CANADIAN JOURNAL of UNDER GRADUATE RESEARCH

Something in the water

Diatoms found in cadaver bloodstreams can help forensic experts identify cases of non-accidental or faked drowning, but existing literature on the technique date back to the 1970's and 80's; a new review is in order *(p.11)*

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Mad world

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Gut feeling

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CANADIAN JOURNAL *of* UNDERGRADUATE RESEARCH

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Letter from the editor-in-chief

It is my pleasure and great privilege to present to you Volume 6 Issue I of the Canadian Journal of Undergraduate Research (CJUR). Supporting undergraduate students across Canada by providing an accessible platform for them to share their research, engage in multidisciplinary dialogue, and experience the peer-review process has always been our number one priority. Throughout the years, we have published a total of 49 manuscripts from universities all over the country. We strive to reach out to more students and we look forward to seeing CJUR grow over the coming years.

2021 marks CJUR's 6th anniversary, and since our founding, we have received immense positive feedback from numerous students, professors, and organizations in the academic community. Throughout the years, we have grown as both a journal and a team. This year, we led a major reviewer recruitment event and onboarded over 100 strong graduate students, post-doctoral fellows, and professors who play a vital role in our peer-review publication process. We have also expanded our content categories to include various new submission types and have worked to make our journal as multidisciplinary as possible by reaching out to academic departments from all fields. This year, our outreach team headed new initiatives with the aim of building relationships with student organizations from universities all across the country. We hosted online workshops and Q&A sessions on academic writing and publishing, reaching out to hundreds of students. During this ongoing pandemic, CJUR believes showcasing students' work is as important as ever, and we will continue to strive to provide an accessible publishing platform for all.

This issue includes submissions from the University of British Columbia, the University of Ottawa, Simon Fraser University, Trent University, and Western University. Each manuscript has undergone two extensive review stages by graduate students, post-doctoral fellows, and professors specializing in the particular field of research. In this issue, we explore various topics, from the effects of antibiotics on gut neurons to the historical perceptions of mental illness. Each manuscript is a reflection of the dedication and work undergraduates put into their research, and I hope you join us in celebrating their work.

As this academic year comes to a close, I reflect on everything our team has achieved. As the CJUR editor-in-chief and chair since 2018, I have had the privilege of working with dedicated and driven students from UBC and other universities from across Canada, who are passionate about research and bringing publication opportunities to the academic community. A massive thank you to the amazing team who has made this journey truly incredible: Derrick Sutanto, Michelle Lisonek, Jashan Saini, Lorenzo Lindo, Ryan Hong, Monica Luo, Uyen Nguyen, Natnaiel Dubale, Tommy Kuo, Jordi Chaffer, Matthew Ma, Claire Cheung, Ryan Chan, and Max Seymour. Although many of us have not been able to meet in person, we have made lifelong friendships and it has truly been an honour to lead this team. I commend everyone who has persevered during this past year, and I applaud all the people that have helped make CJUR the journal it is today. Thank you for your continued support, and I hope you enjoy Volume 6 Issue I.

Yours sincerely,

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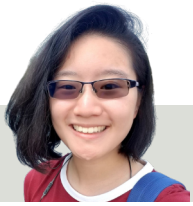

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Historical perspectives on neurodevelopmental disorders: The merging of *us* and *them*

Gillian Shoychet [1]

[1] University of Western Ontario, London, Ontario

Abstract Attitudes towards neurodevelopmental disorders (NDDs) have transformed and fluctuated throughout history, often reflecting the societal culture of the time. Although individuals with NDDs are presently more valued than they have ever been, there are historic and current perspectives and events which have propelled the study of NDDs in both positive and negative directions. The notion of disability originated in Antiquity, where infants with "physical deformities" were deemed unworthy of societal membership. Despite later community inclusion in the Middle Ages, disability was perceived to result from demonic possession or punishment. Subsequently, individuals with NDDs were placed in institutions designed for physical and social isolation. The Enlightenment marked the first positive shift in attitudes and treatment towards those with disabilities, as proponents advocated for the removal of inhumane institutional conditions. The humane movement, moreover, contrasted the long-held belief that individuals with disabilities were "inferior" and "dumb" by illustrating that children with NDDs were capable of learning and worthy of education. The Industrial Revolution reversed these progressive ideas by associating disability with inefficiency. Further, during World War II (WWII), the eugenics movement propagated the belief that NDDs blemished a strong nation, which inculcated widespread support for the forced sterilization and mass murder of individuals with NDDs. Global legislation was instated post-war to protect the rights of those with NDDs. These progressive ideas, originating from the humane movement, fostered the many educational methodologies and opportunities now available for children with NDDs. Though current perspectives towards NDDs have vastly improved, there is greater confusion regarding the moral implications of advancing research in biotechnology for genetic engineering and testing. While benefits exist, there is a potential for the emergence of a modern eugenics movement in the 21st century. The obscurity of this dilemma poses serious moral questions that must be considered within the historical context of perspectives on NDDs.

INTRODUCTION

Neurodevelopmental disorders (NDDs) have been portrayed in various ways throughout history. The American Psychiatric Association ([APA], 2013) published the prevailing view in the *Diagnostic and Statistical Manual of Mental Disorders* (5th ed.; DSM-5), which describes NDDs as an umbrella term for disorders that have an onset in early development and are characterized by impaired personal, social, academic, or occupational functioning. Each NDD has a varying functional impact ranging from mild to severe (APA, 2013). The prevalence of NDDs has increased in the United States (US) from 12.84% in 1999 to ~17% in 2017 (Zablotsky et al., 2019). The latter figure represents approximately one in six children, aged three to 17, diagnosed with at least one NDD (Zablotsky et al., 2019). One possibility for rising diagnostic rates may be a greater awareness of the diagnostic characteristics of NDDs. This increasing awareness may have encouraged heightened acceptance of NDDs in the 21st century, as evidenced by greater societal inclusion and accommodation. Unfortunately, advancements in behavioural genetics may unintentionally diminish the human value of those with disabilities by suggesting that some genes are superior. In pursuit of advancing certain biotechnologies, scientists should caution against restoring negative societal conceptions of disability. Although individuals with NDDs are more valued than they have ever been in society, there are both historic and current events which have

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Address correspondence to cjur.ca

propelled the study of NDDs in both positive and negative directions.

HISTORICAL REVIEW

The concept of disability was introduced in Ancient Greece. However, when examining past accounts of disability, it is important to consider varying conceptualizations throughout history. Past descriptions of disability were broader than current understanding. The term "mental retardation" (currently labelled intellectual disability [ID]) originated in the Therapeutic Papyrus of Thebes in 1552 B.C.E. (Ollendick & Hersen, 1998). The obscure writings described treatments for disabilities of the mind and body resulting from brain damage (Ollendick & Hersen, 1998). The introduction of disability resulted in the establishment of negative attitudes towards those with disabilities. The Age of Antiquity was a tragic period for children born "physically deformed" as infanticide was commonly directed towards infants with congenital disabilities (Patterson, 1985; Schuelka, 2013). Exposure, the most frequent form of infanticide, occurred when infants were abandoned in the wilderness, dying soon after (Patterson, 1985). This was documented in Aristotle's Politics: "As to the exposure and rearing of children, let there be a law that no deformed child should live" (Larson, 2014). In Sparta, Greece's military city, the elders decided whether an infant should live (Arthur, 2020). The criterion was simple: if a child was deemed unfit to fight, that child was unfit to live (Arthur, 2020). From the onset, infants with disabilities were viewed as "inferior", "weak", "burdensome", and "undeserving of societal membership".

During the 5th century, Christian laws prohibited exposure and infanticide as these acts were discordant with religious beliefs regarding the sanctity of life (Gushee, 2013; Mustakallio & Laes, 2011). Rather, two themes prevailed in describing disability: disability as possession and disability as punishment (Mustakallio & Laes, 2011). Children with disabilities who experienced various symptoms (e.g., panic attacks or muteness) were perceived to be possessed by demons (Mustakallio & Laes, 2011). Moreover, disability was viewed as a punishment for a person's sin (Mustakallio & Laes, 2011). However, treatment towards disability in the Middle Ages was mixed: families or the Church usually cared for children with disabilities as it was deemed a Christian duty (Mustakallio & Laes, 2011). However, sometimes these children resorted to begging due to the economic burden they caused their families (Mustakallio & Laes, 2011). Although individuals with disabilities were judged quite negatively, they were permitted to live in medieval society.

The Enlightenment of the 18th century promoted reason, progress, and the scientific method (Brown & Radford, 2015). This cultural climate facilitated the work of French physician Philippe Pinel in establishing the moral treatment movement, which argued for the end of harsh treatment towards individuals with disabilities living in asylums/institutions in favour of more humane and compassionate models of care (Brown & Radford, 2015). The moral treatment movement coincided with humanitarianism (i.e., the commitment to improving the lives of everyone in society, especially those who required the most support [Brown & Radford, 2015]), and together they arguably propelled the conceptualization and treatment of individuals with NDDs. Pinel improved treatment towards the "mentally ill" in the Bicêtre asylum by removing physical restraints, prohibiting physical abuse, and encouraging empathetic communication with patients (Trent, n.d.). Moreover, Pinel replaced the view that disability was caused by demonic possession with the notion that social stress, hereditary conditions, and physiological impairments were the actual cause of symptoms ("Philippe Pinel", 2001).

The moral treatment and humanitarian movements also promoted improvements in education for individuals with disabilities. Jean Marc Gaspard Itard is eminent for his work in the early 1800s with Victor of Aveyron, the "feral child", who lived isolated in the woods until he was approximately 12 (Brown & Radford, 2015). Without human exposure, Victor never developed language, nor knowledge of societal norms (Brown & Radford, 2015). Many physicians deemed Victor an "incurable idiot" (Ferguson, 2006); however, Itard was devoted to teaching Victor language and social skills. Itard was the first person to attempt special education on a child with an ID (Ferguson, 2006). This work was highly progressive for its time, though most professionals viewed Victor's training as a futile pursuit. Itard's work inspired his student, Edouard Séguin, to continue working with individuals with IDs: in 1839, Séguin opened the first school for children with severe IDs ("Edouard Séguin", 2020). Séguin further developed a sensory training method as he believed that IDs were caused by a fragility of the nervous system ("Edouard Séguin", 2020). Itard and Séguin's commitment to educating children with IDs propelled the study of NDDs by emphasizing that individuals with disabilities are not only worthy of education but also capable of learning.

Humanitarianism began to wane towards the end of the 19th century with the rise of industrialization, which transformed agricultural-based economies to manufacturing-based ones (Larkin, 2011). Society represented a giant efficient machine (Larkin, 2011). Individuals with disabilities were unsuited for urban life, as they were perceived as "inefficient" in meeting factory production demands (Larkin, 2011). Consequently, those with disabilities were dismissed and placed in asylums to become "a productive cog in the greater societal machine" (Brown & Radford, 2015, p. 8). Asylums originally emphasized education and development prior to industrialization but abandoned this focus to reflect society's new beliefs on disability (Brown & Radford, 2015). Disability transformed within this cultural context by pathologizing both intellectual and physical impairments (Roeher Institute, 1996). Advances in biomedicine also assisted in the emergence of the dominant medical model of disability, which believed impairment was rooted in the body and therefore required medical treatment (Roeher Institute, 1996). ¬¬¬Once again, although the perceived causes of disability changed from divine to physiological ones, people with NDDs were still disconnected from society, thereby perpetuating the distinction between us and them.

Attitudes towards disability worsened at the end of the 19th century with the advent of eugenics. Francis Galton was inspired by Charles Darwin's theory of evolution via natural selection and was especially intrigued by Darwin's chapter on selective breeding of animals and the implications this could have on humans (Gillham, 2001). In 1883, Galton coined the term eugenics, originating from the Greek word eugenes ("good birth"), to describe his goal of improving the human gene pool through selective breeding (Gillham, 2001). The notion that human abilities are hereditary was the premise of Galton's theory (Gillham, 2001). The eugenics movement progressed in two directions – positive eugenics and negative eugenics (Grodin et al., 2018). Galton advocated for

positive eugenics, which promoted the reproduction of desirable traits (e.g., encouraging highly able individuals to produce more offspring than those "less able"; Brown & Radford, 2015; Grodin et al., 2018). Conversely, negative eugenics favoured restricting the reproduction of negative traits (e.g., via sterilization; Grodin et al., 2018). The eugenics movement gained widespread popularity as countries desired strong and progressive populations (Brown & Radford, 2015; Grodin et al., 2018). Henry Goddard was one such advocate of eugenics, in the early 20th century, aspiring to prevent the breeding of more "feeble-minded" (those with IDs; Grossberg, 2011). Goddard translated Alfred Binet's intelligence test to study "feeble-minded" children in the US (Green, 2019). Goddard introduced three terms to describe lower degrees of mental development: "moron" (IQ = 51-70), "imbecile" (IQ = 26-50), and "idiot" (IQ = 0-25; Green, 2019). Goddard assisted in the screening of immigrants for an intelligence testing program on Ellis Island, as there was a law banning the entry of immigrants deemed "feeble-minded" (Green, 2019). Goddard used the Binet test and his classification system to determine individuals' degree of mental development to separate the "feeble-minded" from American society.

The eugenics movement is arguably one of the most detrimental events to occur in the history of NDDs. This is evidenced by Nazi Germany's adoption of negative eugenics. Adolf Hitler aimed to create an Aryan master race by sterilizing those with any disease or disability to prevent the reproduction of inferior genes (Grodin et al., 2018). However, as the war progressed, euthanasia ("good death") replaced sterilization (Hudson, 2011). Euthanasia supposedly describes "mercy killings" (i.e., assisted dying for those who are terminally ill and suffering). However, under the Nazi regime it was a euphemism for murder (Grodin et al., 2018). The first systematic euthanasia program in the Holocaust targeted children with disabilities (Hudson, 2011). Doctors were compelled to report infants born with an illness or children up to the age of three with an existing illness (Hudson, 2011). These children were murdered in clinics and their parents would then receive letters indicating that they died from natural causes (Hudson, 2011). The criteria for children with disabilities later expanded to include children up to 17 and those with less severe disabilities (United States Holocaust Memorial Museum, 2020). Approximately 5000 to 8000 children were murdered under this euthanasia program (Hudson, 2011). In 1939, the Nazis expanded their program to include adults (Grodin et al., 2018). Aktion T4 was the sterilization program that used poisonous gas to mass murder people with disabilities (Berenbaum, 2020). By the end of the Holocaust, approximately 275,000 victims with disabilities labelled "unfit" for German life were murdered (Berenbaum, 2020). After WWII and the fall of Nazi Germany, the eugenics program lost considerable momentum as countries wanted to distance themselves from anything associated with the Nazis. The eugenics movement significantly halted progress in improving attitudes towards and enhancing the lives of individuals with disabilities.

CURRENT PERSPECTIVES

Prior to WWII, state sovereignty was the dominant governing approach. However, the monstrosities of WWII and the Holocaust accentuated the need for international regulations to protect citizens from their countries' unrestricted state sovereignty (Kanter, 2015). Although international human rights became a topic of chief importance, people with disabilities remained relatively invisible (Kanter, 2015). However, attitudes towards NDDs in the 21st century significantly improved with the increasing demand for further international protection for people with disabilities by individual advocates and organizations (Kanter, 2015). In 2006, the United Nations (UN) adopted the Convention on the Rights of Persons with Disabilities (CRPD), which was the first human rights treaty concerning people with disabilities (Series, 2015). Due to the all-encompassing nature of this legal document, this treaty included, but was not limited to, individuals with NDDs (Kanter, 2015). The CRPD contains 50 articles outlining inclusion principles for people with disabilities, such as the right to accommodation, accessible environments, and inclusive education (Series, 2015). This convention was revolutionary for enhancing the prominence of disability in global legislation (Kanter, 2015). The acknowledgment that individuals with disabilities should be included, as well as given equal status in the community, is not only a recently established movement within disability history, but also brings individuals one step closer towards merging societal attitudes of us with them.

Since the establishment of the CRPD, inclusive education is one remarkable area of growth for children with NDDs. Inclusion in educational settings refers to the integration of both children with and without NDDs in a regular classroom (Lamport et al., 2012). "Co-teaching" commonly occurs in inclusive classrooms where a general teacher and special education teacher both provide instruction in order to adapt to students' educational needs (Lamport et al., 2012). When inclusive classrooms are implemented as recommended, both children with and without disabilities benefit. For example, research demonstrates that inclusive classrooms enable children with disabilities to develop basic communication and motor skills from the prompts and cues provided by other classmates (Hunt, Staub, et al., 1994). Moreover, studies reveal that inclusive classrooms enhance engagement on the part of students with disabilities (Hunt, Farron-Davis, et al., 1994; Mirenda & Katz, 2002). Prolonged engagement most likely occurs as a child with an NDD is commonly accompanied by a typically developing peer (Hunt, Farron-Davis et al., 1994). Although a common complaint is that typically developing children will receive less attention and thus be unable to fulfill their academic potential, studies indicate no academic disadvantages for any student and that all children profit socially (Salend & Garrick Duhaney, 1999). Including students with disabilities in regular classrooms may also shed light on pedagogical issues. Chandler-Olcott and Kluth (2009) provide a scenario where a teacher in an integrated classroom explains a comprehension activity. The researchers propose a situation where most of the students in the classroom misunderstand the activity yet attempt to complete it regardless. A child with autism spectrum disorder, however, may brusquely shout that the activity is useless. The researchers explain that this so-called "challenging behaviour" (i.e., abruptly calling out) can actually expose common problems that everyone in the class experiences, thereby enhancing the likelihood of elucidating confusion and improving learning outcomes. There are various social and behavioural advantages which accompany inclusive classrooms for all children. Although challenges still emerge, the development and improvement of inclusive classrooms continue. It is important to consider the origins of this ongoing and gradual positive shift in attitudes and treatment towards people with NDDs. These changes began with bright-minded individuals who, despite their dissenting opinions, believed that children with disabilities were capable of learning. The early proponents of

humanitarianism, Itard and Séguin, contributed to the development of inclusive classrooms by pioneering education for children with NDDs.

Although attitudes and treatment towards those with NDDs have improved, some still equate disability with suffering (Reinders et al., 2019). This representation of disability as a "pitiful condition" appears recurrently within NDD discourses (Reinders et al., 2019). In turn, some people's perception of disability regresses to reflect periods in history where disability was viewed as a "life not worth living". Critics have argued that society created a "new" eugenics in the 21st century due to the popularity of behavioural genetics (Reinders et al., 2019; Rembis, 2009). Behavioural genetics examines the influence of genes on behaviour, dispositions, and mental traits (Rembis, 2009). Preimplantation genetic diagnosis, prenatal genetic screening, and prenatal genetic diagnosis are three methods, associated with behavioural genetics, which examine the genetic makeup of a fetus (Grodin et al., 2018). These methods assess either the risk or presence of a genetic condition in the embryo or foetus, and produce test results that assist in pregnancy decisions (i.e., whether to have an abortion; Reinders et al., 2019). For instance, the Icelandic government requires physicians to inform pregnant mothers about available screening tests. This legislation likely contributes to Iceland approaching a down syndrome (DS)-free society with approximately only two to three children born with DS yearly (Reinders et al., 2019). It is quite striking that almost 100% of Icelandic women who have a positive test result for DS choose to abort their pregnancies. Although theoretically parents possess "free choice" in pregnancy decisions, these choices are often influenced by systematic bias (Rembis, 2009). Quality of life (QoL) for the child is a main consideration in making these decisions (Reinders et al., 2019). However, physicians and other healthcare professionals often view QoL in a medical context, equating NDD with suffering (Reinders et al., 2019). Contrastingly, in a support-services context, QoL consists of broader domains, including relationships, inclusion, and rights (Reinders et al., 2019). "Designer babies" are another recent concern for opponents of behavioural genetics, as advancing biotechnology opens the possibility of selecting a child's genetic makeup prior to implantation (Grodin et al., 2018). This practice merits careful consideration as it may result in some genetic traits being viewed as superior, which has serious implications for the eradication of certain traits (Grodin et al., 2018). Specifically, NDDs may be viewed by society as "undesirable," thereby potentially resulting in reduced mental wellbeing for those already living with an NDD.

In making prenatal decisions, the narratives of individuals with NDDs and their families are often missing from discussions with healthcare professionals. A recent study of 294 individuals living with DS in the US revealed that approximately 99% of respondents disclosed that they were happy with their lives, satisfied with who they are, content with their appearance, and loved their families (Reinders et al., 2019). Moreover, parents of children with NDDs have reported that factual information is scarce when expectant parents receive their test results (Reinders et al., 2019). Although there is no correct decision on whether to bring a child into the world, they should be presented with balanced information before choosing. In considering the evolving progression of behavioural genetics, scientists should remember the detrimental impact of eugenics on individuals with disabilities. Although behavioural genetics should not be equated to the Nazis' euthanasia program, the latter should remind society that "small steps along a slippery slope can lead to crimes against humanity" (Grodin et al., 2018, p. 55). The Nazis did not immediately begin murdering individuals with disabilities; this only occurred gradually after repeated propagation of their ideology that disability weakened German society (Grodin et al., 2018). Thus, continued research in behavioural genetics warrants caution as these practices may, at some point, breach moral boundaries.

CONCLUSION

Individuals with disabilities were historically perceived as "weak", "burdensome", "demonic and sinful", and "medically impaired". Current representations view those with disabilities as valued members of society. Moral treatment and humanitarianism were critical movements in gradually attaining equal rights for individuals with NDDs, especially where education is concerned. However, notions of eugenics remain present in biotechnological advancements. The goal of eugenics is to improve the human gene pool by either restricting or encouraging the reproduction of specific genes. This core eugenic principle must not be forgotten when continuing to pursue scientific research. Although attitudes and treatment towards individuals with NDDs have significantly improved, people should continue to fight for the rights of those who need greater and individualized support, while remaining cautious of scientific advancements in the field of behavioural genetics.

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Diatom analysis: Reviewing the strengths, weaknesses, and impacts of modern research

Jacob Caine [1]

[1] Simon Fraser University, Burnaby, British Columbia

Abstract The purpose of this paper is to review the theoretical and laboratory techniques which underpin diatom analysis and its ability to help determine death by drowning in a forensic context. Diatom analysis involves the recovery of diatoms (unicellular algae present in most natural bodies of water) which may be inhaled as part of a drowning medium (water) by an individual who has drowned prior to death. This literature review evaluates the strengths and weaknesses of the diatom analysis technique in the forensic context and evaluates recent research to discern whether it can be considered a reliable and valid forensic technique today. It is important to establish clarity as much of the original research conducted throughout the 1970's, 80's, and 90's have conflicting conclusions regarding the validity and reliability of diatom analysis. This literature review will show that modern research techniques used as part of the diatom analysis method have been able to reduce false positive results, increased the ability to distinguish between true and false positives, and found ways to mitigate many of the weaknesses noted in earlier research. Although some weaknesses such as diatom introduction into bodies prior to death and some details surrounding false positive results remain outstanding concerns, it can be seen in the literature that, when combined with existing strengths like seasonal variability and environmental specificity, diatom analysis is a valuable forensic tool, whose reliability has been strengthened by modern research, and can be relied upon to establish a definitive diagnosis of death by drowning.

INTRODUCTION

The forensic technique of diatom analysis is specific and uncommon but is a necessary and important diagnostic due to the complexities involved in determining death by drowning (Jian et al., 2019; Piette & De Letter, 2006). As Yukawa et al. (2013) note, diatom analysis is often considered to be "the gold standard" (p. 1) amongst the many laboratory tests to conclude death by drowning. Over 150,000 diatom species (unicellular algae) exist which can be found wherever sufficient light exists to support photosynthesis in most natural water bodies (Hendey, 1973). Diatoms preserve well, occur in high numbers, are detectible in almost all environments, and are environmentally specific (Horton et al., 2006; Pollanen et al., 1997). The main principle is that diatoms are present in the medium where drowning occurred and they will therefore also be present in body tissues (Horton et al., 2006; Krstic et al., 2002; Pollanen, 1998).

Diatom frustules, the subject of analysis, have hard box-like silica skeletons which are resistant to acid digestion and are almost indestructible, being soluble only in strong acid solutions (Fucci et al., 2015; Hendey, 1973; Seo et al., 2013), making post-mortem analysis possible (Pollanen et al., 1997). Diatom analysis allows forensic pathologists to determine the specific medium which the victim drowned in (e.g. fresh water, sea water, or lake water) because different diatom frustules can be found where environmental conditions differ due to narrow tolerances for temperature, light, salt content, environmental pollution, and pH levels (Levin et al., 2017; Tavassi et al., 2008). Thus, different species will be found in different bodies of water around the world (Auer, 1991; Coelho et al., 2016; Ludes, 2013; Peabody, 1977). Different species will also be present at different depths due to varying photosynthesis abilities (Auer, 1991).

Diatoms can be recovered from fresh, decomposed, and burnt tissues (Fucci et al., 2015)

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Address correspondence to cjur.ca

because prior to drowning, the victim would still have a beating heart which would allow diatoms to travel throughout the body via the circulatory system (Ago et al., 2011). Combining the inhalation of water and a beating heart allow diatoms to pass into the bloodstream as part of the oxygen exchange process (Rohn & Frade, 2006). As part of this process, diatoms then penetrate the lungs when water is inhaled, become lodged in alveoli, and can also be carried to internal organs such as the kidneys, heart, brain, and bone marrow (Ago et al., 2011; Antonenko & Ferris, 1987; Horton et al., 2006; Krstic et al., 2000; Pollanen et al., 1997). Thus, it is unlikely diatoms would be found in the internal organs or bone marrow of bodies disposed of in water post-mortem (Krstic et al., 2002). This makes diatom analysis helpful in determining whether a victim drowned or was disposed of in water post-mortem (Gruspier & Pollanen, 2000). In rare cases however individuals suffer rapid heart failure prior to drowning preventing circulation of diatoms and their entrance into internal organs (Antonenko & Ferris, 1987; Piette & De Letter, 2006).

Diatom analysis however remains controversial because diatoms can be found in tissues of people who died of causes other than drowning due to the widespread distribution of diatoms through the environment in soil, food, and air (Lucci et al., 2008). Diatoms can also implant passively into the lungs, stomach contents, and upper airways leading to unreliable analyses (Ago et al., 2011; Coelho et al., 2016). Diatom analysis remains in use however, because the diagnosis of drowning is highly complex (Ludes et al., 1999; Stephensen et al., 2019; Timperman, 1972), and other signs of drowning can in many cases can be insignificant or non-existent and may dissipate after death (Badu et al., 2015). Diatom analysis was recently used in a United Kingdom animal cruelty case which determined that an owner drowned their dog in a canal (BBC News, 2019; Johnson, 2019). As opinions on its usefulness remains controversial (Yukawa et al., 2013), it is important to evaluate the technique in greater detail.

Through evaluation of older and more recent literature, this paper aims to review the strengths and weaknesses of diatom analysis and determine whether it can still be considered a reliable technique in analysing possible cases of death by drowning. Much of the older literature on diatom analysis has been included as it is foundational to the evolution of the technique. Literature for this paper was collected through library resources at Simon Fraser University and additional online searches for related peer-reviewed literature. As reliability of analysis remains controversial amongst the scientific community (Carballeira et al., 2018), and testing historically laborious (Zhou et al., 2020), it is important to analyse the literature to determine whether the development of new techniques has increased the reliability. It will be seen in the literature that although weaknesses still exist, modern research and laboratory techniques have strengthened the reliability of diatom analysis showing that it should still be considered a strong forensic tool.

DISCUSSION

Strengths

Coelho et al. (2016), note the scientific principle of environmental specificity establishes the validity of diatom analysis. As specific diatom species are found in different water bodies, the presence of diatoms in the deep tissues or bone marrow of victims can lead to a definitive diagnosis of death by drowning (Auer, 1991; Pollanen, 1998). The diatoms present in bone marrow and other tissues can be compared to samples taken from the medium to determine an approximate drowning site to see if similar diatom species are present (Auer, 1991; Hürlimann et al., 2000; Pollanen, 1998). Environmental specificity makes diatom analysis a good indicator to use in suspicious death investigation when drowning is not suspected, because diatoms present in the bone marrow will match those found in the body of water where the victim was found (Pollanen et al., 1997).

When diatoms are present in high quantities, it is possible to pin-point the exact location where drowning took place (Peabody, 1977), but only if the drowning area is localized such as in a pond or a ditch (Auer, 1991). It is possible however to approximate the location due to environmental specificity (Auer, 1991); diatoms would need to be sampled from surrounding areas and waterbodies if the exact location is unknown (Coelho et al., 2016).

Determining which diatom species are present in body tissues can also help establish the potential drowning location and the time of death due to seasonal variability. The prevalence of diatoms in waterbodies also varies seasonally (Hendey, 1973; Pollanen et al., 1997). Diatom blooms (when concentrations are highest) follow seasonal patterns with diatom concentrations spiking in April before decreasing throughout the summer and occurring in lower numbers through the winter months (Pollanen et al., 1997). Seasonal variability can be used to scientifically determine an approximate time of death as the diatom density in bone marrow would reflect water concentrations at the time of death (Hürlimann et al., 2000). As the success of diatom analysis is correlated with diatom concentrations in the environment, diatom analysis is most successful in summer months and least successful in winter months when concentrations are low (Pollanen at al., 1997).

The presence of even a few diatoms in organs other than the lungs can be a reliable indicator of death by drowning (Hendey, 1973). Peabody (1977) suggested that their discovery in bone marrow is an even better indicator. This is supported by Auer (1991), who specifies that analysis of diatoms found in bone marrow is the only definitive way to diagnose death by drowning in decomposed bodies. This is because diatoms can enter internal organs during the body decomposition process sometimes making analysis unreliable (Coelho et al., 2016; (Pollanen et al., 1997). Diatom analysis may be the only way to determine death by drowning after attempted resuscitation or post-mortem mutilation (Timperman, 1972). Lungs are excluded from analysis if the chest has been torn open and lungs have contacted water. Hendey (1973) and Pollanen et al. (1997), suggest that analysis of bone marrow from intact long bones like the femur are preferred. The absence of diatoms does not eliminate drowning as the cause of death, as it is possible that drowning may have occurred in water containing few or no diatoms (Pachar & Cameron, 1993; Peabody, 1977).

To solve this issue, minimum quantitative thresholds have been established and more research has been done regarding diatom density in different body tissues. Pachar and Cameron, (1993), established minimum thresholds for diatoms within a victim's organs and tissues to diagnose death by drowning, only making positive diagnoses when a significant difference existed between the number of diatoms in the lungs and those in closed organs such as bone marrows. In a study by Ago et al. (2011), drowning patterns were assessed based on diatom density in the drowning medium

suggesting that diatom concentrations will be the highest in the lungs and will decrease as the diatoms circulate into deeper organs such as the bone marrow. The higher the diatom concentration is in the drowning medium, the greater the likelihood of positive results (Ago et al., 2011; Antonenko & Ferris ,1987).

Weaknesses

Although there are many strengths with the diatom analysis technique, it is highly important to also discuss the various weaknesses. Diatoms can be introduced into the body through regular activities such as breathing, eating or drinking small diatom cells (Krstic et al., 2002). If exposure to materials containing diatoms occurs, the victim's organs cannot be used as evidence of drowning due to the pre-exposure (Taylor, 1994). Issues arise as marine diatoms can be well preserved in limestone, become aerosolized, and then inhaled, allowing them to enter the bloodstream (Moshkovitz et al., 1983). The lungs therefore are not good organs to analyse due to potential contamination (Horton et al., 2006; Pachar & Cameron, 1993). Inhalation however may be negligible (Antonenko & Ferris, 1987), as the body can naturally eliminate some foreign particles and prevent them from entering the organs (Pachar & Cameron, 1993). Thus further research is needed to analyse potential diatom build up in organs (Law & Jayaprakash, 2007).

Issues may arise however when victims are found in drowning mediums they were previously exposed to. Taylor (1994) notes a specific case where a victim was discovered in a body of water that they swam in every day for 15 years. Due to prior exposure, forensic scientists were unable to distinguish whether the victim last entered the water body alive or was environmentally predisposed to the same diatom species (Taylor, 1994). Further, little consensus exists regarding whether diatoms are accurate indicators of death by drowning, as diatoms can be found in swimmers or fishermen who were previously exposed to diatoms (Diaz-Palma et al., 2009). Thus, analysts must remain prudent in analysing victim records for previous diatom exposure events (Antonenko & Ferris, 1987).

Lab contamination can also impact findings in non-drowned victims as diatoms may be present in water used to wash lab instruments (Pollanen et al., 1997). Diatoms from instruments could mix with the bone marrow solution resulting in false positives (Pollanen et al., 1997). Lunetta et al. (2013) suggest that flasks and equipment be cleaned every 24 hours using sodium hydroxide and that old flasks with minor irregularities be replaced to prevent contamination, arguing that contamination is not an impediment once proper controls are implemented. Contamination however remains inevitable (Ago et al., 2011) although it can be mitigated through environmental specificity (Pollanen et al., 1997).

Diagnosing death by drowning is difficult in a body that has been severely dismembered or fragmented due to environmental exposure (Lunetta et al., 2013; Peabody, 1980). During decomposition, diatoms can passively enter soft tissues and organs (Hendey, 1973; Krstic et al., 2002), making the presence of diatoms in the lungs and stomach of potential cases of drowning inconclusive (Ago et al., 2011; Coelho et al., 2016). Large lacerations can also allow diatoms passive entry into the body, impacting the reliability of analysis (Hendey, 1973). As previously noted, diatoms can also passively enter airways through other means even when a victim did not drown (Bortolotti et al., 2011). Experts must therefore proceed with caution when interpreting diatom results from a decomposing body (Lunetta et al., 2013). Diatoms however are found in much higher concentrations in the bodies of drowning victims than of those disposed of in water post-mortem (Lunetta et al., 2013), and modern research techniques have been created to distinguish between true and false positives (Shen et al., 2019).

Rapid death can also prevent diatoms from circulating through the blood stream for a variety of reasons (Antonenko & Ferris, 1987; Piette & De Letter, 2006). Therefore, in some cases, other investigative methods must be used. Further, although finding high diatom concentrations may indicate voluntary water inhalation (Auer, 1991), lungs are not always a good organ for analysis as diatoms can passively enter them (Ago et al., 2011; Coelho et al., 2016; Pachar & Cameron, 1993; Timperman, 1972). In some cases, diatoms may only be found in the lungs and not other body tissues, meaning it would not be possible to definitively diagnose drowning as the cause of death (Bortolotti et al., 2011; Pollanen et al., 1997).

Horton et al. (2006) also suggest that it may be difficult to conclude if a death was a homicide or a natural drowning, as similar diatom species can be found in different water sources. For example, if a body was found in a lake that the city used for its water supply, it would be impossible to conclude whether the individual drowned in the lake or in a bathtub as the diatom species in either waterbody would not be distinct. Further, due to the low occurrence of positive tests, the likelihood of a false positive is relatively small as well, however this rate can be reduced through quantitative analysis (Ludes et al., 1999; Ludes, 2013; Lunetta et al., 2013; Pollanen et al, 1997). The establishment of a minimum threshold can help distinguish deaths by drowning due to much higher concentrations of diatoms often found in drowning victims (Shen et al., 2019). As Shen et al. (2019) found, significant statistical differences exist between false-positives and true cases of drowning lessening their impacts on analysis.

The main disadvantage of the diatom test however, is its sensitivity (Pollanen, 1998). The study by Pollanen et al. (1997) only obtained a positive result in 30% of freshwater drownings, meaning that diatom frustules will not be detectible in the majority of cases. As can be seen by the cited literature however, much of the original foundational analysis was completed in the 1970's, 80's, and 90's. It is therefore important to analyse the impact that modern techniques have had on diatom analysis as a technique to determine cause of death. Liu et al. (2020) note that the substantial technological changes in recent decades have led to the development of new techniques that have increased the accuracy and reliability of diatom analysis. These methods, as Carballeira et al. (2018) note, have helped further validate it in the forensic sciences.

Impact of modern research advances

Although the weaknesses of diatom analysis are significant, it can be seen that modern research from 2013 to present shows that the reliability of diatom analyses can improve with new techniques and that controversies surrounding it have been reduced.

Many digestion techniques have been developed to separate the diatoms from organs.

Analysis can be completed with a much higher sensitivity using modern microwave digestion techniques, vacuum filtration, and

high-resolution electronic microscopy (Hu et al., 2013). Fucci et al. (2015) also found that using hydrogen peroxide led to the discovery of nine new diatom species compared to the traditional hydrochloric acid method. Zhao et al. (2017) developed a new microwave digestion technique using scanning electron microscopy which led to a 97% positive test rate when evaluating closed organs (liver and kidneys). This was a was an approximate 70% increase from the 27% positive test rate from Pollanen et al. (1997), showing that reliability of diatom analysis has increased substantially with the development of new techniques which produce more accurate and reliable results.

Researchers have also found ways to reduce issues surrounding false positive results which is the greatest threat to diatom analysis reliability (Shen et al., 2019). Shen et al. (2019) found that statistical differences in diatom concentrations in bodily tissues existed between cases of true drowning and false positive results. Although diatoms can be found in victims who did not drown due to passive entry, the number of diatoms found in all organs are significantly greater in cases where victims were known to have drowned (Shen et al., 2019). This new method developed by Shen et al. (2019) shows that parameters can be set to allow forensic pathologists to distinguish between true and false positive cases and to better determine when false positive results emerge. Research by Li et al. (2019) further highlights the use of the ratio of diatom concentration in the lung tissues to the diatom concentration of the drowning medium to increase reliability.

To reduce the impact of contamination, Seo et al. (2013), used DNA-binding properties to isolate the diatoms from the heart blood sample after chemical digestion, which allowed for the separation of diatoms from other impurities, easing detection of diatoms. Seo et al. (2014) also developed a technique using silica-coated magnetic beads to separate diatoms from contaminants which showed significant improvements over previous methods. Zhou et al. (2019) also discovered that artificial intelligence (AI) could be used to automatically identify diatoms on a slide which increases the efficiency of analysis. As diatom analysis remains "time consuming and burdensome" (Zhou et al., 2019, p. 1), the discovery of the reliable use of AI as a rapid and objective method is encouraging to the forensic sciences. Zhou et al. (2019) note that additional research is still necessary to further explore the use of AI. Kakizaki et al. (2019) also worked to develop a new diatom digestion method using papain – a vegetable enzyme from papaya – instead of the more laborious and hazardous traditional acid-digestion method. They noted that this method was promising and that additional development should be undertaken. These studies show that additional research can further refine diatom analysis methods so external factors no longer present grave impediments.

CONCLUSIONS

Although the reliability of diatom analysis has been questioned due to the prevalence of false positive results and past issues with sensitivity in low diatom concentrations, it can be seen that modern research and laboratory techniques have addressed many of the existing concerns (Shen et al., 2019). Modern research has helped strengthen diatom analysis and it remains an important and necessary forensic tool, as diagnosing death by drowning can be highly complex. Along with its existing strengths, which include the presence of diatoms in almost every drowning medium, environmental specificity, seasonal variability, and significant quantitative differences between drowned and non-drowned victims, the reliability of diatom analysis continues to increase due to the use of modern research techniques and additional research. Since the foundational research of the 1970's and 80's, many significant developments have improved the reliability of diatom analysis and reduced many of its grave weaknesses including the ability to better distinguish between true positive and false positive results. As diatom analysis remains one of the only definitive ways to determine death by drowning due to the lack of alternative methods, the literature shows that additional refinement and research can continue to improve the technique, making analysis easier, less hazardous, and less time consuming, so that forensic pathologists continue to have this tool at their disposal and that its important role in forensic sciences can continue.

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ApoA-I deficiency enhances acute inflammatory responses after experimental Traumatic Brain Injury

Honor Cheung [1] Wai Hang Cheng [1 2], Emily B. Button [1 2], Asma Bashir [1 2], Carlos J. Barron [1 2], Anna Wilkinson [1 2], Cheryl L. Wellington [1 2]

[1] University of British Columbia, Vancouver, British Columbia, [2] Djavad Mowafaghian Centre for Brain Health, Vancouver, British Columbia

Abstract Cerebral vascular injury is a common phenomenon after traumatic brain injury (TBI), with complications including vascular inflammation, decreased cerebral blood flow, and increased vessel tortuosity. Promoting cerebrovascular health may therefore be a useful therapeutic intervention after TBI. ApoA-I, the major protein constituent of circulating high-density lipoproteins (HDL) are an attractive target due to its vasoprotective and anti-inflammatory roles in periphery vessels, but little is known on whether these benefits extend to the brain. To address this gap in knowledge, this study was designed to test the novel hypothesis that ApoA-I deficiency would worsen acute vascular and inflammatory outcomes in mice after Closed-Head Impact Model of Engineered Rotational Acceleration (CHIMERA) TBI. ApoA-I Knockout (ApoA-I KO) and WT mice underwent single moderate-severe TBI. Histopathological outcomes at 6hr and 2 days (2D) post-injury were assessed. ApoA-I KO mice exhibited greater Intercellular Adhesion Molecule 1 (ICAM-1), a marker for vascular permeability, in the cortex at 2D post-TBI compared to WT controls, and a subtle increase in brain pro-inflammatory cytokine levels. These results suggest the role of ApoA-I in protecting against TBI induced inflammation.

INTRODUCTION

Cerebral vascular injury is a nearly universal event in traumatic brain injury (TBI), where a functional deficit of the neurovascular unit (NVU) is sustained either directly due to the initial physical injury or occurs over time as part of the following secondary inflammation cascade. (Kenney et al., 2016). The NVU is comprised of endothelial cells, basal lamina covered by smooth muscle cells (replaced by pericytes in capillaries), astrocytes, neurons, and the extracellular matrix, which interact as a major conduit in maintaining central nervous system (CNS) homeostasis, among other functions (Salehi, Zhang, & Obenaus, 2017; Kenney et al., 2016; Muoio, Persson, and Sendeski, 2014).

In particular, damage to the cerebral vasculature often results in neuroinflammation (Schimmel, Acosta, & Lozano, 2017; Elder et al., 2015). Damage to the vasculature can alter the extracellular matrix, promoting the infiltration of peripheral leukocytes into the CNS and activating resident glial cells, including astrocytes and microglia (George & Geller, 2018). Chronic activation of glial cells in the brain contribute to elevated pro-inflammatory cytokine levels, including IL-6 and TNF-α (Schimmel, Acosta, & Lozano, 2017; Elder et al., 2015), and secondary cell death in the cascade of TBI sequelae (Schimmel, Acosta, & Lozano, 2017). These elevated pro-inflammatory cytokines in turn lead to an increased expression of adhesion factors, such as ICAM-1 in astrocytes, microglia and endothelial cells alike, which enables increased leukocyte binding to the endothelium. The increase in leukocyte binding lead back to increased efflux of immune cells from the blood vessels to the brain parenchyma, completing the vicious cycle of transendothelial leukocyte extravasation and pro-longed neuroinflammation (Lutton et al., 2017; Balabanov et al., 2001; McKeating & Andrews, 1998; Shrikant et al., 1995).

The strong association of vascular injury with TBI has prompted an exploration into the linkage between general vascular health and injury outcome. High-density lipoprotein (HDL) has well -defined vasoprotective effects in peripheral blood vessels outside of the brain , with low plasma levels of HDL firmly established as a predictor for cardiovascular disease (Besler, Lüscher, & Landmesser, 2012; Mahdy Ali, Wonnerth, Huber, & Wojta, 2012). ApoA-I, the major protein component of circulating HDL in plasma, has been shown to reduce inflammation

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Address correspondence to cjur.ca

through the inhibition of macrophage chemotaxis and reduced monocyte recruitment from the circulation to the tissue (Iqbal et al., 2016; Yin et al., 2011). It does this by the suppression of adhesion molecules expressed on endothelial cells (Calabresi et al., 1997). In addition, it promotes endothelial nitric oxide (NO) synthase activity, thereby reducing inflammation via downregulation of the pro-inflammatory NFκB signalling pathway (Boyce, Button, Soo, & Wellington, 2017).

ApoA-I is produced by hepatocytes and enterocytes in the liver and small intestines respectively. Although ApoA-I mRNA is not expressed in the brain (Elliott, Weickert, & Garner, 2010), its protein is detected in the brain and in cerebrospinal fluid (CSF). While it is unclear whether the protective effects of peripheral ApoA-I is shared in the brain, recent evidence supports the role of HDL in reducing neuroinflammation in mice models. This includes the finding of increased vascular ICAM-1 expression as well as increased vessel-associated astrogliosis among ApoA-I KOmice when compared to mice hemizygous for ApoA-I (Button et al., 2019).

While these results suggest that peripheral ApoA-I may also exhibit vasoprotective properties in the CNS, whether ApoA-I can offer protection from TBI-induced brain changes has not been tested. To investigate the protective role of ApoA-I, we utilized a previously published CHIMERA (Closed-Head Impact Model of Engineered Rotational Acceleration) model of TBI, developed by our group, (Cheng et al., 2019; Vonder Haar et al., 2019; Namjoshi et al., 2014, Bashir et al.,2020) to induce a closed-head, surgery-free and clinically relevant TBI to ApoA-I Knockout (ApoA-I KO) and wild type (WT) mice. In this study, we tested the novel hypothesis that ApoA-I KO would worsen acute vascular and inflammatory outcomes in mice after CHIMERA TBI as indicated by increased pro-inflammatory brain cytokine levels and vascular adhesion factors. Overall, this study helps shed light on the function of ApoA-I in the brain and by extension, uncovering HDL as a potential therapeutic in improving post TBI inflammatory outcomes.

METHODS

Animals and chimera procedure

All procedures involving animals were approved by the Canadian Council of Animal Care and the University of British Columbia Animal Care Committee. Male and female ApoA-I KO [B6.129P2-Apoa1tm1Unc/J] and WT [C57BL/6J] mice (total N=64) were purchased from The Jackson Laboratory. Animals were 3.5-5 months of age at the time of impact and were randomized into sham or TBI groups of 6hr or 2D endpoints. The TBI group received an impact energy of 2.5 J whereas the sham group received anesthesia and restraint but no impact. A schematic of the experimental timeline is shown in Figure 1. The CHIMERA model was used to induce TBI as described previously (Bashir et al.,2020). Two ApoA-I KO mice (10% of injury group) died right after CHIMERA procedures due to apnea and no WT animals expired prematurely. The groupings for all animals kept for final analysis were: WT-6hr (Sham:N=6 (3 Male, 3 Female), TBI:N=8 (5 Male, 3 Female)); WT-2D (Sham:N=6 (3 Male, 3 Female), TBI:N=7 (4 Male, 3 Female)); ApoA-I KO-6hr (Sham:N=7 (3 Male, 4 Female), TBI:N=9 (5 Male, 4 Female)); ApoA-I KO-2D (Sham:N=8 (4 Male, 4 Female), TBI:N=11(5 Male, 6 Female)) respectively.

Tissue collection

Mice were fasted for 4 hours prior to anesthetization by intraperitoneal injection of 20 mg/kg xylazine (Bayer, Whippany, NJ, USA) and 150 mg/kg ketamine (Zoetis, Florham Park, NJ, USA). Once a surgical plane of anesthesia was reached, mice were perfused for 7 min with ice-cold phosphate-buffered saline (PBS) containing 2500 U/L heparin at 6 mL/min. Brains were excised and bisected in the sagittal plane. Half of the brain used for biochemical analysis was snap-frozen on dry ice and stored at −80°C until use. The remaining half-brain was fixed in 4% paraformaldehyde (PFA) for 2 days at 4°C followed by cryoprotection in 30% sucrose and 0.1% sodium azide at 4°C, after which 20-µm-thick coronal sections were cut using a cryotome (Leica Microsystems, Buffalo Grove, IL, USA).

Cytokine ELISA

Half-brain lysates were homogenized in 8-volumes of ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer containing protease and phosphatase inhibitor cocktails (Roche, Branford, CT), and centrifuged at 9000 rpm for 10 min at 4 °C. The supernatant was extracted and frozen at −80 °C until analysis. Inflammatory cytokines interleukin 6 (IL-6), interleukin-12 (1L-12), keratinocyte chemoattractant/ human growth-regulated oncogene (KC/GRO), tumour necrosis factor α (TNF-α) levels were measured in RIPA lysates using MesoScale Discovery ELISA kit (K15048G, MesoScale Discovery, 1:2) according to the manufacturer's instructions. Data points were interpolated from a standard curve and normalized to total soluble protein concentration measured by bicinchoninic acid (BCA) assay (Thermofisher, 23225) per the manufacturer's instructions.

Histology

Immunofluorescence microscopy was used to visualize intercellular adhesion molecules (ICAM-1) and their colocalization with endothelial cells (CD31). One section from the dorsal hippocampus and one section from the ventral hippocampus were selected from each cyrosectioned half-brain, which were mounted on Superfrost Plus slides. Antigen retrieval was performed with citrate buffer followed by washing with PBS, permeabilizing tissue with Triton, and blocking with 5% donkey serum, 1% BSA and 0.3% Triton X-100 in

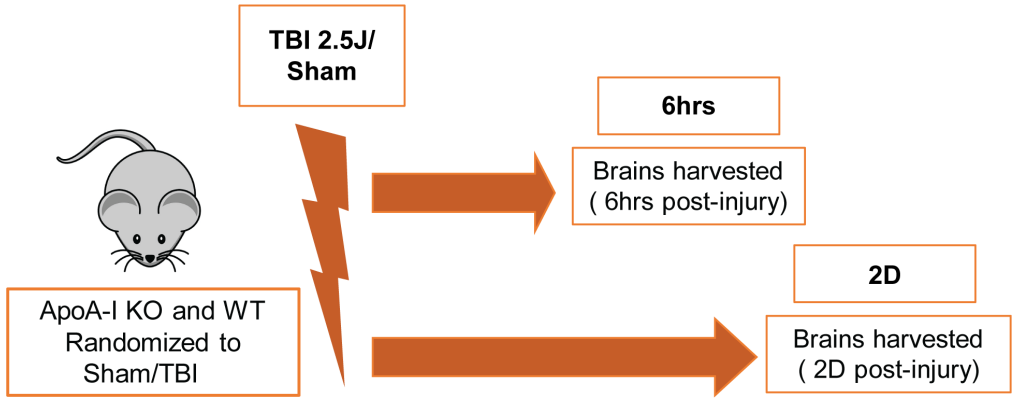

Fig. 1 Experimental Timeline. ApoA-I KO and WT male and female animals underwent a single moderate-severe TBI at 2.5J. Brains from animals were harvested at 6hrs or 2 days (2D) post TBI for histological and cytokine analysis.

PBS for 60 minutes. Primary antibodies, ICAM-1 (R&D, AF796, 1:50) and CD31 (Abcam,ab28364, 1:200) were incubated overnight at 4°C, followed by 3x phosphate buffered saline (PBS) washing steps. Next, incubation with secondary antibodies donkey anti-goat Alexa 594 (Invitrogen, A-11058, 1:400) and donkey anti-rabbit Alexa 647 (Invitrogen, A-31573, 1:400), for ICAM-1 and CD31, respectively at room temperature for 2 hours. The samples were then washed 3x in PBS, cover-slipped and mounted onto slides in Prolong Gold Antifade with DAPI (Invitrogen, P36931). The slides were stored at 4°C until imaging with an Axio Scan.Z1 (Zeiss) slide scanner.

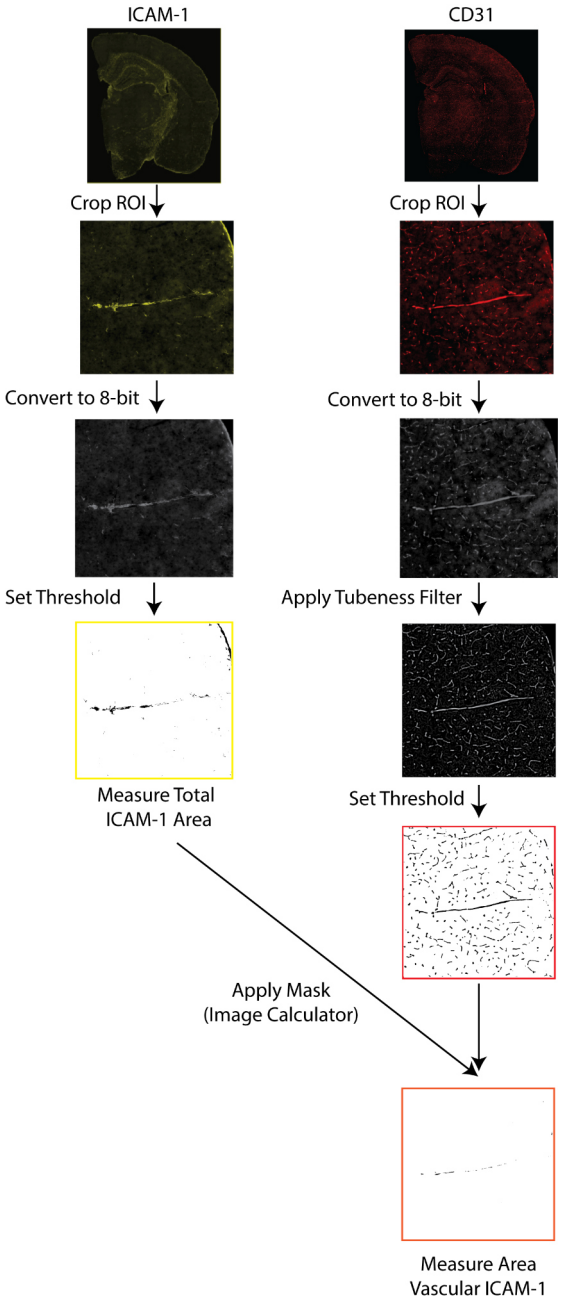

Fig. 2 Schematic diagram of quantification methods used for vascular immunopositivity. Stained coronal brain sections were scanned in respective channels, and ROIs were cropped and converted to 8-bit. The GFAP channel was thresholded to remove background signals, while the ImageJ Tubeness filter was applied to the CD31 channel to isolate the vasculature. Finally, the filtered images were combined by the ImageCalculator function to select the overlapping signals using the "AND" command.

Image analysis

Entire coronal sections were imaged with Zeiss Axio Scan.Z1 (Carl Zeiss Microscopy, Thornwood, NY, USA) at 20× magnification, using fluorescent (ICAM/CD31) imaging. Downstream image analysis was performed with ImageJ (NIH)

A schematic of ICAM-1/CD31 quantification is illustrated in Figure 2. Exported images for each channel were converted to 8-bit black and white images. Cortical regions in the anterior hippocampus and the entorhinal cortex from the posterior hippocampus were drawn manually and saved as regions of interest (ROI) for each sample, using the Allen Mouse Brain Atlas as a guide. ICAM-1 signal was quantified by thresholding and reporting the percentage area containing signal relative to the total cortical area after filtering a background noise <15 pixels. Stained vessels from the CD31 channel were selected with the ImageJ macro Tubeness. The outputs of Tubeness were thresholded and masked, after filtering of background noise <100 pixels, CD31 percentage area was normalised to selected ROI areas. Vascular ICAM-1 was quantified as the ICAM-1 positive area associated with CD31 in each region where masks of the segmented CD31 image for a section were applied to the segmented ICAM-1 image of the same section, using the ImageJ macro ImageCalculator. The ICAM-1 positive area within the CD31 mask was measured then normalized to the total CD31-positive area within the ROI.

Statistics

All animal groupings were blinded during analysis by using surrogate identifying codes. Statistical analyses were performed using IBM SPSS Statistics version 23 software (IBM, Armonk, NY, USA), and graphs were plotted using Prism version 6.07 software (GraphPad Software, La Jolla, CA, USA). For the analysis of histological data, 3-way ANOVA was used considering ApoA-I genotype as one factor, injury status as another factor and timepoint of injury as the third factor, followed by Sidak's multiple comparisons test if significant factor or interaction effects were observed. For inflammatory cytokine assays, Kruskal-Wallis non-parametric test was used to detect overall effect, followed by Bonferroni corrected Mann-Whitney test to detect meaningful between-group differences.

RESULTS

ApoA-I deficiency enhances the acute inflammatory response after CHIMERA TBI

To assess differences in inflammatory status in brain tissue after TBI, we examined cytokine levels in RIPA brain lysates via Mesoscale cytokine ELISA. IL-6 and TNF-α are recognized markers of acute inflammation following TBI, and are produced by activated immune cells. KC/GRO is involved in neutrophil activation and subsequent activation of cells of the innate immune response. IL-12p70 is produced by dendritic cells, macrophages and neutrophils, and is involved in the activation of naïve T cells to activated T cells. We observed a significant overall effect in all four cytokines by Kruskal-Wallis analysis. Further, Bonferroni adjusted Mann–Whitney post-hoc analysis revealed a significant increase in IL-6 levels for both WT ($p=0.002$) and ApoA-I KO mice ($p=0.008$) at 6hr post TBI (Figure 3b). A trend for a significant injury driven increase was observed for WT TBI animals at 6hrs for KC/GRO, IL-12p70 and TNF-α ($p=0.056$), while no significant increase in measured cytokine levels was observed for ApoA-I KO animals at 6hrs (Figure 3a,c,d).

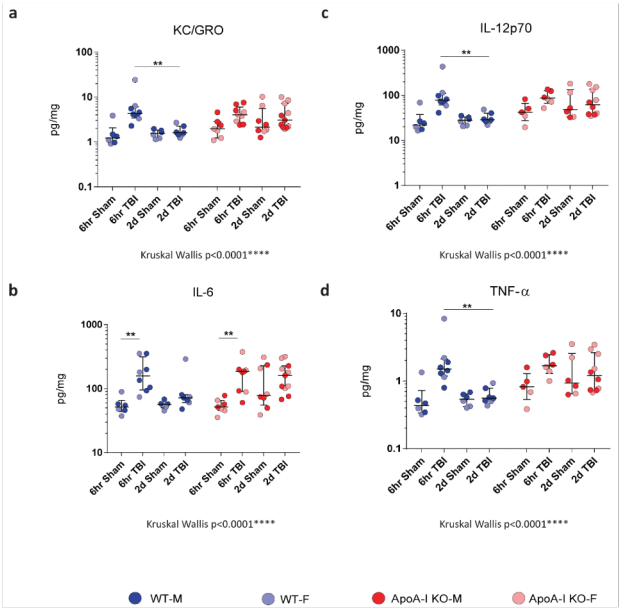

Fig. 3 Altered cytokine profiles in ApoA-I KO animals. Inflammatory cytokine levels were assessed by ELISA at 6hr and 2 day (2D) timepoints post-TBI. Cytokine analysis of (a)KC/GRO, (b)IL-6, (c)IL-12p70, (d)TNF-α levels in half brain lysates normalised to protein concentrations respectively. For KC/GRO and IL-6, Cohort Size: WT-6hr (Sham:N=6, TBI:N=8); WT-2D(Sham:N=6 , TBI:N=7); ApoA-I KO-6hr (Sham:N=7, TBI:N=9); ApoA-I KO-2D (Sham:N=8 , TBI:N=11). For IL-12p70 and TNF-a, Cohort Size: WT-6hr (Sham:N=6, TBI:N=8); WT-2D(Sham:N=6 , TBI:N=7); ApoA-I KO-6hr (Sham:N=5, TBI:N=6); ApoA-I KO-2D (Sham:N=6, TBI:N=10). In all graphs, error bars indicate Median± IQR, y-axis are in log base 10 scale. Light coloured circles indicate female animals and dark coloured circles indicate male animals. All data are analyzed by Kruskal-Wallis test for overall significance, followed by Bonferroni corrected Mann-Whitney U post-hoc analysis. * indicate a significant post-hoc injury effect within a specific timepoint and genotype or a significant genotype difference within a specific timepoint and injury group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

There was a significant decrease in cytokine levels between the WT 6hr TBI group and WT 2D TBI group (p=0.007) for KC/GRO, IL-12p70 and TNF-α (Figure 3a,c,d). No difference of pro-inflammatory cytokine levels between the WT sham and WT 2D TBI group could be detected (Figure 3a,c,d), suggesting that the inflammatory response was resolved by 2D in WT TBI mice. Intriguingly, the same pattern was not observed in ApoA-I KO mice as cytokine levels remained elevated between the 6hr TBI and 2D TBI group (p=0.456, p=1.000, p=0.368, p=0.368 for KC/GRO, IL-6, IL-12p70, and TNF-α respectively (Figure 3a-d). Of note, data points for pro-inflammatory cytokine levels in ApoA-I KO animals were higher and spread over a wider range compared to WT animals regardless of injury status (Figure 3a-d), suggesting a higher baseline cytokine level relative to WT. In addition, while no significant increase was observed in comparing the ApoA-I KO 2D TBI against the WT 2D TBI groups, there was a trend towards significance for KC/GRO (p=0.073), IL-12p70 (p=0.085), TNF-α (p=0.085) respectively (Figure 3a, c, d).

Taken together, while is it clear that WT animals experienced an initial heightened inflammation at 6hr post-injury, this response was mostly attenuated at 2D. This temporal injury-recovery pattern, however, does not appear to be similar in ApoA-I KO animals.

ApoA-I deficiency increases injury driven total ICAM-1 positive area at the cortex at 2D post -TBI

To better analyse specific brain areas affected by TBI, we examined the presence of inflammatory markers through immunofluorescence. ICAM-1 is an adhesion molecule produced by endothelial cells, astrocytes and microglia, and is a prominent marker for vascular inflammation as it promotes leukocyte adhesion to endothelial cells. We quantified the ICAM-1 signal in the entire cerebral cortex, spanning the retrosplenial area to the auditory areas where blood vessels are abundant, and observed a significant injury × genotype × timepoint effect (p=0.035) in total ICAM-1 area by 3-way ANOVA (Figure 4a, c). Sidak adjusted post-hoc analysis revealed increased percentage stained total ICAM-1 between the 2D Sham ApoA-I KO and 2D TBI ApoA-I KO animals (p=0.001), from a mean of 0.343 to a mean of 0.981, while levels in WT animals remained consistent regardless of injury status or timepoint (p=0.863 for 6hr Sham WT vs 6hr TBI WT, p=0.935 for 2D Sham WT vs 2D TBI WT) (Figure 4c). In addition, although main group differences were not significant for vascular associated ICAM-1 (Figure 4c), there was a trend of increased vessel-associated ICAM-1 in 2D TBI ApoA-I KO animals compared to other groups. This finding demonstrated that, compared to WT animals, ApoA-I KO animals with TBI had a stronger induction of cortical ICAM-1, suggestive of a more pronounced inflammatory status. However, specific examination of the entorhinal cortex revealed no significant injury or genotype differences for both total ICAM-1 and vascular associated ICAM-1 in this region (Figure 4d). Together, these data suggest the potential for ApoA-I deficiency to exacerbate TBI-induced inflammation at the cerebral cortex at 2D, although more studies are needed to validate this preliminary observation.

DISCUSSION

This is an exploratory study of the potential role of ApoA-I in acute responses to TBI, with a pre-determined focus on cerebrovascular outcomes as ApoA-I is implicated in vascular contributions to Alzheimer's Disease (AD).

Our results provide proof-of-concept data that ApoA-I deficiency may alter the acute inflammatory response after moderate CHIMERA TBI. We observed a significant increase of cortical ICAM-1 total expression at 2D post-TBI for ApoA-I KO animals only (Figure 4), and an overall increase in cytokine levels independent of injury or timepoint status for ApoA-I KO animals (Figure 3). An increase in total ICAM-1 immunofluorescence was aligned with elevated cytokine levels observed in ApoA-I KO animals as cytokines can lead to endothelial activation, resulting in an increase in expression of adhesion factors (Lutton et al., 2017; Balabanov et al., 2001; McKeating & Andrews, 1998). However, histological data suggest a highly regional-specific difference, as the increase in ICAM-1 expression was only observed in the cerebral cortex at the very acute timepoints studied, though this may be due to how the CHIMERA impact is made beneath the cerebral cortex. Since no baseline differences in ICAM-1 staining between ApoA-I KO and WT animals were observed in this study (Figure 4), ApoA-I deficiency may have a role in the inflammatory response during the recovery process after injury. Supporting this conclusion is the observation that ApoA-I KO animals did not follow the typical injury-recovery temporal response observed in WT animals (Figure 3). Notably, data points for cytokine levels in ApoA-I KO animals were spread over a wider range compared to WT animals regardless of injury status (Figure 3). This may imply that the higher variation in the ApoA-I KO group hindered detection of the cellular response to injury and subsequent recovery. Importantly, these results will need to be confirmed or refuted in future studies with additional animal numbers to provide more statistical power.

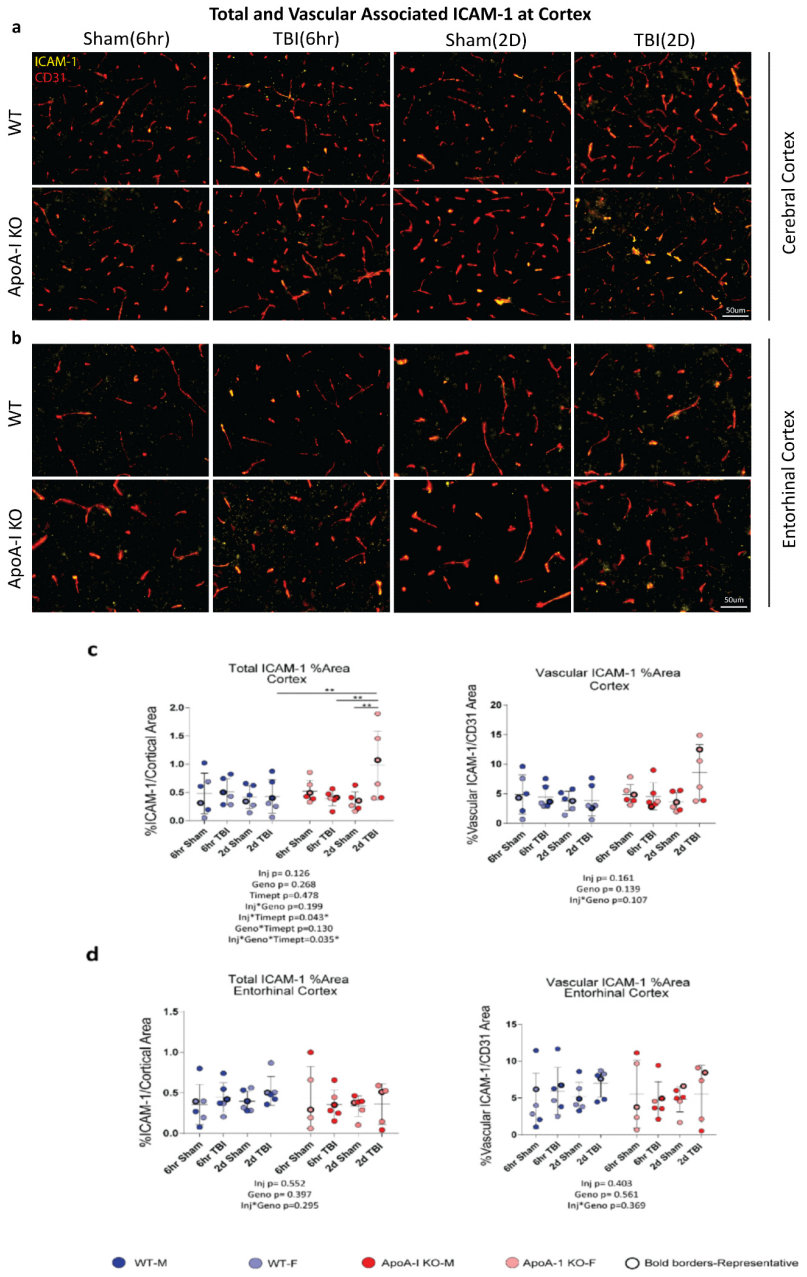

Fig. 4 TBI Induced increased Total ICAM-1 expression at the cerebral cortex in ApoA-I KO animals at 2D post Injury. ICAM-1 expression in the brain vasculature was assessed by ICAM-1 and CD31 co-staining at 6hr and 2D post-TBI. (a)(b) Coronal sections showing total ICAM-1 positive signal and CD31 associated ICAM-1 signal in the cortex of the anterior hippocampus section and entorhinal cortex respectively (c)(d) Quantitative analysis of ICAM-1 staining at the Cortex of the anterior hippocampus and entorhinal cortex respectively. Stained images were quantified by calculating the % of the region of interest (ROI) that were ICAM-1 positive. Vascular ICAM-1 was reported as the ea of ICAM-1 directly overlapping with CD31 signal, normalised to CD31 Area within ROI. Cohort Size: N=6 for all groups, except for the ApoA-I KO-6hr Sham group and the ApoA-I KO-2D TBI group in the entorhinal cortex where N=5. Circles with bold borders in (c)(d) correspond to the representative images shown in (a)(b). Data in (c)(d) were analyzed by 3-Way ANOVA, followed by Sidak post-hoc test. In (c), * indicate a significant post-hoc injury effect within a specific timepoint and genotype, a significant timepoint effect within a specific genotype and injury status, or a significant genotype effect within a specific timepoint and injury status (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

This study agrees with previous results published using moderate CHIMERA, in that cytokine elevation was observed at 6hr only for WT animals, and that histological evidence indicative of inflammation was most robust at 2D post TBI (Bashir et al.,2020).

The most surprising finding was that ApoA-I deficiency appeared to affect grey matter more robustly than the vasculature, as we observed an increase in total cortical ICAM-1 rather than CD31(vascular) associated ICAM-1 following TBI. This result is contradictory to what our group has observed in APP/PS1 mice, a common Alzheimer's Disease (AD) animal model, where APP/PS1 mice deficient in ApoA-I had increased vascular ICAM-1 adhesion as well as increased vessel-associated astrogliosis when compared to APP/PS1 WT animals (Button et al., 2019). This may be due to the fact that APP/PS1 mice express and accumulate A® in the brain parenchyma and in cerebral vessels which triggers the production of increased ICAM-1 in the endothelial cells (Verbeek et al., 1994). Indeed, ICAM-1 is expressed in astrocytes, microglia and endothelial cells alike (Dietrich, 2002). Our results may imply that ApoA-I deficiency enhances ICAM-1 expression more robustly in glial cells rather than in endothelial cells following TBI in a WT mice background, which we used in this study. It will be important to determine whether ApoA-I deficiency and the presence of A® together exacerbates TBI-related pathologies, especially given the interest in understanding TBI in the vulnerable elderly population (Thompson et al., 2006; Haring et al., 2015).

This study has several limitations. We only examined very acute time points of 6hr and 2D.Future studies are encouraged to include post-acute and chronic time points up to 6 months after injury, when potential secondary damage to endothelial cells (Villalba et al., 2017; Andrews, Lutton, Merkel, Razmpour, & Ramirez, 2016; Prakash & Carmichael, 2015) may enable effects of ApoA-I deficiency to be more easily observed. Further, more time points are needed to investigate the extent of modulated cytokine response that we have observed in 2D TBI ApoA-I KO animals, as it is unclear whether this response will persist beyond the 2D timeframe (Figure 3).

Despite these limitations, the study has several strengths, including the a priori blinding strategy used throughout. Based on the inflammatory changes presented thus far, future studies are encouraged to explore the mechanistic relationship between ApoA-I and neuroinflammation further, for example by supplementing ApoA-I KO TBI mice with HDL. To further characterize inflammatory outcomes observed in ApoA-I KO TBI mice, follow up work can be done to uncover transcriptomic changes induced by ApoA-I KO in the brain by techniques such as Single-Cell RNA-Seq (scRNAseq), which would help identify widespread changes in the brain beyond the capabilities of standard histology. Although this work is preliminary, our study shows that ApoA-I deficicency enhances acute inflammatory responses post a single CHIMERA TBI.The results from this study raises the possibility that ApoA-I supplementation may be of therapeutic value in TBI patients.,and shows how additional research in this area would be beneficial in advancing TBI treatment.

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HC carried out all TBI procedures, processed tissue samples, performed histology and analysed all data. WHC, CJB and AB provided technical support for TBI procedures. AW provided technical support for processing biochemistry samples. Cytokine ELISA was carried out by EB. The manuscript was written by HC and critically reviewed by WHC and CLW.

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Effects of antibiotics on colonic neurons of the myenteric plexus in wild *Peromyscus* mice

Jessica Williams Vergara [1]

[1] Trent University, Peterborough, Ontario

Abstract A community of trillions of commensal bacteria inhabit the gastrointestinal tract, collectively known as the intestinal microbiota. The gut microbes are essential for the development and functioning of the enteric nervous system. Approximately two-thirds of the cell bodies of all enteric neurons are gathered in the myenteric plexus, an intricate network of neurons and glial cells that primarily regulate gut neuromuscular activity. Studies in laboratory mice have observed that antibiotic treatment leads to a reduction in microbial abundance and diversity within the intestine, and these findings are correlated with enteric nervous system structural abnormalities. Specifically, antibiotic-treated mice have an abnormal myenteric plexus, which is characterized by a reduction in myenteric neuron numbers and ganglia area. However, it is unknown whether these effects occur in wild *Peromyscus* mice that are exposed to a natural bacterial flora. The goal of this study was to evaluate the effects of antibiotic exposure on the colonic neurons of the myenteric plexus in wild *Peromyscus* mice. Thirty-two wild-caught adult male *Peromyscus* mice were divided into control and antibiotic-treated groups. Whole mount preparations of longitudinal muscle with adherent myenteric plexus were prepared and alterations in colonic neuron and ganglia numbers were assessed by immunohistochemistry analysis. Antibiotic-treatment reduced the total number of colonic enteric neurons/mm2 and the total number of ganglia per myenteric plexus. Our results suggest that antibiotic-induced microbial dysbiosis affects the colonic neurons and ganglia of wild *Peromyscus* mice similarly to laboratory mice. We showed that the antibiotic-driven changes in neuronal density and ganglia arrangement are inducible in wild *Peromyscus* species that are exposed to real world bacteria.

INTRODUCTION

The gastrointestinal (GI) tract is vital for the digestion of food, the absorption of nutrients and water, and pathogen protection (Heijtz et al., 2011, Obata & Pachnis, 2016, Karl et al., 2018). A community of trillions of commensal bacteria inhabit the GI tract, collectively known as the intestinal microbiota. The gut microbiota is an essential component of the GI tract, co-developing a mutualistic relationship with the host. While the host provides both nutrients and a hospitable environment, the microbiota confers many benefits within the gastrointestinal environment, such as metabolic homeostasis. In addition, the intestinal microbiota influences many aspects of host physiology beyond the GI environment itself, including the development and regulation of nervous system structure and activity (Heijtz et al., 2011, Obata & Pachnis, 2016, Karl et al., 2018).

Intestinal neuromuscular activity, secretory and vasomotor control, and gastric peristalsis are primarily controlled by the enteric nervous system (ENS); a major branch of the autonomic nervous system located throughout the entirety of the GI tract (Hyland & Cryan, 2016). This intricate neuronal system consists of millions of enteric nerve cells embedded within the GI tract. The cells bodies of the enteric neurons are gathered into 2 distinct plexi: the myenteric and submucosal plexus. The submucosal plexus is located underneath the submucosa and is fundamental to the regulation of mucosal functions. The myenteric plexus (MP) is located between the circular and longitudinal muscle layers of the gut and contains up to two-thirds of all enteric neurons. The neurons' primary function is to regulate GI neuromotor control (Hyland & Cryan, 2016).

The intestinal microbiota is essential to the proper functioning and integrity of the ENS. Multiple studies have observed structural and functional abnormalities of the ENS in response to microbial depletion (Kabouridis & Pachnis, 2015, Neufeld et al., 2015). The ENS can respond to the microbiota and its components via pattern recognition receptors, particularly toll-like receptors (TLRs). Specifically, TLRs recognize microbial derived components which may stim-

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Address correspondence to cjur.ca

ulate various downstream processes that could influence the structural and functional integrity of the ENS via TLR stimulation (Hyland & Cryan, 2016).

Environmental stressors can cause microbial dysbiosis, a shift in the composition of the microbial communities, which consequently alter the internal GI environment (Karl et al., 2018, Galley & Bailey, 2014). Using antibiotics is a common method to induce artificial microbial dysbiosis in laboratory settings as it produces a germ-free (GF) like phenotype, which allows for the exploration of the effects of the microbiota on ENS structure and function (Yoon & Yoon, 2018). Antibiotic administration leads to a reduction in gut flora diversity and abundance. These alterations in the microbial community may impact the interactions of the microbes with the intestinal environment, possibly altering GI function (Membrez et al., 2008, Vijay-Kumar et al., 2010). In particular, microbial dysbiosis from antibiotic treatment is correlated with macroscopic changes in the intestine and functional gut consequences, primarily GI dysmotility (Caputi et al., 2017, Cao et al., 2017). The functional deficiencies associated with microbial dysbiosis may be due to ENS structural alterations as observed by various studies in GF and antibiotic-treated laboratory mice (Caputi et al., 2017, Hyland & Cryan, 2016). Microbial depletion and dysbiosis are associated with alterations in the MP (Caputi et al., 2017, Collins et al., 2014). These studies highlight the importance of the intestinal microbiota in ENS structural and functional integrity.

In the present study, we sought to evaluate if these structural alterations in the ENS observed in antibiotic-treated laboratory mice translate to wild *Peromyscus* species. Wild mice are exposed to natural bacteria and thus have greater gut flora diversity in comparison with multi-generational laboratory mice that are kept in specific pathogen-free facilities for most of their lives (Wang et al., 2014). Conventional laboratory mice have limited translational research value because they lack real world gut microbe diversity, and so they may not faithfully mirror the physiology of wild species (Rosshart et al., 2019). However, wild mice populations contain natural microbiota and thus may better recapitulate free-living species, including humans (Rosshart et al., 2019).

The present study was designed to evaluate the effects of antibiotics on the colonic neurons of the MP in wild *Peromyscus* mice. It was hypothesized that antibiotic-induced dysbiosis of the gut microbiota would alter the number of colonic neurons within the MP. Specifically, it was predicted that microbial dysbiosis will reduce the number of colonic neurons, which is consistent with findings in laboratory mice.

METHODS

Trapping

Wild *Peromyscus* species were trapped in deciduous forests and abandoned barns around Trent University. The mice were sexed. Adult males were individually housed in a thermoneutral and humidity-controlled room.

Experimental design

The mice were housed for a minimum of 7 days and weighed approximately 18g before being separated into treatment groups. They were separated into two primary treatment groups: Antibiotics (n=20) and Control (n=20). Antibiotic treated mice were given broad spectrum antibiotics (0.5g/L Neomycin Sigma N1876, Lot: WXBB7516V and 1.0g/L Ampicillin Sodium salt Sigma A9518, Lot: 085M4953V) administered through their drinking water and replenished weekly. The experiment duration ran from weeks 0 to 6 monitoring body weight, food intake, and water intake were measured weekly as previously described (Garnett, 2016). Fecal samples were collected from the cages at weeks 1 and 3. At week 6, the mice were euthanized.

Intestinal dissection and preparation for immunohistochemistry

The intestine was removed starting at the lower esophageal sphincter to the rectum. The large intestine segment was separated by cutting the tissue 1-inch below the cecum. The large intestine segments were cut longitudinally at the mesentery, stretched, and pinned out on sylgard resin plates and fixed with 4% paraformaldehyde (replaced with PBS). Using a dissection microscope (LEICA ES2 Stereo Microscope), whole mount preparations consisting of the longitudinal muscle with the myenteric plexus attached (LMMPs) were prepared by carefully removing the mucosal, submucosal, and circular muscle layers. Whole mount preparations were all prepared by the same person to reduce variability.

Cecum content culture

Alterations in microbiota were determined through culturing cecum content as previously described (Garnett, 2016). The contents of the cecum were collected during dissection; 0.04g were placed into 1mL nutrient buffer (1g/50mL), mixed, and placed in a 4°C fridge overnight. Cecal contents were placed at room temperature 2 hours prior to culturing. Culturing was completed on both nutrient agar (Thermo Fisher Scientific, lot: 1800844, 0.028g/mL) and blood agar (0.028g/mL nutrient agar with 0.05mL of sheep's blood per milliliter agar lot: 332503) (Chevelier et al. 2015). 20µL of the nutrient buffer with cecum content solution was placed on each plate. Nutrient buffer with no cecum content added was used as a control sample. The nutrient agar plates were kept in an aerobic chamber at 37°C and the blood agar plates were kept in an anerobic chamber at 37°C. All plates were left in the chamber for 48 hours before recording the approximate abundance of all colonies (Garnett, 2016).

Immunohistochemistry on colonic whole-mount preparations

The whole-mount preparations of LMMP were blocked with PBS-Triton with 4% 100 level blocking serum (Stanbio, Lot: 14860) for 2 hours with gentle shaking every 15 – 20 minutes to reduce non-specific binding of the primary antibody. Next, the LMMP was incubated overnight (21 hours) at 4°C in a 1:1000 concentration of primary antibody HuR (6A97) monoclonal Mouse IgG (Santa Cruz Biotechnology, 71290, lot: C0713) diluted in PBS-Triton with 4% 100 level blocking serum. The primary antibody HuR is a pan-neuronal marker that is specific for HuR, an RNA-binding protein that is found within the nucleus and cytoplasm of all neurons (Phillips, Hargrave, Rhodes, Zopt & Powley, 2003). Following primary antibody incubation, the tissue samples were rinsed sequentially in PBS 3 times for 5 minutes each to remove unbound primary antibody. The tissue samples were then incubated for 2 hours at room temperature in a 1:500 dilution of goat anti-mouse IgG-FITC secondary antibody (Santa Cruz Biotechnology, sc-2010, lot: G2314) in PBS-Triton with 4% 100 level blocking serum. After secondary incubation, the tissue samples were rinsed sequentially in PBS 3 times for 5 minutes each. LMMP samples were then mounted on glass slides using mounting medium (UltraCruz, Lot: HH0813) for

fluorescence microscopy (LEICA DFC350 FX, Leica application suite 2.4.1 build 6384).

Acquisition of images

Images were acquired with a LEICA DFC3650 FX CCD microscope camera using a LEICA DFC3550 FX epifluorescence microscope equipped with a high magnification 40x/1.4 objective and a low magnification 10x/0.22 objective. The IgG-FITC secondary antibody has an emission peak of 525mm (Santa Cruz Biotechnology). Before image acquisition and analysis, all tissue preparations were coded, and the investigator was blinded to the experimental conditions.

Images of each LMMP sample were acquired at both 10X magnification and 40X magnification. Regions of the LMMP with large tears or sections of MP that had been removed during dissection were omitted because they were deemed to be non-representative of the overall tissue. At 10X magnification, images were taken randomly throughout the tissue. These images would be used to determine neuron and ganglion numbers. At 40X magnification, the microscope camera was used to randomly focus on and take pictures of individual ganglions. These images would be used to determine the number of neurons within a single ganglion.

A pilot study was conducted to determine the minimum number of images that would need to be captured per tissue at each magnification to get a representative estimate of the total neuron and ganglia populations in each LMMP tissue. Four randomly selected tissues were chosen for the pilot study. We captured as many microscope fields as possible at both 10X and 40X magnification. Approximately 20 images were acquired per tissue at each magnification. The total number of myenteric neurons and ganglia were counted at both 10X and 40X magnification and expressed as a cumulative average. We plotted the cumulative averages from each tissue count and observed the trends until they reached a plateau. We estimated when we had imaged enough microscope fields when the cumulative average remained relatively flat without any upward or downwards trends.

We determined that we would need to capture 15 images per tissue at 10X magnification for total neuron counts (Figure S2). From these 15 images, we would need to use 10 images for total ganglia counts (Figure S3). We would need to capture 10 images of randomly selected individual ganglions at 40X magnification for total neuron counts per ganglion (Figure S1).

Data analysis

All images were imported into ImageJ to be counted using the "Cell Counting and Marking" plugin. This plugin automatically counts all manually labelled neurons. By labelling a neuron we ensured that each cell was only counted once. Neurons were defined as green fluorescent immunolabeled HuR-positive cells. Neurons bodies that were not completely within the microscope's field of view were only counted if more than 50% of their body was visible. In addition, ganglia were defined as clearly delineated groups of neurons separated by noticeable nerve fibre tracts. If the ganglia were not gathered into noticeable clusters, we defined their boundaries as an area where connecting strands were smaller than the width of 2 neurons. This ganglion definition has previously been described in a similar study (Stenkamp-Strahm, Kappmeyer, Schmalz. Gericke & Balemba, 2013). When counting total neurons within an individual ganglion, we did not include neurons located outside the delineated ganglion structure.

Total neuron and ganglion numbers were quantified and expressed in a variety of ways:

1. Total neuron number per MP was quantified by counting the total amount of neurons in each of the 15 images acquired at 10X magnification per tissue. Total neuron number was expressed in two ways:
    a. The total neuron number was expressed as a cumulative

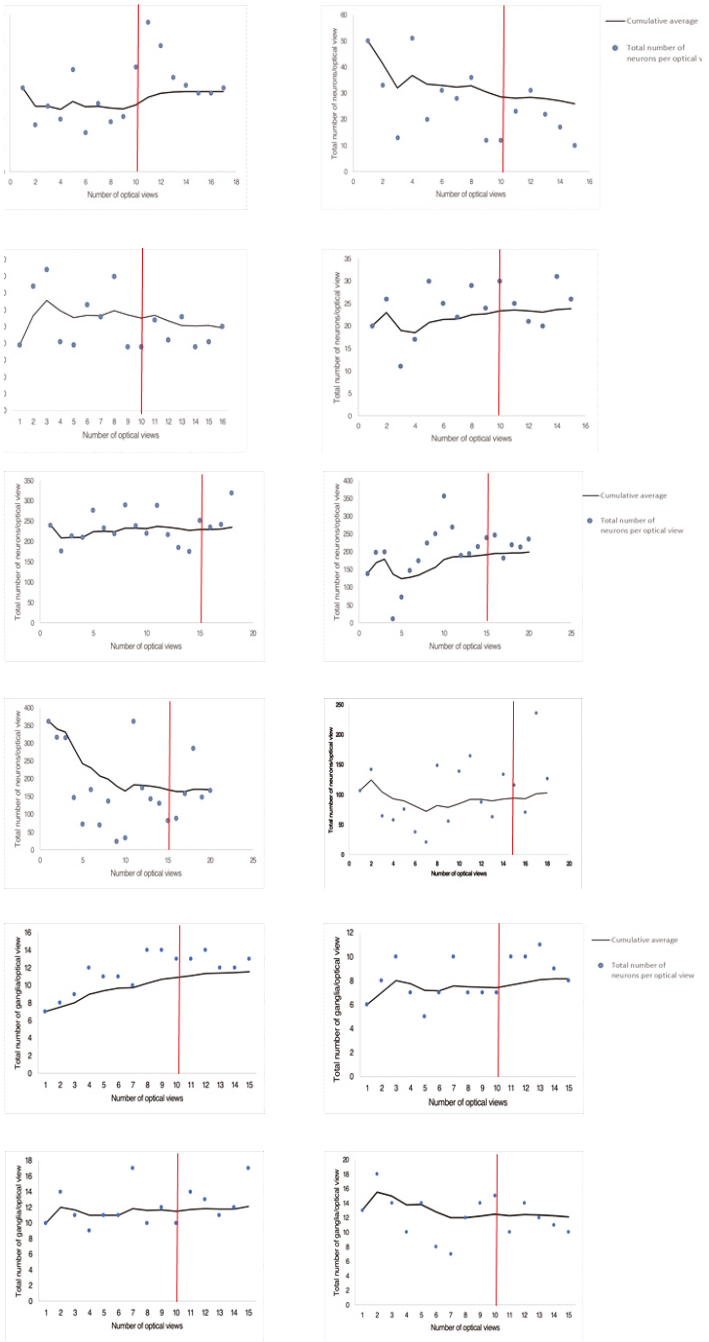

Fig. S1 (top) Determination of optical view number at 40X magnification. The cumulative average of the total number of neurons at 40X magnification flattens at approximately 10 optical views in each pilot tissue. The red line represents where the cumulative average flattens.

Fig. S2 (middle) Determination of optical view number at 10X magnification. The cumulative average of the total number of neurons at 10X magnification flattens at approximately 15 optical views in each pilot tissue. The red line represents where the cumulative average flattens.

Fig. S3 (bottom) Determination of optical view number at 10X magnification. The cumulative average of the total number of ganglia at 10X magnification flattens at approximately 10 optical views in each pilot tissue. The red line represents where the cumulative average flattens.

mean count per MP.
   b. The data was normalized to a total area of 16.8mm2 (total field of view area at 10X magnification) and expressed as a cumulative mean neuron count per total field of view area (mm2).
2. Total ganglia number per MP was quantified by counting the total amount of ganglia in each of the 10 images acquired at 10X magnification per tissue. The total ganglia number was expressed as a cumulative mean count per MP.
3. Total neurons per ganglia was quantified in two ways:
   a. Direct quantification: Total neuron number per ganglia was quantified by counting the total amount of neurons in a single ganglion in each of the 10 images acquired of individual ganglions at 40X magnification per tissue.
   b. Indirect quantification: Total neuron number per ganglia was calculated by dividing the total number of neurons by the total number of ganglia counted per tissue using 10X magnification counts above.

Both quantification methods were expressed as a cumulative mean neuron count per myenteric ganglia.

Statistical analysis

All results are presented as a mean ± standard error of the mean (SEM). Statistical significance was calculated with an unpaired Student's t-test for two-sample comparisons and univariant general linear model for multiple comparisons. Differences between groups were considered significant when $P < 0.05$. N values indicate the number of tissues used per group. One tissue was used per mouse. Discarded tissues were not included.

RESULTS

Consistent body size and weight

There was no difference in mean body weights before the start of the experiment between treatment and control groups. There was no change in body weight from week 0 to week 6 between antibiotic-treated and control mice. Antibiotic administration did not cause a change in food consumption.

Antibiotics quantitatively alter cultured bacterial growth

Cecum bacteria were grown to quantitatively assess colony abundance between antibiotic-exposed and control groups. Antibiotic treatment led to increased bacterial culture growth when compared to control mice (Garnett, 2016).

Antibiotics do not alter total neurons per myenteric ganglia

Antibiotic administration in wild *Peromyscus* mice did not lead to a significant reduction in the average number of total neurons per myenteric ganglia using either quantification method; direct quantification at 40X magnification ($p=0.21$) or indirect quantification calculated from data at 10X magnification ($p=0.44$) (Figure 1a-b).

Antibiotics reduce the total number of enteric neurons in the myenteric plexus.

We found that antibiotic treatment in wild *Peromyscus* mice led to a 24.5% reduction in the average number of total neurons per field of view area (mm2) ($p=0.04$) from a mean of 20.33 total neurons in the control group to a mean of 15.35 total neurons in the antibiotic treated group. In addition, we observed that the average number of total neurons per MP showed a similar trend, though not statistically significant ($p=0.09$) (Figure 2a-b). $P<0.05$ is significantly different from control.

Antibiotics reduce the total number of ganglia in the myenteric plexus

We discovered that antibiotic treatment in wild *Peromyscus* mice led to a 17.7% reduction in the average number of total ganglia per MP ($p=0.05$) in comparison to the control mice (Figure 3a).

DISCUSSION

In the present study, we demonstrated the effects of antibiotic treatment on the colonic neurons of the MP in wild *Peromyscus* mice. This study demonstrated that antibiotic-induced dysbiosis has the following effects: 1) non-significant but trending reduction in the total number of neurons per myenteric ganglia; 2) reduction in the total number of neurons per field of view area (mm2); 3) non-significant but trending reduction in total neurons per MP; and 4) a reduction in the total number of ganglia per MP.

It is assumed that the gut microbiota diversity in the *Peromyscus* mice was altered by the antibiotics, ampicillin and neomycin, similar to lab mice that had received the same doses in previously published studies (Cani et al. 2008). These studies found a 44% similarity in the cecal bacterial communities between antibiotic-treated and control mice. Our cultures of cecal contents from antibiotic treated mice had increased bacterial growth (Garnett, 2016). The broad-spectrum antibiotics may have created opportunities for bacterial growth of remaining bacteria that are normally restricted by microbial competition (Rea et al. 2011).

We observed a significant reduction in the total number of colonic enteric neurons per field of view area in the antibiotic administered mice as suggested by previous studies (Collins et al., 2014, Caputi et al., 2017). We suggest that the observed decreases in total neurons may be due to an antibiotic-induced reduction in microbial abundance and diversity, which may in turn affect colonic neurons. These findings are consistent with data from GF and toll-like receptor (TLRs) knockout mice (Anitha et al., 2012, Brun et al., 2013, Caputi et al., 2017). It is thought that microbial dysbiosis or depletion may be interfering with the cross-talk between microbes and the ENS, and this bi-directional communication may be mediated by pattern recognition receptors, namely TLRs that are capable of directly responding to microbial-derived products. It has been suggested that the maintenance of low doses of lipopolysaccharide, a gram-negative bacterial derived product, may be important in maintaining neuronal survival in adult enteric neurons via TLR stimulation (Anitha et al., 2012). Thus, the structural abnormalities in TLR knockout mice may be associated with a loss in TLR signalling (Anitha et al., 2012). Similarly, microbial depletion from antibiotic treatment may also be interfering with TLR signalling. Our results suggest that these disruptions in TLR signalling could explain the neuronal loss observed in antibiotic-treated *Peromyscus* mice. Other studies have also observed a decrease in the number of neurons per myenteric ganglia in mice treated with antibiotics (Collins et al., 2014, Caputi et al., 2017). Although we did not see a significant reduction in the total number of enteric neurons per myenteric ganglia, the present study shows that the decline in total neuron numbers per ganglia is trending in this expected direction.

We observed a marked decrease in the total number of ganglia per MP in the antibiotic administered mice. We suggest that this may

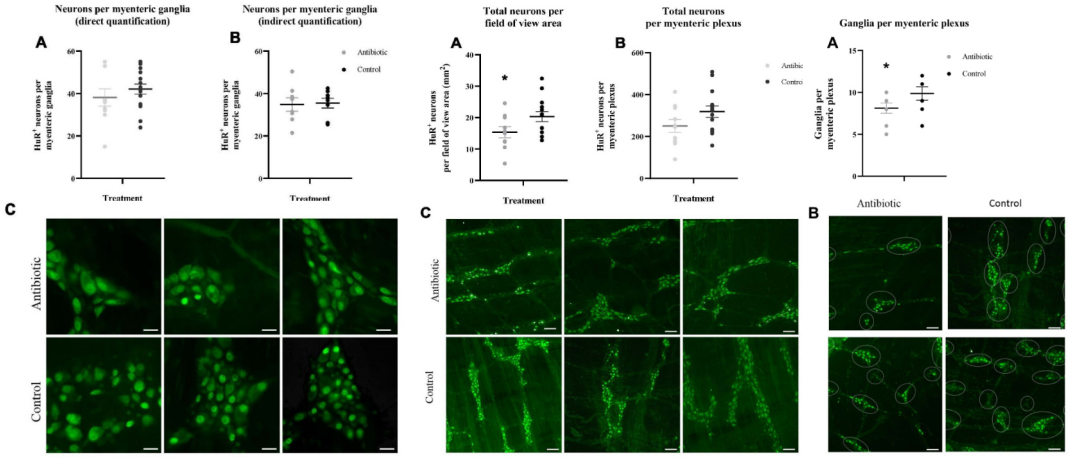

Fig. 1 (left) Effects of antibiotic exposure on the total number of enteric neurons per myenteric ganglia. Antibiotic treatment did not significantly reduce the average number of total enteric neurons per myenteric ganglia in comparison with control mice. (A) Number of HuR$^+$ neurons per myenteric ganglia in LMMP preparations of control and antibiotic-treated mice determined by direct quantification at 40X magnification (p=0.21, N=10-16 per group). (B) Number of HuR$^+$ neurons per myenteric ganglia determined by indirect quantification using 10X magnification data (p=0.44, N==8 per group). (C) Representative microphotographs showing the distribution of HuR$^+$ (green, pan-neuronal marker) neurons at 40X magnification in antibiotic-treated and control mice. Scale bar = 0.25µm. Data are reported as mean ± SEM.

Fig. 2 (centre) Effects of antibiotic exposure on the total number of neurons in the myenteric plexus. Antibiotic exposure significantly reduced the average number of total enteric neurons per field of view area (p=0.04, N=10-14 per group). (A) In LMMP preparations (B) The reduction in the average number of total neurons as determined per myenteric plexus is not significant in the antibiotic-exposed group (p=0.09, N=10-14 per group). (C) Representative microphotographs showing the distribution of HuR$^+$ (green, pan-neuronal marker) neurons at 10X magnification in antibiotic-treated and control mice. Scale bar = 0.75µm. Data are reported as mean ± SEM. * P < 0.05, significantly different from control.

Fig. 3 (right) Effects of antibiotic exposure on the total number of ganglia in the myenteric plexus. Antibiotic administration significantly reduced the average number of total ganglia per myenteric plexus (p=0.05, N=8 per group) (A). (B) Representative microphotographs showing the distribution of myenteric ganglia at 10X magnification in antibiotic-treated and control mice. Circles represent clearly delineated ganglia in antibiotic and control mice. Scale bar = 0.75µm. Data are reported as mean ± SEM. * P < 0.05, significantly different from control.

also be due to reduction in microbial diversity and abundance from the antibiotic treatment. In previous studies, microbial-depletion has been associated with an abnormal MP pattern in GF mice (Collins et al., 2014). Normally, the MP is organized in a stereotypical meshwork of evenly spaced ganglia and interconnecting nerve strands of even thickness. However, in GF mice the MP is less organized, has less abundant nerve fibers, and has unevenly spaced and smaller ganglia compared to conventionally colonized mice (Collins et al., 2014). In addition, a specific marker for enteric ganglion cells, peripherin, has been observed to have an altered expression and distribution in the myenteric ganglia of GF mice, in correlation with a reduction in myenteric ganglia area. Peripherin is a neurofilament protein that forms an important part of the cytoskeleton of various enteric ganglion cells and is often associated with smaller ganglia areas (Brun et al., 2013). We suggest that the microbiota may interact with the ENS to influence ganglia growth and survival via similar mechanisms to those mediating neuronal growth as proposed earlier. Particularly, the administration of the neurotrophic factor, glial-cell derived neurotrophic factor (GDNF) seems to restore peripherin expression levels in the MP and this is correlated with a partial restoration in ganglia size (Brun et al., 2013). Additionally, GDNF has been observed to promote axonal outgrowth and enteric neuronal aggregation in myenteric neuron cultures, mimicking the appearance of ganglions and their associated nerve tracts in vitro (Rodrigues, Li, Nair, & Blennerhassett, 2010). Thus, GDNF is thought to be an important factor in the growth and survival of the myenteric ganglia (Rodrigues, Li, Nair, & Blennerhassett, 2010).

This study has limitations that should be considered when interpreting the results. First, although all attempts were made to acquire microphotographs of whole mount preparations at identical time conditions, preferably after completion of immunohistochemistry, it was difficult to capture all images at a constant time interval. Thus, there may be subtle variations in staining intensity between tissues due to differences in fluorescence degradation over time. This may have affected the quality and clarity of the images acquired days after completion of immunohistochemistry, making it harder to subjectively discriminate among neurons when manually counting those images. In addition, it is important to set microscope settings to capture all images at a standardized minimum baseline fluorescence to reduce unwanted background noise and fluorescence bleed-throughs which can make it hard to distinguish one neuron from another (Stenkamp-Strahm, Kappmeyer, Schmalz, Gericke & Balemba, 2013). Although efforts were made to capture all images using minimum baseline fluorescence settings, it was hard to keep these settings constant for all images due to of differences in staining intensity between tissues. This also may have affected both the the clarity of the images and the manual counting of neurons. Secondly, we did not include immunohistochemistry control experiments, which were imperative to ensure that the antibody staining is accurate and to rule out endogenous staining (Kim, Roh, & Park, 2016). We may have unknowingly included false-positive results during our neuron counts which could have resulted in an overestimation of neuron numbers. Thus, future studies should prepare control immunohistochemistry experiments by omitting the primary antibody in order to rule out artificial background staining from unspecific secondary antibody binding as previously described (Caputi et al., 2012). Finally, all our images were quantified by only one investigator. Although, we were able to re-count all images to ensure consistency in the neuron counts, it is difficult to ensure accuracy in our total count estimates. Future studies may benefit from having 2 or 3 investigators independently counting the neurons and averaging the estimates across the investigators to ensure more accuracy in neuronal counts as previously mentioned (Phillips, Hargrave, Rhodes, Zopt & Powley, 2004).

In conclusion, we demonstrated the effects of antibiotic treatment on the colonic neurons of the myenteric plexus in wild *Peromyscus* mice. Particularly, this study demonstrated that antibiotic-induced dysbiosis is associated with a reduction in the total number of enteric neurons per field of view area, and a similar trend was observed in the total number of neurons per myenteric plexus and per myenteric ganglia. Reductions were also observed in the total number of ganglia in the antibiotic-treated mice. Our results suggest that antibiotic-induced microbial dysbiosis affects the colonic

neurons and ganglia of wild *Peromyscus* mice similar to laboratory rodents.

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"Playing breathalyser roulette": Taking a breath sample without reasonable suspicion and Charter implications

Jacob Caine [1]

[1] Simon Fraser University, Burnaby, British Columbia

Abstract Various organizations and governments throughout Canada have attempted to reduce impaired driving through public awareness campaigns as well as governmental policy. In September 2018 the Government of Canada amended the Criminal Code of Canada [Code] using Bill C-46, increasing the powers of peace officers. Code Section 320.27(2) now allows peace officers to request a breath sample from any lawfully stopped driver without needing reasonable suspicion of impairment. The legislation is aimed at preventing drivers from playing "breathalyser roulette" (Solomon & Chamberlain, 2018, p. 5) and the perception that they can conceal their drinking successfully and thus will be unlikely to be required by a peace officer to take a breathalyser test to assess sobriety. Aspects of the legislation however, may be incompatible with the Canadian Charter of Rights and Freedoms [Charter]. This paper analyses whether or not Code Section 320.27(2) is compatible with Section 8 of the Charter that protects Canadians against unreasonable search and seizure. As a future legal challenge is highly possible, this analysis was conducted to evaluate if the legislation is likely to be upheld by the Supreme Court of Canada (SCC). It was determined after legal analysis that although Code Section 320.27(2) is likely not compatible with the Charter as it fails to pass the reasonableness standard as found in R v Bernshaw (1995), it will probably be upheld as a reasonable limit under Section 1 of the Charter after Oakes Test analysis, a legal test to evaluate whether legislation is a reasonable limit or unconstitutional. The legislation is as minimally impairing on Charter rights as possible and helps to achieve a pressing and substantial legislative objective. Oakes Test analysis however, would be impacted if the Government fails show compelling evidence in justifying why the infringements on Charter rights is necessary for public safety. This legislation, upheld as a reasonable limit, will allow peace officers to request breathalyser samples in more cases, which will hopefully further deter impaired driving.

INTRODUCTION

There has been much debate in the media regarding Canada's amended impaired driving legislation implemented in September 2018 after Bill C-46 was passed on June 21, 2018 (CBC News, 2018; Platt, 2020; Spratt, 2018).). Bill C-46 amended many sections of the Criminal Code of Canada [Code] that involved impaired driving and the obtaining of breath or saliva samples in order to reduce and catch impaired drivers (Act to Amend Criminal Code, 2018). Lawyers argue that the new legislation is "invasive and infringes on citizen's rights" (CBC News, 2018a) whereas those those whose aim is to reduce impaired driving, such as Mothers Against Drunk Driving (MADD,) believe the new legislation will help save lives (CBC News, 2018a; CBC News, 2018b).

Not without controversy, Justice Minister Jody Wilson-Raybould stated in December 2018 that she has "every expectation" the legislation will be challenged in the Canadian court system (Spratt, 2018). This paper thus analyses the amendment to Code Section 320.27(2). The amendment allows peace officers to demand breath samples from motor vehicle operators when they are lawfully stopped, without needing "reasonable suspicion" of impairment (Act to Amend Criminal Code, 2018). As it is no longer necessary for a peace officer to justify why a vehicle search and breathalyser test occurred, it is important to discern whether this amendment is compatible with the Canadian Charter of Rights and Freedoms [Charter].

Some argue that it is unlikely the legislation will be struck down by the Supreme Court of Canada (SCC) as the government objective to stop impaired driving is pressing and substantial (Solomon & Chamberlain, 2018). The targeted societal objective is that many people still drive under the influence causing harm to Canadian society overall, especially as impaired driving remains one of the leading criminal causes of death in Canada (Perrault, 2016; Solomon &

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Address correspondence to cjur.ca

Chamberlain, 2018). The Canadian Medical Association Journal (Wanniarachige, 2015) noted that roadside testing is likely the best deterrent for driving while impaired by drugs or alcohol. The logical reasoning is that increasing the ability of peace officers to use the breathalyser to test for driver sobriety will increase the deterrent effect, dissuading drivers from "playing breathalyser roulette". Deterrence then leads to reductions in impairment related accidents and improves road safety.

Solomon and Chamberlain (2018) however, Professors of Law at Western University, mainly focus on whether or not a substantial and pressing objective exists (part one of the Oakes Test). They complete a basic legal analysis comparing use of the breathalyser to other cases where mandatory screening processes were questioned rather than substantive legal analysis on other aspects related to reasonableness and the Oakes Test. They fail to include other nuances of search and seizure specifically the legal principle of reasonableness which is important in determining whether use of the breathalyser without the need for reasonable suspicion is still compliant with the Charter.

The Oakes test, established in R v Oakes (1986), is a two part legal test to determine whether or not infringements of Charter rights are reasonable and justified in free and democratic societies. The test is used to analyse whether legislation can be deemed a reasonable and justifiable limit using the Charter s. 1 reasonable limits (Johnston, 2009). S. 1 empowers the government to infringe on Charter rights in a variety of circumstances (Johnston, 2009).

Peter Hogg in the 62nd meeting of the House of Commons Standing Committee on September 18, 2017 discusses at 17:04 that although concerns exist regarding the proposed legislation at the time, the legislation would likely be upheld as a reasonable limit (House of Commons Standing Committee [HCSC], 2017). At the 18:00 mark however, Hogg stated that decisions by the SCC can be unpredictable and we will never truly know the result until the SCC officially rules on a challenge (HCSC, 2017). A more detailed legal analysis is thus necessary to add to the scholarly debate and predict with greater certainty whether the new impaired driving legislation will be upheld when challenged in the SCC.

Although a Charter challenge is also possible using Section 9 (arbitrary detention) or Section 10b (counsel upon arrest), this paper will analyse Section 8 and the right to be free from unreasonable search and seizure. According to Solomon and Chamberlain (2019), this section will likely face "the most rigorous challenge" in the SCC (p. 17). This paper sets out to answer the following research question: Would Code Section 320.27(2), be upheld as a reasonable limit using Charter s. 1 by the SCC? A preliminary answer is important to determine what potential legal arguments can be made whether a challenge using Section 8 could be successful.

In answering this question, Code section 320.27(2) will be analysed to evaluate whether or not a Section 8 violation occurs. If a violation is found to occur, the Oakes Test will be conducted to determine whether or not the legislation can be upheld as a reasonable limit using Charter s. 1. It will be demonstrated that the amendment to Code Section 320.27(2) does indeed violate Section 8 as it will likely fail the SCC test for reasonableness using precedent from R v Bernshaw (1995), though it will likely be upheld as a reasonable limit using s.1. This however, is dependent on the Canadian government providing sufficient evidence to justify why the legislation is necessary to improve public safety.

METHODS

A keyword advanced search was conducted on the Lexum SCC database using the words "driving", "Oakes", and "search and seizure" to select the main legal cases. Further, a date range was set to evaluate all decisions from from January 1, 1987 (after Oakes Test implemented) to January 1, 2019. Selection was based on the search list and whether it was applicable to the analysis of Code Section 320.27(2). *Goodwin v. British Columbia* (2015), the main case, along with *R v. Bernshaw* (1995), *R v Hufsky* (1988), and *R v Ladouceur* (1990) were selected. *R v. Dyment* (1988), *R v. Chehil* (2013), and *Hunter et al. v Southam Inc* (1984) were also selected using a snowball sampling method as they were referenced in *Goodwin v. British Columbia* (2015), and *R v. Simmons* (1988) was cited as precedent in the above mentioned *R v. Bernshaw* (1995). These cases helped to clarify important legal aspects. The selected SCC cases were chosen in adherence to Canadian common law principles where previous court decisions set the framework for judgements on similar issues that will be encountered in the future. Only SCC cases were selected at sets the precedent for all lower Canadian courts.

R v Hufsky (1988), and *R v Ladouceur* (1990) specify that stopping vehicles at police checkpoints and requesting basic insurance documentation does not constitute a search under s. 8 because it is not an intrusion on an individual's reasonable expectation of privacy. The search and seizure occurs however, once a breath sample is requested. *R v. Bernshaw* (1995) outlines what is required for a search to be compliant with the Charter and specifies reasonable and probable grounds for a search and seizure. *R v. Simmons* (1988), cited within *R v. Bernshaw* (1995), defines when the reasonable expectation of privacy is expected and that a breathalyser test would not be considered unreasonable. *R v. Dyment* (1988) outlines when and how a search will be considered reasonable and elaborates on *Hunter et al. v Southam Inc* (1984) which established that a balance must be maintained between searching and individual privacy. Legal reasoning from *R v. Chehil* (2013) and *Goodwin v. British Columbia* (2015) are helpful in determining whether the search or seizure was reasonable.

RESULTS & DISCUSSION

Analysis of Section 8

Section 8 provides Canadians with "the right to be secure against unreasonable search or seizure" (Charter, 1982). This right is engaged when the state conducts a search or seizure that interferes with an individual's reasonable expectation of privacy. First, it will need to be determined if the search and seizure is authorized by law and secondly if the law is reasonable and the search is carried out in a reasonable manner.

As noted by Monahan et al., (2017), the federal government has the "exclusive legislative authority" (p. 348) over Canadian criminal law stemming from Section 91(27) of the Constitution Act of 1867 (formerly known as the British North America Act). This means that the first step of Section 8 analysis is satisfied. The second stage is determining reasonableness. As set out in *Goodwin v. British Columbia* (superintendent of motor vehicles), 2015, determining reasonableness requires steps. It must be shown jointly that the law achieves an important public objective, the process is subject to

state review in order to to guard against abuse of power, and that the intrusion goes no further than necessary (*Goodwin v. British Columbia* (superintendent of motor vehicles), 2015, para. 96).

Jurisprudence to determine reasonableness

Hunter et al. v Southam Inc (1984, para. 56) established that the government has to prove that the legislation is reasonable. First, the majority in Goodwin v British Columbia (2015) established that keeping impaired drivers off Canadian roads is an important legislative objective to ensure public safety. The first three lines of the Bill C-46 preamble demonstrate that the legislation is directly aimed at deterring impaired driving behaviour (Act to Amend Criminal Code, 2018) which satisfies the first step of the reasonableness test.

The majority in R v Chehil (2013, para. 3) noted that legislative balance for Section 8 is maintained by ensuring the availability of judicial oversight to correct for potential mistakes made by law enforcement. Canada currently has a sufficient and transparent review process as those convicted can apply online to the minister of justice to review miscarriages of justice or wrongful criminal convictions (Department of Justice, 2016). Further, as of 2018, Canadians convicted of impaired driving offences do not have mandatory minimum prison terms unless they are repeat offenders (Department of Justice, 2018). If we look at additional case law from Lemieux v British Columbia (Superintendent of Motor Vehicles), (2019), we can see that the legislation was amended by the British Columbia legislature so as to place the burden of proof on the driver to prove they were not impaired. This means that the driver can now challenge prohibitions stemming from roadside breathalyser tests. Further, placing the burden of proof on drivers was upheld by the BC Court of Appeals and an appeal was denied leave to the SCC in April 2020 (*Larry Edward Lemieux, et al. v. Superintendent of Motor Vehicles, et al.*, 2020). Although more detailed analysis could be conducted, it seems that the judicial review processes are reasonable.

Justice Karakatsanis, writing for the majority in Goodwin v British Columbia (2015), cited precedent from R v Dyment (1988) noting that the breathalyser test goes much further than simple document search as the state "invades an area of personal privacy essential to the maintenance of human dignity" (Goodwin v British Columbia, 2015, para. 65). The breathalyser test however, is far less intrusive than blood or DNA samples because it has a significantly lesser impact on bodily integrity and privacy interests (Goodwin v British Columbia, 2015, para. 65). Reasoning from R v Simmons (1988) adds that the reasonable expectation of privacy is lower when driving meaning that the breathalyser test can be seen as reasonable. As noted in R v Bernshaw (1995), the breathalyser test does not violate an individual's reasonable expectation of privacy, because it is lower when driving due to the necessary regulation (para. 100). The case of R v Bernshaw (1995) highlights that reasonable grounds are still is necessary to use the breathalyser test at a roadside stop.

In R v Bernshaw (1995, para. 51) however, Justice La Forest, writing for the majority, noted that reasonable and probable grounds to search "is not only a statutory but a constitutional requirement" for a lawful search and seizure to be permitted under Section 8. As peace officers no longer need reasonable suspicion under the amended legislation, it is probable that Code Section 320.27(2) will be found to violate Charter s. 8 in the SCC. Oakes Test analysis will therefore be required to determine if the legislation can be upheld using the Charter s. 1 reasonable limits clause. The accuracy of the breathalyser test itself may also impact the SCC decision.

Although the minimal intrusiveness helps the reasonableness, Justice Karakatsanis cited legal precedent from R v Chehil (2013) noting that "the reliability of the search or seizure mechanism is directly relevant to the reasonableness..." (Goodwin v British Columbia, 2015, para. 67). Therefore, a search method which catches too many innocents cannot be reasonable (R v Chehil, 2013). Researchers at the Croatian Institute for Medical Research and Occupational Health ran a controlled experiment to test the accuracy of breathalysers used by roadside screening officers. They found that roadside screening devices are highly reliable if police officers follow the manufacturer's instructions when measuring (Juric et al., 2018). Breathalyser accuracy however, is heavily scrutinized by lawyers (Weisgarber, 2019) which also impacts reasonableness. Information regarding the accuracy of the breathalyser test is therefore necessary in order to justify the reasonableness of the breathalyser testing procedures and may also be important when considering proportionality aspects of the Oakes Test.

Applying the Oakes Test

The Oakes Test, established in R v Oakes (1986), is the legal test to determine whether or not infringements of Charter rights are reasonable and justified in free and democratic societies. Using this two-part test, the government has a responsibility of proving in court that the legislation is aimed towards a pressing government objective. Secondly the government must show, that the legislation is proportional to the objective showing a rational connection, the infringements of rights are minimized, and that harms are outweighed by the benefits (Johnston, 2009).

In Goodwin v British Columbia (2015, para. 25), all SCC justices agreed with the government that removing impaired drivers from the highway was a pressing and substantial objective. This is consistent with R v Hufsky (1988), and R v Ladouceur (1990). Solomon and Chamberlain (2018) note that impaired driving still is a large societal issue in Canada and the Canadian Centre on Substance Use and Addiction [CCSA] (2019) adds that impaired driving remains "one of the most prominent factors contributing to serious road crashes in Canada" (p. 1).

During this analysis stage, research by Trackman et al. (1998) found that the SCC defers to the government objective in over 90 percent of Oakes Test Cases. Therefore, as long as the government uses appropriate social science statistics, it is highly probable they will likely be able to justify that a pressing and substantial objective still exists. After passing the first stage, we then move on to analysis regarding proportionality.

Proportionality analysis involves asking and answering the following three questions (Goodwin v British Columbia, 2015, para. 79):

1. Does a rational connection exist between the legislation and its aims?
2. Is the legislation as minimally impairing as possible of the right or freedom in question?
3. Do the societal benefits of the legislation outweigh the negative effects of an infringement on the Charter?

Solomon and Chamberlain (2018, p. 5) note the difficulty of perceiving driver impairment as the police officers are solely dependent on "behavioural and sensory observations" and that the "signs

may be difficult to detect in the brief time that motorists are stopped at checkpoints". Further, they cite research by Homel (1990), who notes that "experienced drinkers may be able to conceal signs of intoxication" and that many drivers "play breathalyser roulette" determining that the odds of getting caught whilst impaired are slight, meaning the rewards of driving impaired are greater than its risks (p. 5). Brudner (1998) adds that the SCC has loosened requirements placed on the government when proving a rational connection exists. Currently, as long as the presumed connection is not unreasonable, it can be deemed valid by the courts. Hence, it is likely a rational connection between the amendment and the legislative objective exists.

Although infringing on an individual's reasonable expectation of privacy, a breathalyser test was deemed by the SCC to be minimally intrusive compared to other search methods such as taking blood or saliva samples (Goodwin v British Columbia, 2015, para. 48). It will be important for the Canadian Government to prove that the method is accurate however, so that the intrusion on individual privacy is as minimal as possible. A failure by the government to prove that removing reasonable suspicion infringes on the rights of individuals could lead to Code section 320.27(2) being deemed unconstitutional.

After evaluating research by Wanniarachige, 2015 and Solomon and Chamberlain (2018) and statistics mentioned by Perrault (2016), it seems that impaired driving remains a large societal issue. As impaired driving remains a causal factor in many vehicle crashes, (CCSA, 2019), the Government of Canada will likely be successful in showing that the societal benefits outweigh the infringements on Section 8. Although a failure to demonstrate acceptable evidence would mean the legislation is deemed to violate the Charter, Johnson (2009) notes that the courts sometimes accept evidence that is tenuous. This means that even if the government case may be lacking some support, they could still succeed in convincing the SCC to uphold the provisions as a reasonable limit on S. 8.

Limitations

First, the conclusions depend on the Canadian Government showing ample, reliable, and convincing evidence to prove their case in court. If the government fails to show that the breathalyser test is accurate it would likely be determined that section 320.27(2) of the Code violates Section 8 of the Charter. It is also possible that new SCC justices will have different interpretations and create new precedents if the older ones are deemed to be outdated (Knopff et al., (2017). Principles of SCC legal interpretation can change over time as newer judges create new precedents to make up for mistakes from previous judges or when past decisions no longer align with Canadian values (Knoff et al., 2017). Second, this paper only evaluates the changes to alcohol screening procedures under Code s. 320.27(2) and analyzing other laws regarding driving under the influence warrants separate legal analysis. This is because there are potential differences that exist between the accuracy of testing devices for other substances, specifically tetrahydrocannabinol (THC) (CBC Radio, 2019).

CONCLUSIONS

It can be determined that if the Canadian government uses reliable, plentiful, and accurate social science evidence, it is likely the SCC will uphold the amendments to Code Section 320.27(2) after Oakes Test analysis. Compatibility with the Charter would mean the legislation can be used to try to reduce instances of drivers playing "breathalyser roulette". Although continued debate will occur amongst lawmakers and lawyers, it is ultimately up to the courts to decide whether changes to dangerous driving legislation are compliant with the Charter. Since 2018 when this paper was initially written, the constitutionality of S. 320.27(2) was challenged in R v Morrison (2020) (Saskatchewan Provincial Court). Justice Baniak found that even though a s. 8 violation occurs, the infringements are minimal and justified under s. 1 (paras. 169, 170-175). Although we still do not know if this case will be appealed and granted leave by the SCC (Young, 2020), this provides additional evidence to support the findings and conclusions of this paper that the legislation will be upheld as a reasonable limit on individual Charter rights.

Although the final decision will still be unknown until the legislation goes further throughout the court system and potentially to the SCC, lawyers will continue to argue that the legislation violates the Charter. Additional legal analysis however, remains necessary to evaluate whether Code section 320.27(2) could survive a Charter challenge under other sections including s. 9 or s. 10b. Further, it will be important to also evaluate whether other amendments made to the impaired driving legislation in Bill C-46 could also withstand Charter challenges.

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The effectiveness of orthosis as a treatment for adolescent idiopathic scoliosis

Pan Li Yao [1] Zhang Chen Chao [1]

[1] University of Ottawa, Ottawa, Ontario

Abstract Orthosis is a non-invasive method of treatment for patients with scoliosis, in which prescribed individuals undergo correction of spinal misalignment with the support of a brace. For Adolescent Idiopathic Scoliosis (AIS) specifically, which occurs in youth during critical stages of bone development, it is essential to improve the spine's condition as much as possible before the body fully matures. Although orthosis does not aim to correct the misalignment, it has been proven that bracing will aid in slowing down the progression of deformity. However, a common concern is that the spinal curve will regress back to its original state upon removal of the brace at the end of the treatment period. The aim of this paper therefore is to determine the capabilities of orthosis as a standalone intervention for patients with AIS. A systematic review was performed by consulting the search engines of PubMed Google Scholar and JSTOR, as well as the databases of Medline and OVID. Clinical studies were limited to cohort trials published within the last twenty years which targeted populations of youth to determine the Cobb angle's rate of progression, regression, and spinal curvature of these patients. When comparing orthosis treatment conditions to the absence of intervention in both short-term and long-term cases, it was evident that there are benefits to the corrective forces applied by the brace that outweigh potential drawbacks. Thus, orthosis has been found to significantly decrease the Cobb angle, making it an effective tool for spinal correction.

INTRODUCTION

Characterized as a deformity that causes the vertebrae to rotate and create an irregular curve, scoliosis is a condition in which the body has a spinal angulation of ten degrees or more (Alman and Janicki, 2007). The difference in distance from healthy spinal alignment is measured using the Cobb angle, which determines the severity of scoliosis. This is done by drawing lines from the upper and lower vertebrae of the curve, extending those lines, and measuring the angle at which they intersect (Alman and Janicki, 2007). Deviation from the normal curvature of the spine along the sagittal plane can result in an elongated "S" or "C" shaped curve (Alman and Janicki, 2007). Physical manifestation of the condition includes uneven shoulders and waist, with more prominent ribs, and often with one hip higher than the other (Alman and Janicki, 2007).

The causes of scoliosis can be categorized into three main categories: congenital, neuromuscular, and idiopathic (de Baat et al., 2012). Congenital scoliosis occurs when the condition presents in infants at birth, which is likely inherited from the parents (de Baat et al., 2012). Neuromuscular scoliosis results as a side effect of other conditions such as cerebral palsy or paralysis (de Baat et al., 2012). In most cases, however, the cause of scoliosis is unknown and is thus labelled as idiopathic (de Baat et al., 2012).

One of the most frequent presentations of spinal deformity is Adolescent Idiopathic Scoliosis (AIS), found in patients between the ages of ten and eighteen, with the majority of cases being female (Choudhry et al., 2016). Diagnosis of the condition typically occurs before the onset of menstruation and progresses during puberty. The severity of the condition is determined based on the type of scoliosis, the magnitude of the curve, and the number of years of growth remaining for skeletal bones to fully develop (Choudhry et al., 2016). Although the symptoms of AIS are fairly mild, it is common for patients to experience some level of back pain, and in

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Address correspondence to cjur.ca

more severe cases, further complications related to their internal organs. As such, scoliosis patients should be aware of the treatment options available, which include continuous observation, orthosis, and surgery.

One of the few non-surgical treatments for scoliosis is orthosis, which is correcting disorders related to the misaligned limbs or spine with the use of a brace. This treatment method in the short term provides lower back support and prevents further worsening of the spinal deformity (Grivas and Tsiligiannis, 2012). In general, the aim is to prevent the spine from passing fifty degrees of curvature, and from various clinical studies it has been shown the usage of bracing can halt this progression (Grivas and Tsiligiannis, 2012). However, the efficiency of orthosis treatment can still be controversial as some physicians question the ability of bracing to truly alter the natural course of spinal deformity (Grivas and Tsiligiannis, 2012). The main concern is the possibility of the spine returning to its original state after the treatment period due to a lack of specifically targeted interventions. In this systematic review, an analysis was conducted on the varying treatment strategies using orthosis to determine the effectiveness of braces in both short-term and long-term periods.

METHODS

Clinical and longitudinal studies were analyzed in order to determine the overall effectiveness of orthosis for individuals with AIS. A follow-up period must be included within the study to observe any changes over time. This review was performed on November 21, 2019 by compiling information from various search engines including PubMed, the University of Ottawa library database, and Google Scholar. The final screening consisted of results from the databases Medline, OVID, and JSTOR. Different combinations of keywords (scoliosis, brace, treatment, Cobb angle, progression rate, correction), were incorporated in the electronic search strings. To limit the number of articles obtained, only those that were published within the last twenty years were used.

A total of two hundred fifty-six articles were found on Medline, OVID, and JSTOR with the required search criteria. These were then filtered by the type of study to include cohort studies as the primary design. Of the results available, a screen was conducted based on the title of the articles, followed by a secondary screen based on the abstract provided and whether the full articles were easily accessible. The final screen was performed after reviewing the entirety of the texts, narrowing down the options to five articles that will be referenced in this review.

ANALYSIS

When left untreated, the condition of scoliosis will worsen over time because of the compressive forces of the vertebrae. Intervertebral discs have properties that allow them to resist any one-sided compression force by enlarging the opposite side, resulting in an asymmetrical spine due to the imbalance between the spaces of the discs (Stokes et al., 2011). With the assistance of orthosis however, opposing forces to counteract the stress experienced by the vertebrae will be applied. This theory is the basis for orthosis; the usage of a brace will counteract the compressive forces and prevent progression of the Cobb angle.

In practice, orthosis has been shown to be effective in treating AIS in the short-term. A study conducted on adolescents aged ten years or older with a Cobb angle of twenty-five to forty degrees concluded that the average brace correction rate was 48% (Kuroki, 2018). During this study period, participants were expected to wear the brace for eighteen hours every day and the best outcomes were recorded by those who had their brace on for twenty hours or more (Kuroki, 2018). This confirmed that longer use of orthosis in daily life was more beneficial and had better immediate results. Furthermore, skeletally immature patients who were still developing and thus had more malleable bones experienced a significant decrease in the progression of their scoliosis (Kuroki, 2018). In total, there were six cases where the spinal curve improved and fifteen participants with Cobb angles that remained unchanged—an overall positive conclusion (Kuroki, 2018).

During long-term treatment plans, the issue occurs when the brace is eventually removed, as AIS patients typically only follow orthosis prescriptions until the age of eighteen. It has been recorded that the use of braces in adolescent years does not necessarily provide benefits to an existing case of scoliosis; the condition will continue to worsen once the patient no longer has any external support for their spine (Kotwicki and Cheneau, 2008). However, despite the fact that the Cobb angle may further increase over time, the short-term benefits can still be considered worthwhile for many.

Compared to AIS patients who do not undergo any kind of treatment, the spinal curves of treated patients increase three degrees every year before the age of twenty, and one degree after the age of twenty (Aulisa et al., 2017). Those who experienced further deterioration of their spine and those who had no changes to their condition were only a small percentage of patients. A ten-year follow-up to one study revealed that the average deterioration of spinal curvature between participants was only three degrees (Aulisa et al., 2017). This is significantly lower than the regular rate of decline, suggesting that the use of a brace was overall beneficial. After another five years, fifteen years post the initial date of the treatment, patients' curves did not regress past their original Cobb angles of thirty degrees or more (Aulisa et al., 2017).

The reduced deterioration of spinal curvature in AIS patients who experience scoliosis as they grow through puberty and into adulthood can be accredited to the fact that orthosis treatment is especially effective during the critical period of bone development. By wearing a brace to push against the thoracic and lumbar regions of the spine in the opposite direction of the curve, the pressure applied on the body will balance the forces and aid in stabilizing the spine's misalignment (Cheneau and Kotwiki, 2008). There are two mechanisms that work in combination to properly adjust the acting forces on the spine: the passive and active mechanisms.

The passive mechanism involves pushing perpendicularly on the body's surface against the highest point of convexity, allowing maximum pressure to be directed at the most deformed region of the spine (Cheneau and Kotwiki, 2008). This action of displacing body tissues from the convex to concave parts of the torso is known as tissue transfer, where pushing forces specifically affect the surrounding sections of the thoracic apex including the spinal vertebrae (Cheneau and Kotwiki, 2008). Pressure can also be applied through trunk distraction forces, known as the cherry stone effect, which counteracts the force of gravity in order to elongate the body and straighten the spine (Cheneau and Kotwiki, 2008).

In contrast, the active mechanism consists of forces that are produced from within the body, such as muscle contractions, vertebral growth, and various pressures affecting posture (Cheneau and Kotwiki, 2008). Vertebral growth is an especially important factor for those with AIS as treatment options for this condition depend on improved development of the spine. The critical period for adolescents is during a growth spurt when vertebral remodelling takes place. With continuous spinal reloading, the breakdown of the asymmetric vertebrae is done by osteoclasts, cells that function to break down bone tissue in the process of maintenance and repair (Cheneau and Kotwiki, 2008). Meanwhile, osteoblasts are responsible for synthesizing collagen for the matrix of bone formation and serve to modify the shape of the vertebrae, allowing for the buildup of stronger bones (Cheneau and Kotwiki, 2008). There is also an anti-gravitational effect involved, using the postural control system which shifts one's axis of balance (Cheneau and Kotwiki, 2008) allowing for the locomotor system to regain equilibrium and maintain an upright posture. The active mechanism will help to apply the necessary forces onto patients' bodies and manipulate the balance of the spine (Cheneau and Kotwiki, 2008). This results in successful corrective properties on the spinal misalignment of AIS patients through the use of orthosis.

DISCUSSION

As shown from the clinical studies conducted, the overall effectiveness of orthosis is still a concern amongst many scientists despite having some success in treating scoliosis. Once vertebrae have reached full maturity, the goal is to make the spine strong enough to stand alone without any support from braces. In clinical practice, however, it is still common to see the Cobb angle continuing to increase over time. This regression, although present, ultimately does not take away from the benefits of orthosis overall.

Positive results of long-term studies indicate that patients who wear braces had an increase of spinal angulation by three degrees over ten years (Aulisa et al., 2017). In comparison, individuals without treatment experienced the same change in Cobb angle in only a single year (Aulisa et al., 2018). Thus, the application of orthosis has allowed spinal deformity to degrade ten times slower than its natural rate of development (Aulisa et al., 2017).Therefore, orthosis may be effective both during treatment and over long periods of time, although it is important to remember that these numbers are only an average, and individual outcomes varied greatly.

Overall, there are three possible outcomes of scoliosis treatment: an increase in spinal curvature, a decrease in spinal curvature, and no change in Cobb angle. A major factor determining which category a patient falls under is the lifestyle choices they make over the years (Berdishevsky, 2016). It is often recommended that physiotherapy should be incorporated into the treatment plan and those who attend weekly sessions with exercises specifically for managing scoliosis are likely to notice more improvement in the end (Berdishevsky, 2016). It is also important for patients to be mindful of their body as incorrect posture and excess loading pressure in their daily lives can cause their scoliosis to worsen and speed up the rate at which the condition progresses.

The majority of studies state that the best results of improving spinal curvature through the use of orthosis were obtained by individuals who wore their brace for twenty hours or more every day during the trial period. However, researchers usually do not take into consideration or mention in their reports the patient's compliance toward wearing a brace daily for multiple months. Over time, patients might not keep the brace on for the required amount of time due to lifestyle complications or general discomfort (Weinstein, 2013). Despite orthosis being tailored to each individual, it should be expected that there will always be a level of unease as pressure has to be applied onto the spine in order for the brace to be effective in correcting the misalignment (Weinstein, 2013). Physical alterations such as pressure sores and skin colour changes may occur as well due to friction caused by movements (Weinstein, 2013). All of these physical stressors, combined with psychological factors that could affect the patient's perceived image, will influence how often they wear the brace and should be accounted for when determining the effectiveness of orthosis. For this reason, modern braces are built with consideration of the patient's comfortability in mind, using less rigid materials such as lightweight plastic rather than metal like they did in the past (Fayssoux et al., 2010). With the flexibility of plastic, it allows for partial movement to accommodate for growing adolescent bodies, as well as the development of new and varying orthotic models that encourage patient compliance.

Strain in other areas of the patient's life, such as personal routines and physiotherapy needs specific to each individual, would also impact the outcome of orthosis (Weinstein, 2013). Future prospects to consider should focus on how lifestyle factors influence the effectiveness of the braces and how they can be used to explain the variability of the results obtained. Over time, patients may become less diligent in wearing their brace due to a multitude of reasons and examining this will explain the reasons why some people experience a positive change to their spinal curvature while others remained unchanged. Treatment plans can then be adjusted to give patients a better chance at improving their Cobb angle based on their own circumstances.

CONCLUSION

The use of orthosis yields a positive result in both short-term and long-term treatments, effectively reducing the progression of the Cobb angle in the majority of cases. In this regard, the use of orthosis has demonstrated to be effective in correcting the deformed vertebrae for those suffering from scoliosis, slowing the rate of deterioration of AIS in many patients compared to those without treatment. The active and passive mechanisms of orthosis oppose the compression forces of the vertebrae; this procedure is found to be most beneficial to the youth population as their bones have not yet fully developed, allowing the spine to better adjust to the brace for improved support. Despite this, the process of spinal correction through orthosis is not instantaneous and will require long-term commitment before significant results can be achieved.

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