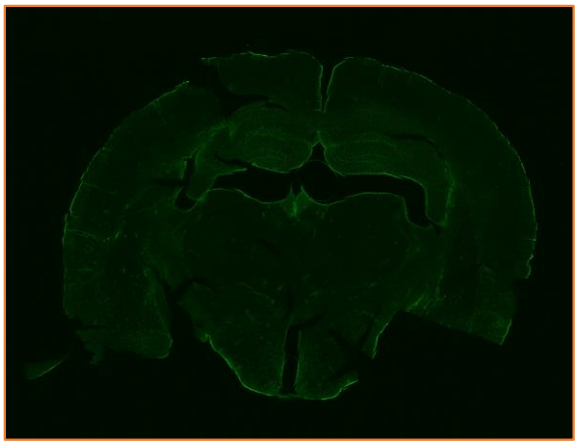

Preliminary work exploring the protective effect of carbon dioxide against traumatic brain injury

ABSTRACT

Traumatic brain injury (TBI) affects millions of people each year and has massive human and economic costs. Particularly prevalent in military servicemembers and contact sport players, TBI can lead to long-term social and cognitive mental consequences. TBI has numerous modalities of injury, many of which can be attributed to the propensity of the brain to move within the skull during rapid acceleration, deceleration, and rotation, which leads to injury from the brain impacting the skull or rotational strain. Successful preclinical and clinical preventative interventions against TBI have implemented slosh mitigation, which minimizes the cranial reserve volume. Preliminary results indicate that carbon dioxide, putatively by its ability to reduce slosh by increasing blood volume in the cranial compartment, is a promising preventative measure against TBI. Our findings show pre-trauma carbon dioxide exposure normalizes several key indicators of TBI in mice. Future work will involve unraveling the biomolecular mechanisms surrounding this effect.

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Introduction and Background

Traumatic brain injury (TBI) is one of the leading causes of death and long-term disability worldwide [1]. In 2014, there were nearly 3 million TBI-related hospitalizations and deaths in the United States alone [2]. Additionally, TBI accounts for roughly 30% of all injury-related deaths in the US [3]. TBI often leads to secondary complications, particularly neurodegenerative diseases such as Parkinson's disease, chronic traumatic encephalopathy (CTE), and Alzheimer's disease [3]. It has been described as the "signature injury" of the military intervention in the Middle East and has high prevalence among contact sports players in addition to its prevalence in the lay population due to accidents [4]. Due to the wide range of demographics TBI effects and the large number of people who experience a TBI each year, there is a large corresponding economic cost. In the US estimates suggest $4 to $15 billion are spent on costs related to TBI each year, with more recent estimates going as high as $17 billion [5] [6]. Much of these costs are related to long-term disability. While the societal and individual cost and suffering is high, there is still no FDA-approved therapy for TBI, though many groups including ours are working toward such ends [7].

Psychiatric disorders after TBI are frequent: up to 61% of all TBI survivors suffer from depression, and of new TBI cases, up to 27% of patients suffer from PTSD, up to 38% from heightened aggression, 9% from mania, and 20% from psychosis [8]. TBI is associated with a much higher risk of suicide: in a study of a Taiwanese cohort, the TBI group had a suicide attempt rate 2.23 times higher than the non-TBI group [9]. TBI also results in social deficits. In pediatric TBI, patients are at risk of lifelong social deficits: decreased social responsiveness, abnormal interactions, communication deficits, and impaired adaptive behavior [10]. TBI survivors may also struggle with behavioral regulation and aggression: in a study of young male offenders, the prisoners had a higher underlying rate and severity of TBI than a college-enrolled control group [11]. Additionally, more numerous and severe TBI was correlated to worse behavioral regulation, higher aggression, and higher hopelessness [11].

A traumatic brain injury is a general term for a variety of modalities and severities of injury that affect the brain. TBI encompasses both open- and closed-skull injuries to the brain, as well as injury due to rotation, acceleration-deceleration, and axonal tearing. Mild traumatic brain injury, mTBI, is a less severe form of TBI also referred to as concussion. mTBI is the most common type of TBI suffered by all groups worldwide [12]. While mTBI, by definition, is not fatal and does not impact life expectancy, it can and does lead to long-term and lifelong disability [4]. Repetitive mTBI is a major cause of CTE in sport athletes [13]. By the Mayo scale, mTBI has shorter and less severe loss of consciousness or amnesia if any, and if there is visible injury or fracture the dura is left intact [14]. A distinct modality of TBI, suffered particularly by military personnel, is blast TBI (bTBI), which can be mild or moderate to severe [4]. In a blast-induced TBI, there are two major mechanisms of injury: mTBI caused directly by a high-explosive shockwave, and mild or moderate to severe TBI caused by being thrown from the blast wind or struck by flying or falling debris [15].

In mTBI, after a sharp impact to the skull, causing rapid acceleration and/or deceleration, injury is caused when the brain impacts the inside of the skull [4]. Multiple injuries can occur both ipsilaterally and contralaterally to impact as the brain rebounds within the skull. This results in contusion and hematoma. Rotational injury also often occurs; rapid rotational movement widely distributes shear and strain throughout the brain and results in axonal injury, particularly strain and shearing in white matter tracts. This phenomenon is referred to as diffuse axonal injury (DAI) [16]. DAI is particularly prevalent in

bTBI [17]. The primary injury from just the high-explosive shockwave would be expected to be DAI due to the shockwave passing through the brain, while being thrown by the blast wind or being struck by debris could cause more diverse injury types: penetration of the brain by debris, contusion and hematoma from the brain impacting the skull, and DAI from rotational strain and shear [18].

The primary and currently only widespread protective equipment against TBI is helmets, which have applications for military and sports. Much research has been done regarding the protective ability of helmets in contact sports, and, despite advances in helmet technology, with novel shapes, shell materials, and padding types continuing to be developed and entering the market, these new technologies have made no improvements over their predecessors in reducing mTBI [19]. Furthermore, current evidence has not indicated that helmets would reduce mTBI in rugby, which is a helmetless sport, although they are still better than no helmets in American football [19][20]. Thus, while helmets are effective at preventing cranial fractures, skull trauma, and penetration of the brain, more widespread use of helmets and more advanced helmet technology are not likely to reduce mTBI [21]. This is for the simple reason that although helmets can reduce the acceleration of the head by a certain amount, they do nothing directly about the relative motion between the brain and the skull.

Injury caused by the brain impacting the skull and by rotational strain both occur because the brain is able to move relative to the skull during acceleration and deceleration [22]. In aerospace engineering, slosh is a studied phenomenon that refers to the propensity for a violently agitated fluid to knock against the walls of its container. This same phenomenon is responsible for the brain's ability to knock against the walls of *its* container, the skull. [22] Therefore, reducing the ability of the brain to slosh within the skull has been identified as a target of injury preventions to reduce the incidence and extent of TBI. By mitigating slosh, acceleration and deceleration of the head would result in both the skull and its contents moving in unison. The brain being fixed with respect to the skull would prevent collisions between the brain and the skull as well as the rotation that could lead to excessive torsion [23].

The reason the brain is able to move within the skull is the compensatory reserve volume (CRV), a small amount of "free" space, which exists because the brain tissue, cerebrospinal fluid, and blood do not completely fill the skull and bony spinal canal [23]. When the volume of the components within the skull and bony spinal canal increase, the CRV compensates at first and the change in volume occurs at relatively constant pressure, but once the CRV is exhausted, any further increase in volume will significantly affect pressure [23]. Filling the reserve volume is thought to hold the brain in place during acceleration-deceleration events and facilitates brain and skull moving in unison, thus minimizing brain slosh and damage.

Of the numerous components that affect intracranial pressure (ICP) the most amenable for intervention is the amount of blood in the intracranial and spinal compartments: modifying blood flow is the simplest, fastest, and most easily reversible method of achieving increased volume and ICP [23]. To date, the most widely studied mechanism of modifying blood flow to the brain for the purposes of reducing free volume is internal jugular vein (IJV) compression, typically achieved using a fitted collar that rests on the neck and applies pressure to the left and right IJV. Ultrasound confirms a significant backfill into cerebral venous capacitance [24]. This results in mild engorgement of the cerebral venous vasculature, which mimics the action of an airbag, reducing the amount of brain movement that is possible [25]. Numerous preclinical studies have found that increasing ICP and reducing CRV by restricting jugular vein outflow significantly reduces markers of TBI in rodent [23] and swine [26] models.

This preclinical work led directly to development of an IJV compression collar for human athletes. Of the numerous clinical trials that have studied this collar, trademarked as the Q-Collar, analysis of efficacy has been done using both diffusion tensor imaging, used to measure disturbance to white matter tracts as a measure of DAI, and functional magnetic resonance imaging (fMRI), which quantifies functional changes between collar and non-collar groups. In hockey, soccer, and American football, these studies have shown there is a significant difference between pre- and post-season white matter integrity in non-collar groups, but no such difference in white matter integrity in the collar wearing groups; this indicates that increasing blood volume in the cranial compartment is a safe and effective way to prevent mTBI [24] [27] [28] [29]. Furthermore, in an fMRI study of working memory in high school football players, the protection from slosh injury afforded by IJV compression was shown to prevent any pre- and post-season difference in working memory in the collar group, while the non-collar group experienced significant change in brain region activation in a working memory task as a function of how recent and numerous collision events were [25].

While the jugular constriction method is proven to be effective in reducing incidence of TBI, there exists an even more powerful method to increase intracranial blood volume and thus reduce CRV and protect the brain from slosh: the partial pressure of carbon dioxide in the blood. Hyperventilation has been studied as clinical intervention to control posttraumatic intracranial hypertension: hyperventilating reduces the partial pressure of CO_2 in the blood, which causes cranial arteries to constrict, reducing blood flow and thus blood volume, ultimately reducing intracranial pressure by up to 40% [30] [31]. Conversely, exposure to a hypercapnic atmosphere significantly and profoundly increases cerebral blood flow by the inverse of the same mechanism: a 5% CO_2 atmosphere can increase the intracranial pressure by up to 300% its physiological level, indicating an exhaustion of the compensatory reserve volume [32]. Given the proven effectiveness of IJV compression in reducing incidence of TBI by preventing slosh by increasing cranial blood volume, carbon dioxide's ability to significantly increase cranial blood volume warrants investigation into its ability to prevent slosh and thus TBI.

Preliminary Work and Findings

Our current work is on investigating the ability of carbon dioxide to reduce the incidence and severity of TBI using a murine bTBI model. We examine the primary and secondary sequelae of TBI with both immunohistochemistry staining and microscopy and transcriptional studies. Our model is a gas-driven shock tube that produces a shockwave scaled for murine subjects and capable of causing bTBI [33]. A slightly angled, tubular chamber known as a driver section, gated by a 76.2 µm thick polyester membrane, is pressurized until the membrane ruptures. This produces an overpressure shockwave, which is carried down a tubular driven section. A more powerful shockwave can be produced using a thicker membrane. At the exit of the driven section is a PVC pipe that shields a subject's internal organs while leaving the head exposed. This model results in a consistent Friedlander waveform, which consists of a sudden overpressure, followed by a millisecond-scale logarithmic decay and then an underpressure phase, which slowly returns to baseline. The key strengths of this model are the protection of internal organs, which prevents confounding systematic injury, and the fact that the rodent's head is unrestrained, which allows for rapid acceleration of the head leading to skull-brain collisions and rotational injury. This ability to replicate many diverse injury types of TBI is not present in models like cortical impact, wherein the exposed cortex is struck, typically by a falling weight. Our ability to model rotational and skull-brain impact injury is particularly relevant for studying the prevention of slosh.

In addition to body temperature, we use behavioral measures to gauge the effects of TBI. To measure the immediate severity of TBI we use righting reflex time (RRT), which is the time it takes for supine anesthetized mice to regain consciousness and right themselves. For mice exposed to our bTBI model, a more severe TBI will lead to longer loss of consciousness and slower RRT. Both mice and humans are social species, and TBI leads to social deficits in both. To measure sociability, we use the Crawley three chamber test for sociability, wherein a mouse is allowed to explore three chambers freely, one of which contains a novel inanimate object, another of which is empty and separates the other two, and a third which contains a mouse in a small enclosure, which the mouse being tested has not seen before and would typically spend time socializing with: the assay measures how much time the mouse spends with the novel mouse vs the novel object. We would expect to see mice given a TBI to have impaired or atypical socialization behavior. As a measure of social dominance and aggression, we use the tube test for social dominance (in males) wherein a mouse is placed in a tube opposing another mouse and a more dominant mouse will advance in the tube, causing the other mouse to back up and be forced out of the tube, and the number of "wins" is used to quantify the social dominance and aggression of the mouse. As a measure of despair-like behavior, we used the forced swim test: mice naturally float in water, but are averse to being in it, so when placed in a shallow basin, they swim in an attempt to find an exit. Mice with despair-like behavior spend less time swimming and more time floating: giving up on finding an exit, which in nature would be a serious threat to survival, is deemed to be a proxy for despair. We also use the open field test (OFT) to gauge motor and arousal deficits of TBI. Mice with motor or arousal deficits spend less time roaming in an open arena and more time stationary, resulting in less overall distance traveled.

Immediately after bTBI, mice in the TBI group display significantly higher righting reflex times than sham mice (Fig 1A). This demonstrates the loss of consciousness and acute neurological deficits that occur shortly after injury. An additional physiological difference observed is that TBI mice experience a significant drop in body temperature in the minutes following TBI (Fig 1C). At the 3-hour timepoint, TBI mice have significantly less locomotor activity in a 30-minute OFT than sham mice, confirming motor and arousal deficits (Fig 1B).

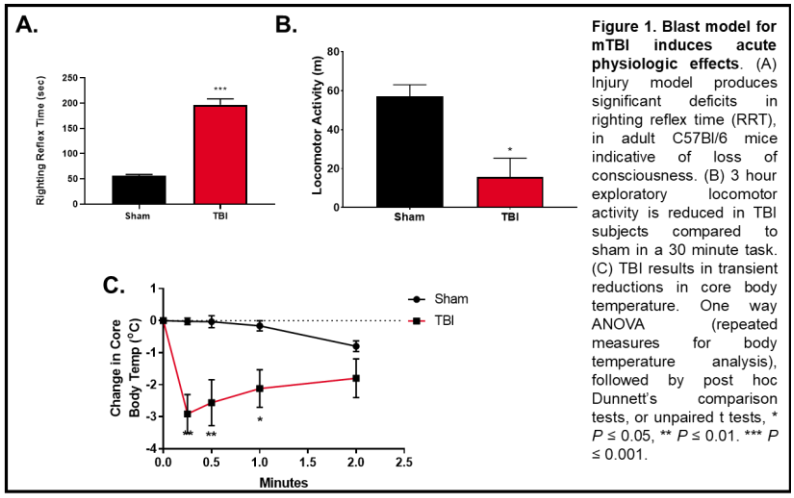

Figure 1. Blast model for mTBI induces acute physiologic effects. (A) Injury model produces significant deficits in righting reflex time (RRT), in adult C57Bl/6 mice indicative of loss of consciousness. (B) 3 hour exploratory locomotor activity is reduced in TBI subjects compared to sham in a 30 minute task. (C) TBI results in transient reductions in core body temperature. One way ANOVA (repeated measures for body temperature analysis), followed by post hoc Dunnett's comparison tests, or unpaired t tests, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Our results for the Crawley three chamber test demonstrate social impairment at the 10-day timepoint after bTBI: TBI mice spend significantly less time near the enclosure of the novel mouse, and where sham mice spend significantly more time in the chamber containing the novel mouse, TBI mice do not display a significant preference for either chamber (Fig 2A-B). From this data, it is clear that our model reproduces the social deficits seen in TBI.

In the tube test for social dominance, at the 10-day timepoint, we observe significantly less wins for the TBI mice, which seems counterintuitive given that TBI increases aggression in humans, but at the 30-day timepoint, TBI mice are more dominant than sham mice (Fig 3A-B). This replicates the pattern of social withdraw and later heightened aggression in some human TBI. In addition to social deficits in TBI mice, we observe despair-like behavior at the 10-day timepoint in the forced swim test: TBI mice spend significantly more time floating immobile than sham mice (Fig 3C).

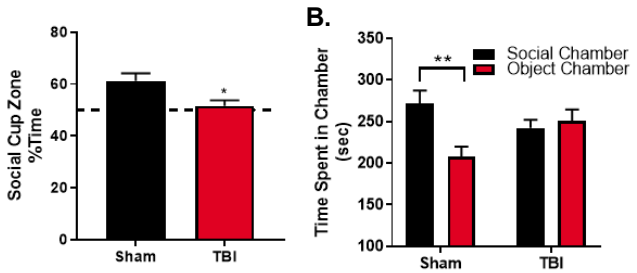

Figure 2. TBI causes depressive like behavior, altered social behavior, and social dominance. (A) Sham mice exhibit a preference for spending time in the social cup zone (the area immediately around the novel animal) and (B) the social chamber in the Crawley three chamber test for sociability 10 days post injury, an effect that is not observed in TBI subjects.

We have run several preliminary experiments to determine the protective effects of CO_2. Before being exposed to bTBI, mice are exposed to CO_2 using a specialized chamber that diffuses the gas flow through an aquarium stone to prevent them from noticing and becoming anxious. We have found that CO_2 exposure normalizes righting reflex time almost back to baseline: there is no significant difference between air-exposed sham mice and CO_2-exposed TBI mice or CO_2-exposed sham mice and CO_2 exposed TBI mice (Fig 4A). Furthermore, CO_2 exposure attenuates differences in distance traveled in the open field test between sham and TBI mice: we find no significant difference between either sham group and the CO_2 TBI mice (Fig 4B). Additionally, CO_2 reduces temperature fluctuations that occur after TBI (Fig 4C). This preliminary data shows that CO_2 exposure *per se* can almost completely recover several primary indicators of TBI in mice. The fact that both RRT and body temperature are normalized immediately after TBI before significant changes in protein

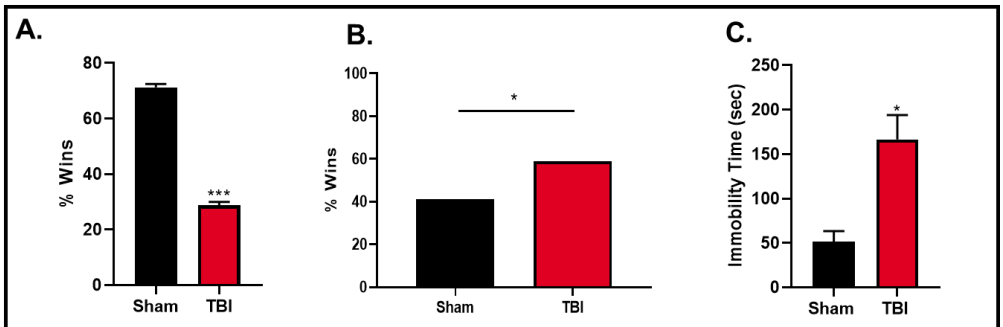

Figure 3. TBI causes depressive like behavior and altered social dominance. (A) In the tube test for social dominance sham subjects win significantly more comparisons 10 days post injury compared to TBI subjects, indicative of a reduction in social dominance in TBI animals. (B) In contrast to the 10-day time point, TBI subjects win significantly more comparisons 30 days post injury compared to sham subjects. (C) Mice subject to TBI spend more time immobile in a 3 minute forced swim test 7 days post injury, indicative of despair like behavior. Importantly there are no differences in locomotor activity at this time point (not shown). Two-way ANOVA followed by post hoc Bonferroni's comparison tests, unpaired t tests, or Fisher's Exact Tests (tube test data) * $P \leq 0.05$, ** $P \leq 0.01$. *** $P \leq 0.001$.

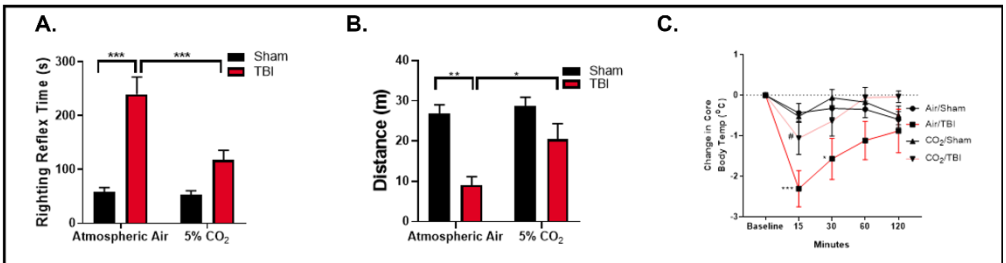

Figure 4. CO_2 pre-exposuremitigates acute physiologic effects elicited by mTBI. (A) CO_2 mitigates deficits in RRT, an indication of unconsciousness, induced by TBI. Two-way ANOVA, followed by post hoc Bonferroni's multiple comparison tests; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. (B) Pre-exposure with 5% CO_2 attenuates reductions in exploratory locomotor activity 3 hours following mTBI. (C) TBI causes transient reductions in core body temperature relative to Air-Sham, an effect that is blocked when subjects are exposure to 5% CO_2 immediately prior to injury. Two-way ANOVA, followed by post hoc Bonferroni's multiple comparison tests or unpaired t test (freezing time data); # $P \leq 0.05$ (body temp data, CO_2-TBI vs Air-TBI)

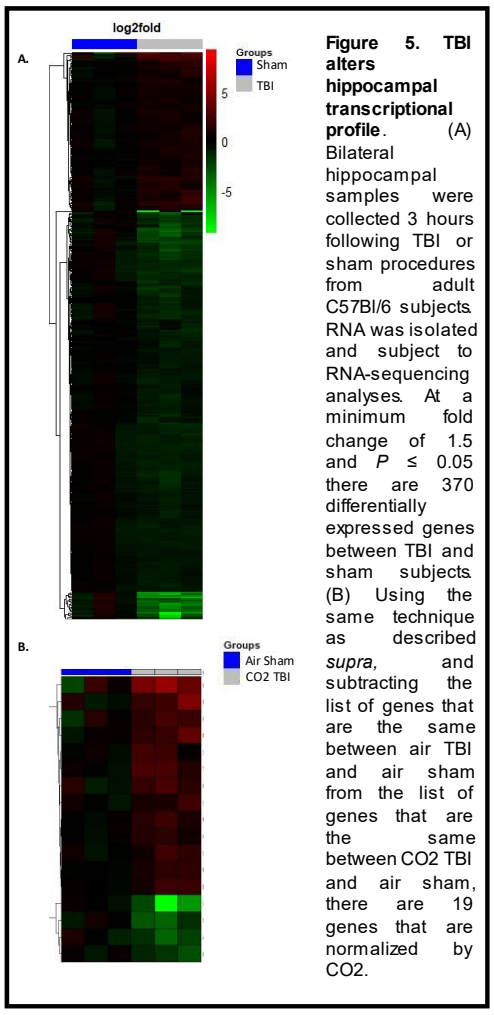

Figure 5. TBI alters hippocampal transcriptional profile. (A) Bilateral hippocampal samples were collected 3 hours following TBI or sham procedures from adult C57Bl/6 subjects. RNA was isolated and subject to RNA-sequencing analyses. At a minimum fold change of 1.5 and $P \leq 0.05$ there are 370 differentially expressed genes between TBI and sham subjects. (B) Using the same technique as described *supra*, and subtracting the list of genes that are the same between air TBI and air sham from the list of genes that are the same between CO2 TBI and air sham, there are 19 genes that are normalized by CO2.

expression take place indicates that these effects are due to the ability of CO_2 to affect the volume of blood in the cranial compartment and prevent slosh.

Using RNA sequencing, we have identified 370 genes that are differentially expressed in the hippocampus at the 3-hour timepoint between air sham and air TBI mice (Fig 5A). Further, of these 370 genes, 19 are recovered by CO_2 (i.e. are differently expressed between air sham and air TBI but not differently expressed between air sham and CO_2 TBI, Fig 5B). This demonstrates that CO_2 can reverse both physiological and molecular indicators of TBI and is promising for its potential as a preventative measure. Interestingly, CO_2 completely spared subjects from TBI; we should expect to see no difference in any of these genes between air sham and CO_2 TBI mice. CO_2, for all its ability to attenuate the effects of TBI, may not normalize all genes TBI affects and may have different effects on other genes that TBI *per se* doesn't affect.

Future Work

While slosh mitigation is putatively the primary mechanism by which CO_2 attenuates the effects of TBI, compared to IJV compression, CO_2 implicated in a much wider range of gene cascades throughout the body due to its physiological presence. It is therefore of interest whether CO_2 might prevent some sequelae through molecular mechanisms rather than slosh mitigation. Additionally, since gene expression is a valuable tool to detect types or localizations of injury, it is also of interest more generally to see which specific genes CO_2 changes the expression of in the context of TBI. This knowledge would help determine how much injury CO_2 prevents, which types of secondary sequelae CO_2 prevents, and how it does so.

Future work will be dedicated to finding the differentially expressed genes within the context of TBI and CO_2 exposure. Given the importance of immunoreactive secondary injury, we will be looking specifically at glial expression in addition to whole-brain expression of genes. We also hope to examine whether CO_2 prevents neurodegeneration and will be using tissue staining and microscopy to determine whether the microstructure of the brain is more intact and whether we observe less neurodegeneration in CO_2-preexposure TBI. In addition to the physiological markers of TBI, we also intend to run the same behavioral assays we have for TBI alone with CO_2-preexposure TBI to determine whether in addition to immediate physiological consequences, CO_2 can prevent long-lasting social and behavioral deficits. Finally, we intend to explore the dose and time dependence of CO_2's protective effects against TBI.

Given the proven efficacy of the Q-Collar, the promising preclinical results of CO_2 in mitigating TBI, and the similarity of their putative mechanisms of action, our work may lead to the development of future preventative measures against TBI. If the lines of research mentioned previously show CO_2 can reliably prevent primary and secondary injury in TBI, we will collaborate with biomedical engineers and clinical

researchers on interventions to leverage our preclinical CO_2 research into meaningful reductions in the incidence and severity of TBI.

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