Repetitive sub-concussive impacts induce inflammation-implications for innocuous head impacts in sports

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ABSTRACT

A sub-concussive impact is defined by Center for Diseases Control as a bump, blow or jolt to the head not resulting in any apparent symptoms. The current effort was conducted to determine the effects seen in repetitive sub-concussive impacts in comparison to a single sub-concussive impact. This pilot study attempts to assess the neuropathological changes in rats subjected to repetitive sub-concussive impacts. Nine Sprague Dawley rats were divided into three different groups (n=3 each): sham, sub-concussive (SC) and repetitive sub-concussive (RSC). They were subjected to SC or RSC using a modified Marmarou impact acceleration injury model under anesthesia. SC or RSC impacts consisted of dropping a 50-gram brass weight from a height of 40 cm onto a metal disc (helmet) taped to the scalp. The animals were sacrificed 7 days post-impact, and their brains were perfused with 4% paraformaldehyde. Representative sections encompassing the hippocampus were processed by immunohistochemistry for assessing microglial and astrocytic proliferation changes using IBA (Ionized Calcium-binding Adaptor molecule) as the marker. Four sections from each brain 500 microns apart were taken from the left and right sides of each section encompassing the CA1 region of the hippocampus. Our results support that RSC induced a significant increase in microglial proliferation compared to SC and sham animal groups (p< 0.05).

INTRODUCTION

Traumatic Brain Injury (TBI) is the damage caused to the brain by an external non-penetrating force causing rapid acceleration and deceleration of the brain (Finnie, 2001; Ma, Aravind, Pfister, Chandra, & Haorah, 2019; Xiong, Mahmood, & Chopp, 2013). It can be caused by such things as sports impacts, motor vehicle accidents, or blast waves. Within U.S., about 1.7 million people suffer from TBI annually and 5.3 million people suffer from TBI associated effects (Chiu et al., 2016). About 80-95% of the cases are mild and moderate. Recent gene expressions have shown there is an upregulation of Parkinson's and Alzheimer's disease from mild TBIs. TBI consists of the primary and secondary phase. The primary phase begins with the initial insult to the head causing laceration and/or contusion, diffuse axonal injury, intracranial hemorrhage resulting in tissue death (necrosis). The secondary phase is a result of cascaded effects initiated to restore the cellular homeostasis of the damaged tissue. This process can last weeks or even months. This process is not very well controlled and can cause worsening of the primary effects resulting in progressive neurodegeneration and delayed cell death. Pre-clinical and clinical studies of TBI

patients have shown that glial cells are a central component in the white matter degenerative process.

Efforts have been made to improve protective gears to mitigate the energies of impacts delivered in combat sports such that receiving a single impact is usually not fatal. It is the multiple impacts that represent a unique risk for neuropsychologic problems for the athletes {Mendez, 1995 #80} {Stojsih, 2010 #73}. Mild Traumatic Brain Injury (mTBI) or a concussion as it is commonly called results in long term neurological effects in about 15% of the cases {Alexander, 1995 #286}. A primary concern associated with mTBI is the susceptibility of the injured person to a secondary injury. This is especially true in sports-related concussions. Players are often returned to play without proper time for a complete recovery to occur. Secondary Impact Syndrome (SIS) and cumulative neuropsychological deficits were first identified in 1973 by Schneider et al. after two football athletes died after receiving a second head injury on the field (Cantu & Gean, 2010). It is believed to be caused due to loss of autoregulation of cerebrovasculature leading to increase in the intracranial pressure. This leads to swelling and herniation eventually causing death of the brain cells.

Sub-concussive head impacts are impacts that do not result in a clinically diagnosed concussion (Davenport et al., 2016). An athlete, such as a boxer or football player, may undergo multiple sub-concussive impacts during one game/event. These impacts are overlooked because a single one does not have clinical noticeable effects. However, when there are multiple insults over a short period of time, the effect may be additive.

In order to study TBI and mTBI, small animal models are often used. In these studies, usually rodents are chosen since their brain mimics humans to a certain extent and the low cost associated with such experiments and their volume. Multiple blunt impact-causing methods are used such as weight drop (WD) models, fluid percussion (FP), controlled cortical impact (CCI) (Chiu et al., 2016). Several studies have been done using rats and mice to assess the effects of single and repetitive (single impact/day over a few days) mild or concussive head impacts on these animals.

There are several ways to detect the level of damage done due to an impact event. In experimental models, one of the most common is the use of histology. Histology allows for the underlying pathology to be quantified. There are several histological analyses that can be performed after the induction of an impact. Microglial proliferation has been found to increase in the animals with multiple impacts over a few days (Gao et al., 2017; Klemenhagen, O'Brien, & Brody, 2013; Mannix et al., 2014; Petraglia et al., 2014; Robinson et al., 2017; Semple et al., 2016; Shitaka et al., 2011; Tyburski, Cheng, Assari, Darvish, & Elliott, 2017; Xu et al., 2016).Microglia comprise of 10% of the cells in the brain and are the first to react in cases of an injury, inflammation, stroke or neurodegenerative diseases in the brain (Augusto-Oliveira et al., 2019). They migrate and accumulate around the site of traumatic brain injury (TBI) to establish a protective environment to mitigate effects of the injury (Donat, Scott, Gentleman, & Sastre, 2017). As macrophages, the function of microglia is to get rid of cellular and molecular debris generated due to the blunt impact.

The current effort was conducted to determine the effects seen in repetitive sub-concussive impacts in comparison to (non-repetitive) sub-concussive impacts. This pilot study attempts to assess the neuropathological changes in rats subjected to repetitive sub-concussive impacts.

MATERIALS AND METHODS

Nine Sprague Dawley male rats were used for this study (weight 306 ± 25 grams). They were ordered from Charles River Laboratories (Wilmington, MA) and the study was conducted in accordance with Institutional Animal Care and Use Committee (IACUC), Wayne State University, Detroit, MI. They were housed in a vivarium with unlimited access to food and water in a 12-hour light/ 12-hour dark cycle and given 7 days acclimatization time before starting the experiment. Basic behavioral cognitive testing was conducted (right reflex, radial arm maze), however this paper will only focus on the histological results. The rats were randomly divided into three experimental groups (n=3 each): sham, sub-concussive (SC) and repetitive sub-concussive (RSC). The rats were subject to impacts using a modified Marmarou model with a 50 grams weight being dropped from a height of 40 cm which resulted in .20 joules of impact energy versus the typical 8.82 joules when using the original 450 grams dropped from 2 meters. The experimental setup is depicted in Figure 1.
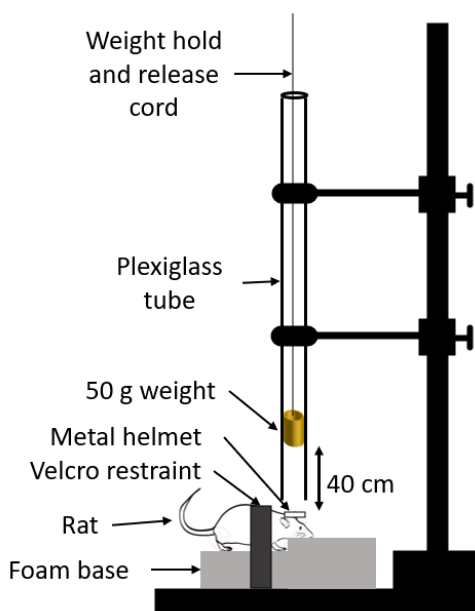

Figure 1: Schematic of the weight-drop modified Marmarou model. A 50 g brass weight is dropped down a hollow plexiglass tube onto a metal plate fixed on the rat's head.

The animals were anesthetized using 4 % isoflurane and 0.5 L/min oxygen and subjected to head impacts. The sham group rats were only anesthetized and placed under the impactor setup on the foam bed but not impacted. A 12x12x43 cm polyurethane foam bed was placed in a plexiglass box of similar dimensions under a 2.5 m long transparent plexiglass tube inside which weights would be dropped from. The weights were tied and held at the 40 cm mark measured from the top of the rat's head on the polyurethane foam. The rats were strapped in place with their front

limbs splayed to the side on the foam bed. The SC group was impacted once and the box was moved out of the way to avoid a second impact. The RSC group were impacted 10 times with 10 seconds between each impact. All rats were placed in a secondary enclosure for about 2 minutes till they regained consciousness and then released in their original cages.

The rats were sacrificed 7 days after the impact. They were transcardially perfused with Phosphate Buffer Saline (0.1 M PBS, 7.4 pH) followed by 4% paraformaldehyde (PFA). The brain was carefully retrieved and postfixed in 4% PFA. To section the brains, they were cryoprotected in 15% and 30% sucrose, frozen in plastic blocks in OCT (Optimal Cutting Temperature) solution in -80° C freezer and sectioned in a cryostat (Leica Biosystems, Vista, CA) at 40 microns thickness between bregma -1.5 to -4.5 to retrieve sections consisting of the hippocampus and stored in multi-well plates.

The sections were first washed in PBS, followed by incubating in citrate buffer solution and 0.6% hydrogen peroxide solution. They were then incubated for 3 days with 1:2000 dilution ratio IBA antibody (Vector Laboratories) in Bovine Serum Albumin (BSA) at 4° C. After 72 hours, they were incubated in secondary antibody goat-anti rabbit (Vector Laboratories) 1:200 at room temperature. They were then incubated in avidin-biotin peroxidase solution (Vectastain ABC kit, Vector) and final staining was done in diaminobenzedene (DAB) in distilled H_2O. All sections were washed 3 times five minutes each in between each step. All sections were mounted onto glass slides and left to air dry for two days after which they were dehydrated using 100% alcohol and Xylene and coverslipped using permount as the mounting medium (Ghatak & Combs, 2014; Hovens, Nyakas, & Schoemaker, 2014).
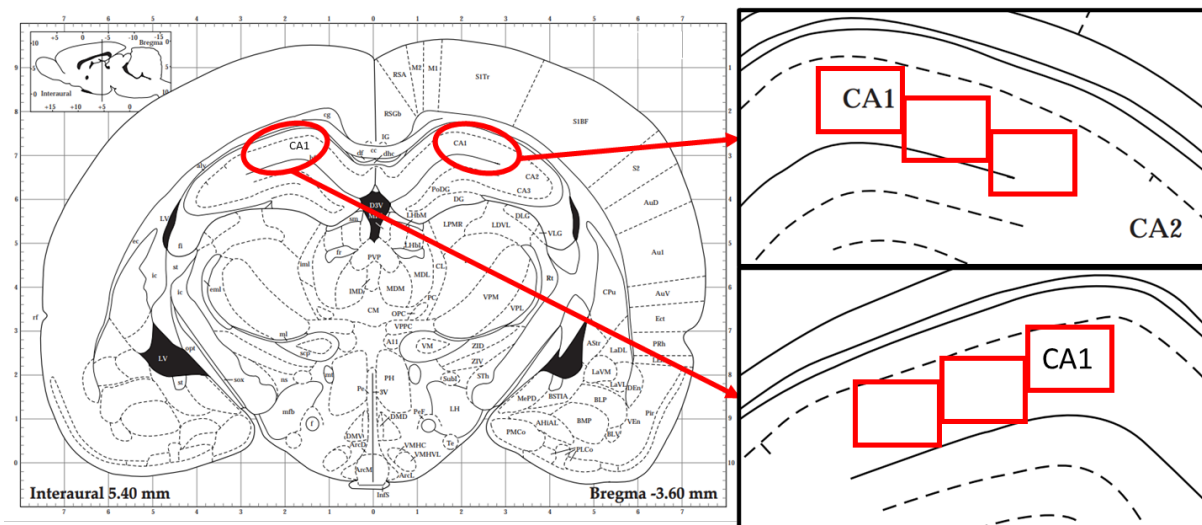

Figure 2: Diagrammatic representation of the areas captured in the CA1 region in the hippocampus at -3.6 mm bregma in the rat brain on the right and left side (Paxinos & Watson, 2006).

The sections were observed under an EVOS XL Core microscope (Thermo Fisher Scientific). Four sections per brain were observed and three images (each 500x400 microns^2) from the left and right side of each section were captured encompassing the CA1 region of the

hippocampus as shown in Figure 2. Twenty-four images were analyzed for each brain (4 sections x (3 left + 3 right-side images)). The number of microglial cells were counted by the author J.V. and M.H. (blinded during the counting) on ImageJ software (NIH, Bethesda, MD). The total number of cells on each side of the hippocampus were averaged and one-way ANOVA was performed in GraphPad Prism (GraphPad software, San Diego, USA) to check out the difference between means of the three experimental groups- sham, SC and RSC.

RESULTS
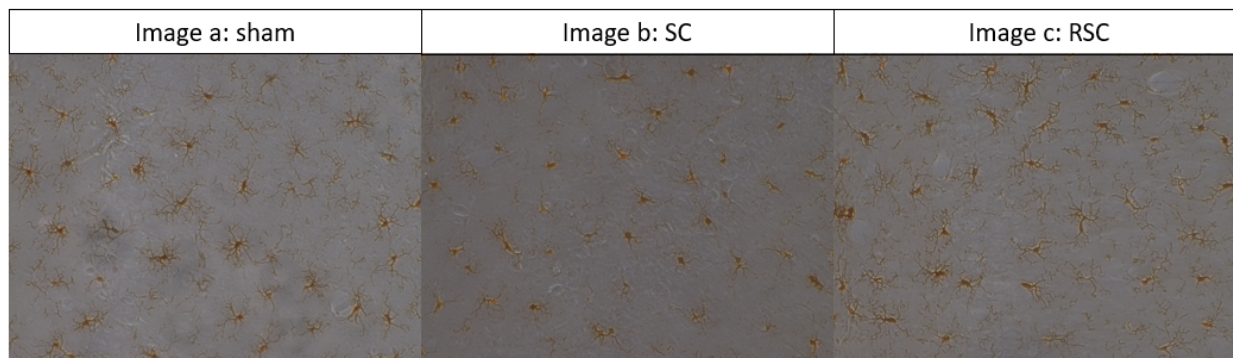

| Image a: sham | Image b: SC | Image c: RSC |

Figure 3: Examples of microglial cells in each image: Sham= 32 cells, Sub-concussive (SC)= 33 cells and Repetitive Sub-concussive (RSC) group= 40 cells. Each Image is 500x400 microns.
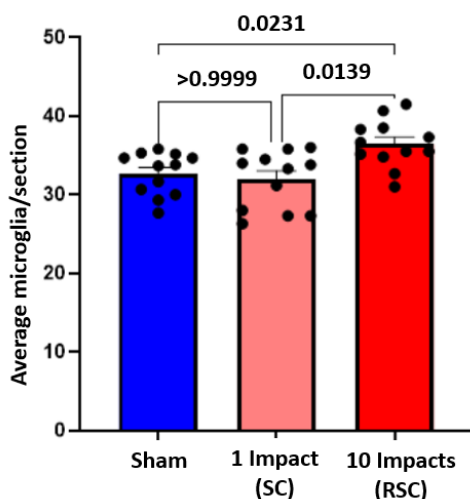

Figure 4: Graph of average number of cells varying across the three experimental groups- sham, SC and RSC (n=3 each).

Figure 3 shows examples of the cells observed in the sham, SC and RSC groups in the CA1 region of the hippocampus. Figure 4 shows the number of cells observed in the three different experimental groups. Visually, a difference is seen in the mean of RSC compared to both the sham and SC groups while no appreciable difference is seen in the sham and SC groups. For the RSC group, the average number of microglial cells per section 36.15 ± 3.04 were higher than both the sham and SC group where 32.71 ± 2.73 cells and 31.96 ± 3.73 cells were observed respectively.

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Statistical analysis also supported a significant difference between these groups. RSC induced a significant increase in microglial proliferation compared to SC and sham animal groups ($p< 0.05$). There was no significant difference between the number of cells in the sham and SC group ($p>0.9999$).

DISCUSSION

This study focused on the effects of repetitive sub-concussive impacts analogous to the intensity of impacts athletes face in contact sports like boxing and soccer. They received multiple impacts the effects none of them causing symptoms of an mTBI. Together, the effects of repetitive impacts are additive as observed by the results in this study. The significant difference in the number of microglial cells observed in the RSC groups in comparison to the sham and SC group proves two theories- a) A single subconcussive impact is not deleterious to the neuronal structure and causes no appreciable changes in the microglial proliferation and b) although a single sub-concussive event is not harmful, the increase in the number of microglial cells indicates that its effect is additive and causes neuronal cell damage.

The goal of this study was to create a model of subconcussive impacts to determine long term, additive effects. There are multiple studies that report on the number of concussive events in sports, however athletes also are receiving large numbers of subconcussive impacts. Stojsih et al. reported the HIC (Head Injury Criterion) values of 134 punches from 73 boxers and only one of them was over the concussion threshold indicating most of the impacts in boxing lie below the threshold level and do not cause any external symptoms {Stojsih, 2010 #73}. Jansen et al. also reported that over 90% of the head impacts in boxing and Mixed Martial Arts fell below the threshold which has a 25% probability of causing mTBI {Jansen, 2021 #284}. These studies demonstrate that most (over 90%) of the head impacts in contact sports fall in the sub-concussive range.

There is a need to further refine this model. In the current study, all of the specimens were sacrificed 7 days after the impact whereas most of the previous work sacrificed specimens within 72 hours of impact. Robinson et al. also state the difference between the impacted mice 24 hours and one week after the injury is different (Robinson et al., 2017). Since this study sacrificed specimens one week after the impact, the number of microglia appearing between 1 and 7 days after the injury might be higher. Hence, a higher difference could have been observed if the specimens were sacrificed earlier. Future work will include the evaluation of multiple time points to determine when the microglia activation peaks.

In addition, the number of RSC impacts needs to be explored. Previous work has investigated time periods of 24 hours or more between impacts. For the current model, impacts were delivered every 10 seconds. This was based on data collected from the field. Jansen et al.'s study reported an average of 12.3 ± 10.4 head impacts per match for amateur boxers {Jansen, 2021 #284}. A boxing match runs for a total of 6 minutes i.e. 360 seconds. Based on time scaling of 100:1 for rats versus humans, each impact will be performed every 1 second. The timing and number of impacts needs to be further investigated in order to set thresholds of injury.

The main limitation of this study is the small number of specimens. However, statistically significant differences between the SC and RSC were noted. Future work will expand on the number of specimens in each group along with varying the timing and number of impacts.

CONCLUSION

The modification of the well-established Marmarou technique for TBI allowed us to create a model that produced a subconcussive event, i.e. the single impact events that were not statistically different from the sham animals as validated by the histological assessment (p=>0.999) and the repetitive sub-concussive impacts being statistically higher than the sham and single impact events (p<0.05). This will allow for future research to assess the cumulative effects of sub-concussive impact events and ultimately provide guidelines for sports concussion assessment.

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