by an electroencephalogram [34]. Physical side effects include musculoskeletal/dental injury, superficial skin burns, oral lacerations, and persistent myalgia (nausea, headaches and muscle aches are common post treatment). Cognitive adverse effects relate to temporary amnesia of autobiographical information [34]. High relapse rates (50-95% within six months) prevent ECT from being a sustainable treatment option for the majority of patients [3].

High Frequency Repetitive Transcranial Magnetic Stimulation (rTMS)

rTMS uses a wand to provide repeated subconvulsive magnetic stimulation to the left dorsolateral prefrontal cortex [19]. The theory underlying rTMS is based on the belief that using brief high intensity magnetic fields stimulates cortical neurons, thereby ameliorating depressive symptoms [14]. rTMS is administered by turning an electrical current on and off to produce magnetic fields generally lasting 150 microseconds, producing frequencies from 1-20 Hz repetitively over the course of 30 minutes [14, 19]. The magnetic pulse causes depolarization and stimulation of neurons to a depth of 2 cm below the cerebral surface. The magnetic field is too weak to affect subcortical cingulate or limbic structures, thereby reducing the risk of adverse effects [3]. rTMS has been shown to be as effective as ECT in treating non-psychotic patients with TRD [3]. Its non-invasive nature and mild profile of side effects (headache) make it an attractive alternative to other neuromodulation therapies [19].

Vagus Nerve Stimulation (VNS)

VNS was originally designed to treat treatment refractory epilepsy [19]. VNS is performed by surgically inserting an electrical wire into the neck. The electrical wire is coiled around the left vagus nerve and connected to a stimulator in the chest wall [19]. The stimulator delivers irregular but continuous electrical signals to the vagus nerve. VNS was reported to have a 40% response rate in patients suffering from TRD in an open trial [19]. However, in a double blind randomized control trial, active VNS was not shown to be more effective than the placebo [3]. Although the FDA has already approved its use, these conflicting results question the efficacy of VNS. Aside from potential operational hazards, VNS has mild side effects including voice irritation and modification [3].

Deep Brain Stimulation (DBS)

DBS is currently being researched as a neuromodulation therapy [19]. DBS was first used to treat Parkinson's disease by stimulating the subthalamic nucleus, thereby reducing the severity and frequency of tremors [19]. DBS works by applying electrical stimulation through neurosurgical implantation of electrodes on bilateral regions of the brain [22]. This treatment arose from a hypothesis of depression that implicated abnormalities in neurocircuitry in explaining the disorder [25]. To date, there is no consensus on which region of the brain DBS should target. However, functional imaging studies suggest that the subgenual cingulate Brodmann area 25 (BA25) should be targeted for bilateral stimulation [22]. Studies have shown heightened activity in BA25 in depressed patients and in healthy controls during times of sadness [22]. Furthermore, healthy controls during times of self-described happiness, and depressed patients undergoing adequate treatment, both displayed decreased activity in BA25 [3]. DBS works by electrically stimulating this region, consequently depolarizing it and inhibiting hyperactivity [25]. Positive results have been shown in recent studies, and have led clinicians to research the effects of stimulating the anterior limb of the internal capsule or the nucleus accumbens [22, 25].

Alternative Therapies

Alternative therapies for the treatment of depression are controversial. Some are convinced that herbal remedies can replace modern therapies and professional care. On the opposite end of the spectrum are practitioners so embedded in the paradigms of their fields that they are unable to accept the possibility of effective alternative treatments. The patient is ultimately harmed by the acceptance of either of these extremes. The more prudent direction would be an amalgam of the two, a moderate willingness to research alternatives without the naïveté of forsaking modern medicine.

Hypericum perforatum

H. perforatum is an herbal remedy used to alleviate "melancholia" [19]. A meta-analysis done in 2001 was a testament to the efficacy of *H. perforatum* in treating moderate depression [19]. Since then, two methodologically sound trials in the U.S. have shown it to be no more effective than a placebo [19]. The efficacy of the remedy is still in question, but it is believed to exert antidepressant effects by inhibiting the function of serotonin and monoamine oxidase transporters, as well as g-aminobutyric acid (GABA) receptors [19].

Omega-3 Fatty Acids

Research indicating depressed patients have phospholipid abnormalities has led some clinicians to believe that correcting such abnormalities would alleviate depressive symptoms [13]. Since then, practitioners have added the important fatty acid eicosapentaenoic acid to antidepressant medications and have reported it to exert a therapeutic effect on previously unresponsive patients [19].

Light Therapy (LT)

LT has proven to be useful in treating seasonal winter MDD. Patient response rates have been recorded to be greater than 60%, making it an effective and viable antidepressant therapy [19]. Research has indicated LT may also be useful in treating non-seasonal depression, but due to lack of proper experimental conditions, conclusive evidence has still not been found [13]. Some evidence indicates LT is useful in combination with other therapies and is more effective than monotherapy [19].

Total Sleep Deprivation (TSD)

TSD involves subjecting patients to abnormal sleep behavior by forcing them to stay awake for 36 hours, then sleep for 12 [8]. Despite its efficacy in ameliorating symptoms, it is an unsustainable treatment with a high relapse rate [8]. However, TSD may be effective as a preliminary treatment to TRD patients whose symptoms are so severe that they are unresponsive to other treatments. TSD has a great potential to decrease the depressive symptom profile temporarily, making it easier for practitioners to begin administering more long-term treatments to patients initially unwilling to receive therapy [19].

Exercise

Prior research has indicated that exercise is a viable treatment for MDD [5, 6, 23, 37]. The exact causes of its efficacy are unknown, but it is believed to have both physiological and psychological effects [6]. Observed psychological effects of exercise include increased self-efficacy, a sense of mastery, distraction, and changes of self-concept [5]. Physiological changes include increased central norepinephrine neurotransmission, changes in the hypothalamic adrenocortical system, increased secretion of atrial natriuretic peptide and amine metabolites, increased metabolite and serotonin synthesis and metabolism, increased neuropeptide Y transmission in the hippocampus, and increased brain derived neurotrophic factor in the hippocampus [9, 11, 12]. These findings suggest that exercise not only alleviates symptoms of depression, but also targets root physiological causes. In 2004, Craft and Perna showed exercise to reduce symptoms as measured by the HAMD

scale (Hamilton Depression Rating Scale) by 70%, a remarkable result given that remission is defined by reduction of 50% [19]. Furthermore, Blumenthal et al. showed that exercise had significantly smaller relapse rates (8%) compared to sertraline (38%) and ECT (70%), suggesting it is a viable long-term treatment strategy [5]. Running was shown to be as effective as psychotherapy in the treatment of depression, which makes it far more viable given its low cost. A large-scale study in the European Union including 55,000 adolescents concluded that exercise not only reduced symptoms of depression, but is also correlated with overall improved mental health [23]. The process of neurogenesis, stimulated by exercise, in the adult rat hippocampus, may explain why exercise improves general mental health and results in increased resilience to depression and other psychiatric ailments [11].

Conclusion

Despite evidence suggesting that exercise therapy is an effective therapy for depression, the medical community has not yet adopted it as a treatment approach. This is because past studies using exercise as an intervention were methodologically flawed, compromising the experimental integrity and subsequent results [23]. Therefore, further research that corrects past methodological flaws is needed to validate prior findings. Performing neurological scans (i.e. computed topography and magnetic resonance imaging) to pinpoint underlying physiological changes attributable to exercise (i.e. alterations in neurotransmitter concentrations, changes in BDNF levels, stimulation of neurogenesis, modification in the structure of the subgenual prefrontal cortex, and limbic cortical metabolic changes) will further our understanding of the mechanisms underlying the antidepressant effects of exercise.

The physiologically beneficial effects of exercise, low costs, high remission, and low relapse rates make it a prime option for TRD patients. The main hurdle confronting the psychiatric community is not only achieving remission for TRD patients, as currently developing neuromodulatory techniques promise to increase in efficacy, but doing so in a way that is non-invasive, cost efficient, and resilient to relapse.

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JULS REVIEWS

Sodium Alginate – A Potential Tool for Weight Management: Effect on Subjective Appetite, Food Intake, and Glycemic and Insulin Regulation

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Abstract

In light of the rising incidence of obesity and type II diabetes, identification of foods and dietary practices that contribute to a healthy body weight and adequate glycemic control is of high importance. A number of soluble viscous dietary fibres have shown promise in controlling food intake and glycemic responses. One type of soluble fibre, sodium alginate, possesses several advantages over others, including thermal stability and improved palatability. This review presents a critical evaluation of alginate's efficacy as an aid in weight control, in terms of its ability to regulate satiety and food intake and modulate glycemic and insulinemic responses. Despite a number of conflicting findings, the literature largely suggests that sodium alginate possesses significant potential as a weight control agent, with possible implications in obesity and type II diabetes treatment.

Introduction

Alginate is a polysaccharide that is abundant in nature, as it is synthesized by brown seaweeds and by soil bacteria [1]. It is widely employed in the food processing industry, often as a thickener or emulsifion stabilizer [2]. Sodium alginate is the most commonly used alginate form in the industry, since it is the first byproduct of algal purification [3]. Sodium alginate consists of α-l-guluronic acid residues (G blocks) and β-d-mannuronic acid residues (M blocks), as well as segments of alternating guluronic and mannuronic acids (GM blocks) (Figure 1). The guluronate residue blocks allow alginate fibres to form gels by binding Ca^{2+} ions and stomach H^{+} ions, which cross-link the fibres into a viscous polymer matrix [3].

Viscous fibres have been generally recognized for their ability to reduce energy intake and attenuate postprandial glycemic responses [4, 5]. Such properties are of interest for their use in products assisting in weight management. However, poor palatability mostly resulting from increased product viscosity is a serious technical challenge [6]. Since sodium alginate forms gels mainly upon entering the acidic gastric environment [7], rather than in the oral cavity, it may not be burdened with the same problem. In addition, unlike other viscous fibres, sodium alginate has the capacity to withstand moderate amounts of thermal stress as might be encountered during boiling or cooking of the product [3].

Despite sodium alginate's theoretical potential to aid in weight management, a number of conflicting findings exist as to its physiological role in the modulation of satiety, energy intake, and glycemic and insulinemic responses. This review presents a critical evaluation of these findings, concluding that, at appropriate chemical and physical parameters, alginate treatments are indeed able to attenuate glycemic excursions (by slowing gastric emptying and/or intestinal nutrient absorption rates), reduce insulinemic responses, enhance satiety, and subsequently decrease energy intake (Figure 2). Altogether, these findings support the significant potential of sodium alginate in weight management and glycemic control in obese and diabetic individuals.

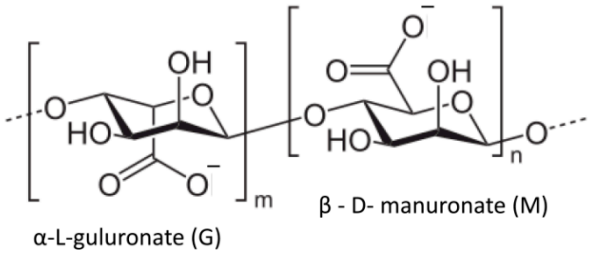

Figure 1: Basic structural unit of alginate polymers, consisting of α-L-guluronate (G) and β - D- manuronate (M) residues.

Energy Intake

Nutrient intake reduction is one of the primary goals in management of obesity and type II diabetes. While it has been postulated that soluble viscous dietary fibres modulate energy intake [8, 9], studies evaluating this property in sodium alginate-enriched products diverge in their findings.

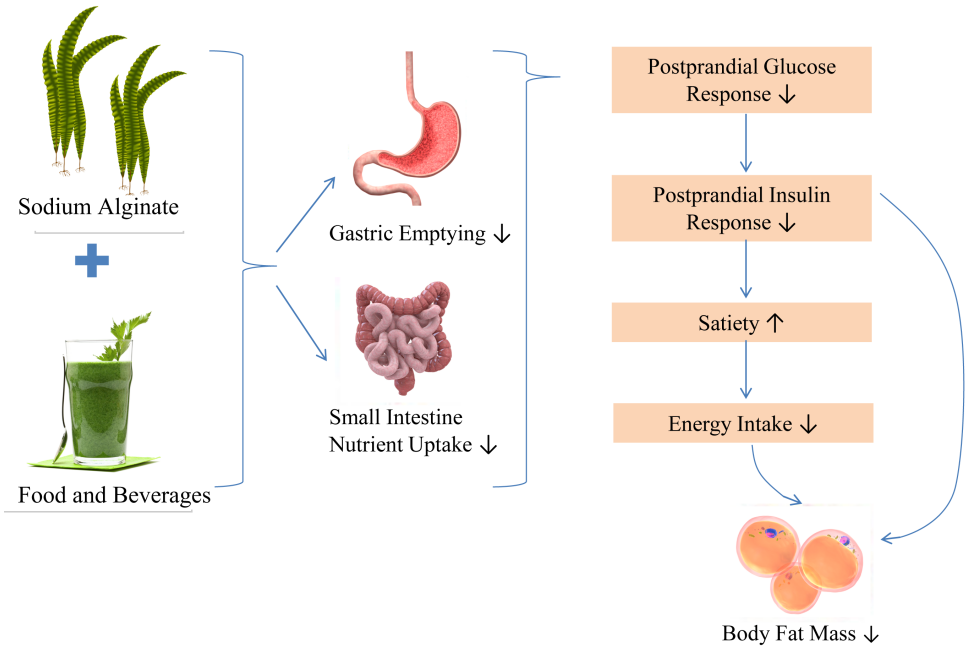

Figure 2: Possible mechanisms of sodium alginate's physiological effects. Images by Eduard Härkönen, Sebastian Kaulitzki, Shawn Hempel, Photos.com.

In advocacy of alginate fibres' capacity to attenuate nutrient consumption, Paxman et al. showed that subjects who ingested a daily preprandial sodium alginate beverage showed a 134.8kcal reduction in energy intake [10]. As the authors pointed out, the potency of this reduction becomes apparent when one considers Hill's estimate that the majority of Americans can prevent weight gain by reducing their daily energy intake by only 100kcal [11]. In a similar vein, consumption of a sodium alginate beverage in a seven day intervention trial induced a reduction in energy intake at dinner in obese women without severe dietary restriction habits [12]. Jensen et al. further confirmed the efficacy of preprandial alginate treatment on reducing energy intake by observing the effect of a 330 ml alginate-based beverage on healthy subjects [13].

On the other hand, select findings suggest that alginate treatments may not have an effect on energy intake. However, these findings are likely artefacts of suboptimal experimental design parameters, rather than valid evidence of alginate's inefficacy. Jensen et al. [13] were unable to observe an effect of a 500ml alginate beverage on food consumption. This seemingly contradictory result can be attributed to the fact that the beverages were not supplemented by divalent ions that are normally added to induce cross-linking and gellation [3], relying entirely on stomach acid for this function. Since the 500ml beverage likely diluted the stomach acid [13], sodium alginate may not have achieved sufficient gel formation which may be necessary for the alginate's physiological effects of increasing satiety and attenuating glycemic responses [7, 14].

Similarly, Mattes and colleagues [15] found that consumption of a 55g solid bar containing 1.1g sodium alginate had no significant effect on energy intake. However, these findings can once again be attributed to the failure of the experimental set-up to produce the conditions needed for the functionality of the sodium alginate gelling complex. The amount of alginate used (1.1g) may not have been sufficient to induce gellation: the majority of the studies that have been able to demonstrate significant physiological effects of alginate treatments have used quantities such as 2.8g [12], 3.25g [7] 3.73g [16, 17], 5g [10], and 9.9g [13]. Alternatively, the low water content of the bar may have limited the capacity for gellation [18]. Yet another explanation put forward by French [19] suggests that the differences between the alginate treatment and control were masked by the small volume and energy content of the bar or the significant or exigenic effect of the control bar.

In sum, the above data suggests that when properties of sodium alginate products are optimized, they are likely to be successful in attenuating energy intake. The studies that failed to demonstrate an effect on energy intake likely used alginate treatments with suboptimal parameters, such as dose or water content, and therefore should not prompt one to discount alginate's potential in weight management.

Mechanism

In general, soluble dietary fibre modulates food intake through enhancement of both immediate satiation and prolonged satiety feelings [20, 21]. Below is an evaluation of sodium alginate's capacity to increase postprandial satiety, which is likely mediated through attenuation of postprandial glycemic and insulinemic responses.

Satiety

Whether acutely or repetitively consumed, sodium alginate's capacity to increase postprandial satiety is evident in several studies [7, 14, 18, 22]. However, as is the case with observations regarding energy intake, specific physico-chemical characteristics of sodium alginates must be optimal for the satiating effect to be evident.

Two studies have demonstrated that when the structural composition of alginate allows the formation of a strong gel, such as sodium alginates with high guluronic acid content (at least 70% G-residue content [7]), postprandial fullness is enhanced [7,14]. On the other hand, consumption of a weak gelling (40% G-residue content) alginate failed to produce any significant changes in satiety [7]. Similarly, Peters and colleagues found that alginate treatments have no effect on satiety below minimal gel strength (1.8N) [14]. Clearly, the chemical composition of alginate fibres must be optimized to produce sufficient gel strength and sufficient effect on postprandial satiety. Since lower scores of hunger and higher scores of fullness result in better weight loss outcomes [23, 24], the effects of alginate treatments on postprandial satiety make them an appealing candidate for facilitating obesity treatment.

Mechanism

Sodium alginate treatment may be increasing satiety in several ways. Firstly, as Hoad et al. suggested after observing alginate-induced gastric lumping by MRI, the gel lumps may be promoting satiety via antral distention [7]. Secondly, alginate-induced gel formation may increase satiety by attenuating glycemic and insulinemic responses, as described below.

Regulation of postprandial glycemic and insulinemic responses

Glycemic Reponse

Postprandial hyperglycemia has long been recognized as a contributing factor to type II diabetes and obesity. Ideally, a treatment for these conditions should have the capacity to attenuate the glucose surge after a meal. Although sodium alginate's efficacy in producing this effect varies among studies as a function of experimental-related parameters, data as a whole suggest that it may have significant potential in dampening glycemic responses.

By far the most drastic reduction effect in postprandial plasma glucose levels was observed in Williams' study [25], where pre-prandial consumption of alginate-containing bars yielded a significant reduction in blood glucose levels at 15, 30, 45, and 120 minutes post-feeding concomitant with a 33% reduction in the positive incremental area under the glucose curve (AUC). It should be noted, however, that these effects may not be attributed with certainty to sodium alginate: the experimental products' total fibre and guar content exceeded that of the controls, and an increase in either of these parameters is able to dampen blood glucose level excursions independently [26].

Nonetheless, Wolf et al. [27] demonstrated sodium alginate's ability to downregulate the postprandial glycemic response independently of total fibre content. The alginate beverage produced lower postprandial glucose levels than a control treatment of equal fibre content. However, as one might expect, the reduction was not as dramatic as in Williams' [25] study: the difference between blood glucose excursions was only significant at 60 minutes. Furthermore, Murray and colleagues [28] found that an alginate-containing diet reduced the postprandial glycemic response in dogs to a greater extent than diets containing an equivalent amount of oat and soy fibre by weight, as well as diets containing more than twice the weight content of soy fibre. Taken together, these two studies suggest that alginate's beneficial effects on postprandial glycemic responses are at least as good as, and in some cases exceed, that of other dietary fibres.

Interestingly, Paxman et al. [10] demonstrated a correlation between body fat percentage and area under the glucose response curve post meal consumption. When overweight or obese subjects consumed a drink containing strong-gelling sodium alginate before their meals, this correlation was abolished [10]. The authors pointed out that the ability of alginate to restore glucose uptake in these individuals to the levels of healthy participants was indicative of the therapeutic potential of the alginate products.

Mechanism

In general, viscous dietary fibres dampen the rise in blood glucose levels following food intake by delaying gastric emptying [16, 29, 30] and slowing the absorption of nutrients in the small intestine [31, 32]. Whether one of these mechanisms or both of them enable sodium alginate's effect on glycemic response attenuation is unclear.

Wolf et al. [27] attribute the observed effects of alginate on the glycemic response in their study to the alteration of gastric emptying rate, rather than intestinal absorption rate. Due to the pH sensitivity of the alginate complex used in the study, the alginate was expected to lose its viscosifying properties upon entering the duodenum, suggesting that its effect was entirely dependent on gastric emptying and independent of action in the small intestine. Similarly, Torsdottir et al. [16] observed a decrease in the gastric emptying rate after consumption of an alginate-containing meal.

In contrast, two studies concluded that alginate's mechanism of action is not dependent on gastric emptying rate. Hoad et al. [7] measured gastric emptying by magnetic resonance imaging, and found no difference between the rates induced by alginate-containing beverages with strongly- and weakly-gelling alginates, guar-containing beverages, and milk. Paxman et al. [10] concluded that sodium alginate was unlikely to act through slowing gastric emptying, since it did not alter the time taken to reach peak glucose levels and peak cholesterol levels in the plasma. Thus, the authors attributed these physiological effects to small intestinal absorption rate alterations. It should be noted, however, that the latter speculation is not supported by empirical data. Furthermore, the observation that the time taken to reach peak glucose levels did not differ between alginate-containing treatment and control might simply have been due to the capacity of alginate complexes to buffer initial pH changes, which has been observed elsewhere by Wolf [27]. The buffer capacity might have prevented the complex from reaching optimal pH for viscosification until a certain time after ingestion, thus preventing the alginate from exerting its effects on glucose absorption until after the peak glucose levels were attained.

In order to determine if the glucose small intestinal absorption rate was indeed influenced by sodium alginates, Kimura et al. [33] perfused alginate- and glucose-containing solutions through the jujenal segment of rat bowels, and found that alginates had no effect on glucose absorption into the mesenteric artery. Since the perfusates were not gelled, as the alginate complex is expected to be upon passing through the acidic stomach environment, these findings cannot conclusively exclude the possibility of the alginate's action at the level of the small intestine. Indeed, the authors suggested that sodium alginate may play a role in inhibiting Na^+ absorption from the small intestine based on Kato's [34] finding that alginic acid enhanced Na^+ excretion into feces in rats, subsequently inhibiting glucose transport via the Na^+-dependent glucose transporter.

Insulinemic Response

Obesity is generally characterised by hyperinsulinemia and insulin resistance [35], which progressively increases with obesity [35, 36]. Sodium alginate was found to reduce insulinemic responses as a consequence of attenuated glucose excursions [16, 33]. A limited number of studies have described attenuated postprandial insulinemic responses following sodium alginate consumption. Kimura et al. [33] found that feeding milligram quantities of alginates to rats dampened the insulinemic response below that of animals fed glucose alone at select time points. More convincing evidence was provided by Torstodir et al. [16], who showed that sodium alginate-containing meals attenuated the insulinemic response for 180 minutes post treatment consumption, with significant differences from the control at 15, 60, and 90 minutes. In contrast to the above studies, Wolf et al. [17] did not observe any significant changes in the postprandial insulin response AUC between alginate treatments and control. However, Wolf's study differs from others in that the control consisted of a treatment of equal fibre content (gum arabic and guar mix) as the alginate beverage. The study, therefore, does not exclude the possibility of alginate's efficacy in regulating the postprandial insulin response, rather suggesting that it may not exceed that of other fibres.

Interestingly, Wolf et al. reported an attenuated glucose response accompanied by an increase in insulin mean peak incremental change from baseline following consumption of an alginate-containing beverage [17]. The authors suggested that this contradictory finding may point to the alginate's ability to elicit secretion of glucagon-peptide 1, a potent insulin secretagogue whose levels increase in response to some dietary fibres [37]. Its insulinotropic action would account for the lack of attenuated insulin levels in response to an attenuated glucose response.

Concluding Remarks

Sodium alginate appears to have a particular value in appetite and food intake regulation in addition to glycemic control. However, whether these beneficial metabolic impacts are maintained following chronic consumption of sodium alginates is still not clear and requires further analysis. Regardless, investigation of foods and beverages with similar properties may prove to be instrumental in identification of conjunctive therapeutic agents in treatment of obesity and type II diabetes.

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JULS LETTERS

Modulation of DNMT3A Expression and Nuclear Localization in Bladder Smooth Muscle Cells in Response to Obstructive Stimuli

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Abstract

Purpose: Recent preliminary studies (unpublished) indicate that epigenetic changes, specifically DNA methylation via DNA Methyltransferase 3A (DNMT3A), may be an important factor underlying obstructive urinary pathologies, which are characterized by bladder smooth muscle cell hyperplasia, hypertrophy, and de-differentiation. A number of pharmacologic agents were tested for their ability to modulate the levels of DNA methylation by regulating the expression and nuclear localization of DNMT3A. Materials and Methods: Bladder smooth muscle cells (BSMCs) of early passage numbers (0-2) were plated on 12-well culture plates pre-coated with either native collagen (NC) or denatured collagen (DNC) obtained by heat denaturing Type I bovine collagen. Compounds being tested were added 2-3 hours after plating. Cells were incubated in the presence of inhibitors for 48 hours under standard culture conditions, then fixed and stained for DNMT3A (anti-mouse, red). Results: AG490 treated cells showed significantly reduced DNMT3A nuclear localization (P<0.001) and no effect on BSMC proliferation and de-differentiation. Conclusions: AG490, a STAT3 inhibitor, was able to significantly reduce DNMT3A expression and nuclear localization in BSMCs in response to obstructive stimuli.

Abbreviations

BSMC	**Bladder Smooth Muscle Cell**	**MMP**	**Matrix Metalloproteinase**
DNMT	**DNA Methyltransferase**	**mTOR**	**Mammalian Targets of Rapamycin**
DNC	**Denatured Collage**	**NC**	**Native Collage**
ECM	**Extracellular Matrix**	**αSMA**	**Alpha Smooth Muscle Actin**
ERK1/2	**Extracellular Signal-Regulated Kinase 1/2**	**STAT3**	**Signal Transducer and Activator of Transcription 3**
JAK	**Janus Kinase**		

Introduction

Obstruction to the bladder detrusor muscle can arise from traumatic injury to the brain or spinal cord, exposure to heavy metals, and misuse of the bladder [1]. In general, when the bladder fails to empty properly, the increase in pressure excessively stretches the detrusor muscle wall and cause hypoxia, mechanical strain, and matrix degradation [2]. These pathological consequences can lead to the progressive loss of architectural and functional integrity of the bladder smooth muscle extracellular matrix (ECM). The ECM of bladder smooth muscle cells (BSMCs) is an important modulator of bladder function. Major components of the ECM include proteins, proteoglycans, and glycosaminoglycans [3]. Fibrillar collagens (collagen I and III) constitute the bulk of the scaffold proteins in the ECM and are remodelled for experiments in this study to mimic conditions of denatured ECM. Upon obstruction, the ECM changes in structure and composition due to increased pressure or volume, and BSMCs respond to these changes with hyperplasia, hypertrophy, and de-differentiation [4]. Recent studies (unpublished) suggest that remodeling of the ECM can influence epigenetic mechanisms such as DNA methylation, which may be the underlying reason for the irreversibility of some pathological conditions even with the removal of obstruction.

Epigenetic processes alter the expression and function of genes without corresponding alterations in their DNA sequences. Epigenetic modifications are heritable characteristics that can occur via a number of processes including histone modification, DNA methylation, and RNA interference [5]. DNA methylation is considered to be the most stable epigenetic phenomenon and is generally associated with transcriptional repression. It is mediated by DNA methyltransferases which catalyze the addition of methyl groups to

Table 1: Chemicals tested and their targets of action and concentrations used.

Chemical	Target	Concentration
PD98059	Erk Kinase	20µM
Lithium Chloride	Gsk 3 Beta	10µM
Rapamycin	mTOR	15ng/ml
Actinomycin D	Translation	0.5µg/ml
Cyclopamine	Primary Cilia	40µM
AG490	**STAT3**	**2.5µM**
AG1478	EGFR Kinase	50µM
PD153035	EGFR Kinase	50µM
Doxycycline	MMP	10µM
Ly294002	PI3 Kinase	5µM

cytosine residues at stretches of DNA rich in cytosine and guanine dinucleotides (CpG islands) [6, 7]. There are three major types of DNMTs known in mammals: DNMT1, DNMT3a, and DNMT3b [5]. DNMT3a and 3b are responsible for catalyzing new methylation patterns, while DNMT1 acts to maintain these patterns [6]. Aberrant DNA methylation has been implicated in a number of diseases and can be a hallmark of cancer [8].

The goal of this study is to identify agents that would reduce the expression or localization of DNMT3A in response to harmful stimuli such as denatured matrix. Assuming that DNA methylation is an important epigenetic phenomenon underlying the pathological state of the bladder under obstruction, inhibition of DNMT3A nuclear localization or expression, and subsequent reductions in DNA methylation levels, may serve as a feasible therapeutic target for obstructive urinary diseases.

In order to test this theory, a number of chemical inhibitors (Table 1) that target pathways implicated in BSMC hyperproliferation or de-differentiation in response to obstruction were tested for their ability to modulate DNMT3A levels.

Methods and Materials

Rat BSMC Culture

Bladders were extracted from neonatal Sprague-Dawley rats (2-5 days old) and smooth muscle cells were isolated as described in Herz et al.(2003) [9]. Cells of early passage (0-2) were used for experiments in this study. Cells were maintained in Eagle's minimum essential medium (EMEM; Multicell Technologies, Woonsocket, Rhode Island) containing 10% fetal calf serum (Invitrogen Canada, CA) and antibiotics (Multicell) on 10 cm tissue culture plates at 37°C and 95% O_2/5% CO_2. Once cultures reached confluency, cells were harvested by brief incubation with 0.25% trypsin+0.053mM/L EDTA (Multicell), washed with PBS, extracted into 10ml tubes, pelleted (3min at 600xg), and resuspended in EMEM+6% fetal calf serum at a concentration of $3x10^4$ cells/ml. Cells were serum starved for 24 hours prior to use in order to synchronize BSMCs to G_0.

Collagen and Treatment Preparation

Type I bovine collagen solution (Elastin Products Company, Owensville, Missouri) was neutralized with 0.1 mol/L NaOH in 1X PBS (Multicell) as described in Aitken et al. (2006) [4], and gelated at 37°C. Denatured collagen was obtained by boiling the type 1 bovine collagen (30min) and then neutralized. NC and DNC were loaded onto 12-well plates with sterile saline-coated glass cover slips. NC-loaded plates were incubated at 37°C for polymerization to occur. DNC-loaded plates were left at room temperature to dry. Both DNC and NC gels were washed with EMEM before cell plating to ensure neutral pH. 1ml of BSMCs, at a concentration of $3x10^4$ cells per ml, was transferred to each well. Chemicals compounds (Figure 1) were added 2-3 hours after plating (n=3) and cells were incubated for 48 hours at 37°C and 95% O_2/5% CO_2.

Immunoassays

After 48 hours, cells plated on NC and DNC were washed with PBS and fixed with 4% paraformaldehyde. After 20 min, fixed cells were washed with PBS and then permeabilized with 0.02% Triton X-100. Blocking solution (10% normal goat serum and 3% bovine serum albumin in 1X PBS) was applied for one hour to reduce non-specific binding of antibodies. Anti-mouse antibodies for DNMT3A and anti-rabbit antibodies for αSMA (Abcam) were then added (1:200 in 5% normal goat serum) and incubated overnight at 4°C. After washing, fluorescent secondary anti-mouse antibodies (Cy3-red; Jackson Immunolabs) and anti-rabbit antibodies (Cy2-green; Jackson Immunolabs) were added one hour (both diluted 1:200 in 5% normal goat serum), followed by a PBS wash and Hoechst staining (5 minutes). Cover slips were mounted on glass slides with Dako mounting media.

Statistical Analysis

Cells were visualized on the deconvolution microscope using Volocity 5.0; and the amount of fluorescence was quantified with Image-J. Proliferation was assessed by cell counting; DNMT3A expression and BSMC differentiation were assessed by the amount of red and green fluorescence respectively. Statistical significance was determined by two-tailed t-tests (n=9) for the comparison of two means at α=0.05.

Results

The majority of inhibitors tested did not show significant modulation of DNMT3A expression or nuclear localization compared to BSMCs plated on DNC alone (vehicle control). Cells plated on DNC had significantly increased DNMT3A nuclear localization. AG490, a STAT3 inhibitor, is one inhibitor that was able to decrease DNMT3A nuclear localization compared to the vehicle control (P=0.001). However, it did not affect BSMC de-differentiation or proliferation. On the NC control, DNMT3A expression or localization, BSMC proliferation, and the level of differentiation were not affected by AG490 (Figure 1).

Discussion

Preliminary data (not published) have shown that DNMT3A responds to strain-induced matrix degradation by increasing in expression levels and localizing to the nucleus. In addition, removal of stimuli does not necessarily result in the return of normal phenotypes [4], which further supports the hypothesis that stable epigenetic changes may have occurred in response to obstruction. In this study, chemical compounds were used to inhibit signalling pathways implicated in the hyperproliferative or dedifferentiation responses of BSMCs under strain in order to test if there is corresponding inhibition of DNMT3A nuclear localization and/or expression. This study found that the inhibition of STAT3 by AG490 leads to decreased nuclear levels of DNMT3A, but no significant reduction in de-differentiation or proliferation.

AG490 Inhibition of STAT3 decreased DNMT3A localization but not de-differentiation or proliferation. STAT3 is a transcription factor which is primarily phosphorylated and activated by Janus kinase (JAK 1 and 2). STAT3 responds to a variety of signal molecules including growth factors and cytokines. Following activation, STAT3 translocates to the nucleus where it binds cis regulatory elements and induces the transcription of genes associated

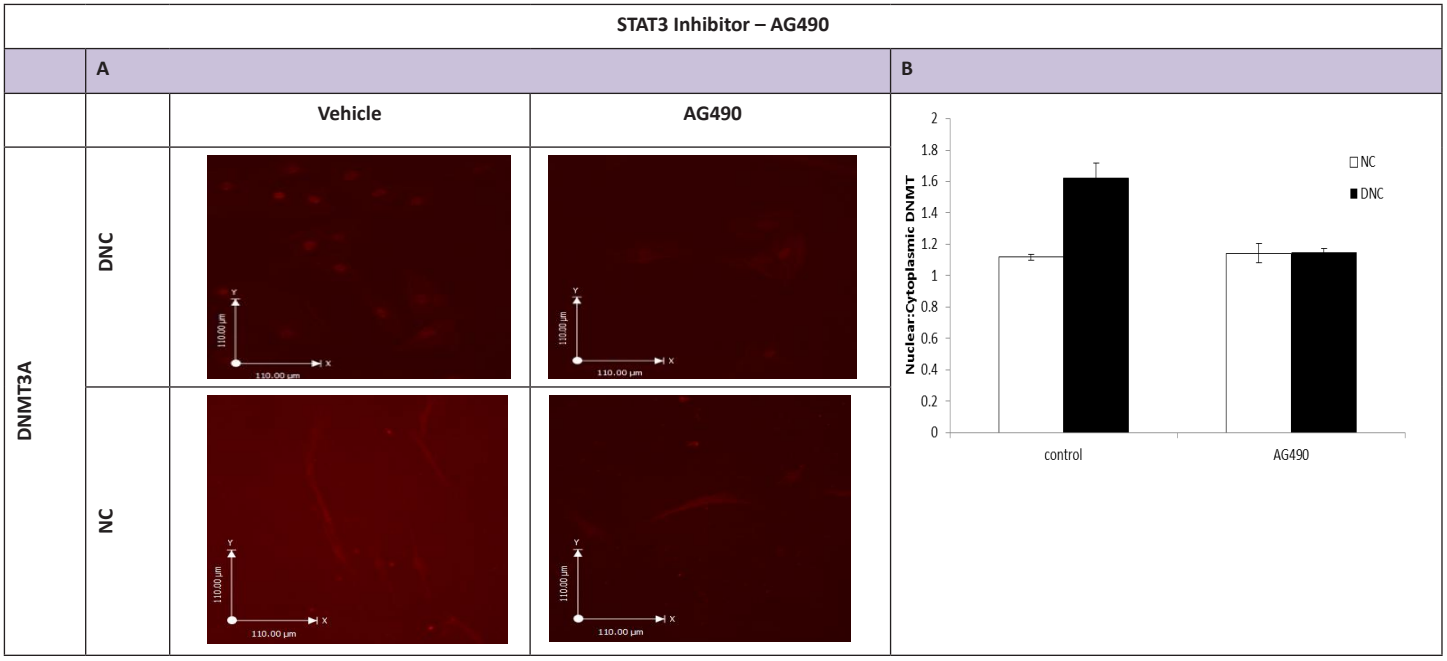

Figure 1. **(A)** Reduced nuclear DNMT3A fluorescence in AG490 treated cells compared to vehicle. **(B)** Treatment with AG490 significantly reduced the nuclear to cytoplasmic ratio of DNMT3A in BSMCs plated on DNC. P-Value=0.001, n=9

with growth and inflammation [10]. One way to account for how obstructive stimuli could induce STAT3 activity is that increased strain and pressure may upregulate matrix metalloproteinase (MMP) activity, leading to matrix remodelling and the exposure of neoepitopes and growth factors previously sequestered within matrix components [5, 10]. Some of these cryptic epitopes may act to induce the activation of STAT3 and its downstream targets, some of which may influence proliferation and dedifferentiation.

The finding that STAT3 inhibition decreased nuclear localization of DNMT3A, but not the de-differentiation or proliferative response, does not necessarily invalidate DNA methylation as a mechanism through which pathological phenotypes persist in BSMCs following exposure to denatured ECM. More sophisticated or alternative methods may be needed to assess proliferation and dedifferentiation. These may include assessing proliferation by ^{3}H thymidine incorporation for quantifying proliferation or by using late differentiation markers such as Calponin and SM22, which are differentiation markers, as opposed to αSMA which is an early maker of differentiation [11].

Conclusion

Findings in this preliminary study are not sufficient to deduce the role of DNA methylation in BSMCs under obstruction. Nevertheless, it does provide evidence that DNMT3A expression and localization is affected in BSMCs in response to ECM degradation, although the mechanisms through which DNA methylation can induce proliferation and dedifferentiation in BSMCs remain poorly understood. The possibility that DNA methylation may promote phenotypic changes in BSMCs under pathological conditions provides grounds for further research into the role of epigenetic modifications in urinary pathologies.

Pharmacotherapies currently available for the treatment of obstructive urinary pathologies that target muscarinic receptors and other second tier medical treatments are associated with a number of side effects and have shown limited efficacy [12]. Findings in this preliminary study suggest that chemical inhibitors are able to modulate the expression and/or localization of DNMT3A, which has been shown to change in response to obstructive stimuli. Presently, STAT3 inhibitors are under investigation for the treatment of breast cancer and chronic myeloid leukemia for its ability to decrease tumour cell proliferation. If future research demonstrates DNA methylation to be an important epigenetic process underlying the pathological state of BSMCs under strain, pharmacologic agents which target epigenetic processes may emerge to be a more effective alternative for the treatment of urinary pathologies.

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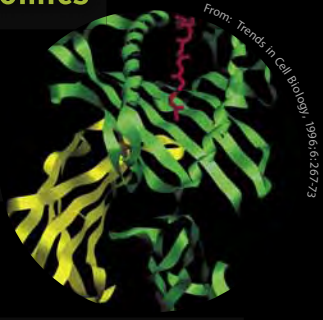

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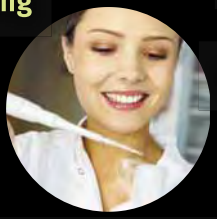

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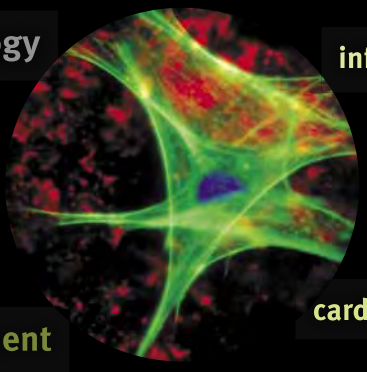

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