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Original Research

Western diet induces mild metabolic impairment and aggravates neuropathology in an experimental mouse model of traumatic brain injury

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ABSTRACT

Traumatic brain injury (TBI) and lifestyle habits such as Western diet (WD) consumption represent two risk factors that affect an individual's health outcome globally. Individuals with TBI have a greater risk of mortality from associated chronic diseases than the general population. WD has been shown to impair cognitive function, decrease the brain's capacity to compensate for insult by affecting recovery as well as induce metabolic syndrome (MetS) which may be a risk factor for poor TBI prognosis. Hence, this study aims to investigate the impact of WD on TBI behavioral outcomes and neuropathology. Eight-week-old male C57BL6 mice were fed either WD or normal chow for 4 weeks prior to TBI induction. At week four, mice underwent either an experimental open-head TBI or a sham procedure. Mice continued their respective diets for four weeks after brain injury. Metabolic, cognitive function, and molecular assessment were performed four weeks after TBI. Results showed that while WD significantly increased fat percentage and elevated plasma cholesterol, there was no change in blood glucose level or body weight, indicating an early stage of MetS. Nevertheless, this was associated with neuroinflammation and impaired cognitive functions. However, there was no significant impact on cardiovascular function and mitochondrial bioenergetics. Importantly, the mild MetS induced by WD triggered basal motor, cognitive deterioration and exacerbated the long-term neuropathology of TBI. Taken together, our work highlights the magnitude of the contribution of lifestyle factors including the type of diet, even in the absence of overt metabolic consequences, on the neurobehavioral prognosis following TBI.

1. Introduction

Traumatic brain injury (TBI) is a significant global neurological disorder and the leading cause of mortality and disability among individuals under the age of 40.[1] The most common causes,

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according to the Centers for Disease Control and Prevention (CDC) in the United States (US), are violence, accidents, and sports.[2] There are approximately 288,000 TBI hospitalizations each year, with males accounting for 78.8%.[3] TBI can be classified based on type, severity, location, injury mechanism, and physiological response.[4] The injury severity significantly impacts the clinical symptoms and functional outcome of TBI.[3] TBI is a pathological change that affects the entire body on multiple levels. Due to the heterogeneity of TBI, targeting a single pathway may be obscured by the activation of multiple cascades.[5] The key roadblock restricting therapeutic advances in TBI research is the complexity of the biochemical and molecular signaling linkages involved in secondary injury.[6] Even with all the omics studies invested in understanding TBI pathophysiology and biomarkers,[7–10] there are no FDA-approved treatments for TBI to date. However, lifestyle factors such as diet intake have been shown to impact TBI outcomes.[11–14]

Current lifestyle trends such as the increased consumption of the Western diet (WD), a modern dietary pattern rich in fat, and simple sugars, and low in dietary fibers, have been associated with the incidence of health conditions such as dyslipidemia, hyperglycemia, hypertension, and obesity. A cluster of these conditions is referred to as metabolic syndrome (MetS).[15] WD is a major cause of obesity and a risk factor for dementia and other neurodegenerative disorders.[16] Regular consumption of WD has been linked to numerous physical and mental health issues.[17] In a recent exploratory study, WD decreased motor-muscular and sensory functions, increased inflammatory iNOS + Microglia, and increased amyloid-β peptide and phosphorylated Tau.[5] Several diet compositions have been employed in animal studies. Usually, diets containing 35%–60% fat, with a high concentration of saturated fatty acid (SFA), cholesterol, and significant levels of simple sugar and salt are often employed.[18] Individuals with TBI have a greater risk of mortality from associated chronic diseases such as diabetes, heart disease, and hypertension than the general population.[19]

Diets rich in fat such as high-fat diet (HFD) have been shown to suppress the brain's capacity to compensate for insult by decreasing brain-derived neurotrophic factor levels in the hippocampus,[20] impair cognitive and working memory in a controlled cortical impact (CCI) rat model,[21] and worsen hippocampal injury.[22] In studying the impact of diet on TBI outcomes, it is important to note that several diet formulations such as HFD (≥30% of energy from fat),[23] high fructose diet,[24] and high fat/sucrose (WD)[20] have been employed in several TBI models. WD alone can affect several organ systems in the body including the brain. When superimposed with brain injury, WD increases brain vulnerability and alters recovery patterns.[21] As such, varying dietary compositions led to different metabolic end-points, and thus there was no clear delineation of whether the impact on TBI was related to subtle direct neurometabolic changes or ensued as a complication of overt systemic metabolic disruption such as diabetes and/or obesity. Thus, it is crucial to understand the interplay between the early consequences of WD and TBI prognosis.

Both WD consumption and TBI were reported to be associated with a state of altered cerebral energetics.[14] Additionally, both involved a disruption of the gut barrier precipitating cerebrovascular as well as cardiovascular dysfunction.[25] Our recently published work showed worsening neuropathology and increased predisposition to neurodegeneration in mice fed high-fat (60% Kcal) and subjected to TBI.[13] However, HFD-fed mice in the former study developed clear signs of obesity (increased body weight) and type 2 diabetes manifesting as an increased blood glucose levels. Stemming from the interdependence between dietary components, a high-fat, high-carbohydrate diet is expected to culminate in a different cellular metabolic state and consequently alternate metabolic outcomes and possibly a disparate effect on TBI as opposed to a diet high in fat only.[26] Indeed, our previous work on animal models fed WD revealed an early state of metabolic deterioration characterized by subtle, yet consistently detrimental pathological signaling affecting the cardiovascular and cognitive function.[27,28] In the present study, we investigated the impact of a diet that mimics the Western diet (WD) by using a sucrose-supplemented HFD diet. We hypothesize that the combination of high fat and high sugar would precipitate a mild state of metabolic dysfunction, resulting in aggravation of long-term secondary injury associated with TBI possibly through the alteration of cerebral mitochondrial energetics.

2. Materials and method

2.1. Animals and experimental design

Animals were obtained from the animal care facility at the American University of Beirut (AUB), and all experimental procedures were performed in compliance with an experimental protocol (# 21-09-589, August 2020) approved by the AUB Institutional Animal Care and Use Committee (IACUC). Eight-week-old C57BL6 mice were housed in a temperature-controlled environment with 12 hr light/dark cycles. At the beginning of the experiment, mice were randomly divided into two groups: one group was fed normal chow (NC) and the other group was fed WD. The NC is composed of 14% kcal fat, 32% kcal protein, and 54% kcal carbohydrate (Teklad 8604 Envigo), and the in-house prepared WD is composed of 42% kcal fat, and 34% sucrose by weight, as used in the previous studies.[29] Mice were fed continuously with their respective diet for eight weeks. After 4 weeks of feeding, mice in each group were randomized 1:1 to either an open-head TBI or a sham procedure. Open-head TBI was induced using the CCI model. Afterward, mice were placed on their respective diets for an additional 4 weeks. Twenty-four hrs (24 hrs) after injury, 5-Bromo-2'deoxyuridine (BrdU) was administered intraperitoneally at a dosage of 100 mg/kg for three consecutive days. Mice were sacrificed 28 days after the last BrdU injection as shown in Fig. 1.

2.2. Power analysis and experimental groups

In the experimental design of this study, the number of animals per group for each experiment was selected to achieve a 90% power to detect a difference with a 95% confidence level. MWM is particularly prone to variation. Hence, based on our previously published studies, the optimal sample size required for this test is 9 mice per group.[13,30] For grip strength, rotarod, and pole climbing, a minimum of 8 mice per group was required to detect a difference among groups. For cardiovascular assessment, a minimum of 6 mice per group were required to obtain 90% power. In addition, a minimum of n = 3 per group was used for all molecular tests. A total number of 40 animals were used for this study: NC SHAM (n = 10); NC TBI (n = 10); WD SHAM (n = 10); WD TBI (n = 10). The results of all experiments were reported as mean ± SEM (standard error of the mean). The appropriate statistical test was done as described in the relevant Fig. legend using GraphPad Prism 8 and a $p < 0.05$ was considered significant.

2.3. Controlled cortical impact (CCI) injury procedure

Open-head TBI was performed using a CCI model. CCI is a highly reproducible model for brain injury, that uses an electromagnetic impact device to induce head injury. The injury was performed as discussed in our previous work.[21,13,31] Briefly, mice were anesthetized by an intraperitoneal injection of Ketamine and Xylazine mixture (50 mg/kg Ketamine and 15 mg/kg Xylazine). Upon loss of

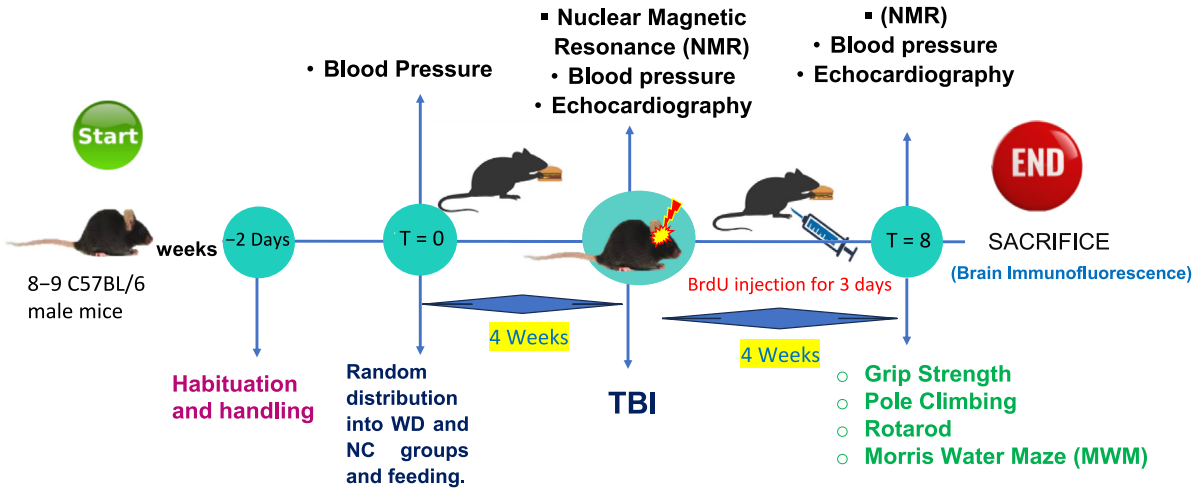

Fig. 1. Experimental timeline. 8–9 weeks old C57BL/6 mice were randomly assigned into two groups normal chow(NC) or western diet (WD), and fed continuously for 8 weeks. Blood pressure was initially measured before the distribution. At week 4, body fat composition, blood pressure, and echocardiography measurements were taken before half of the mice were subjected to an open-head Traumatic Brain Injury (TBI). After TBI, the mice were injected with BrdU for three consecutive days. At week 8, body composition and cardiovascular parameters were assessed again. Also, behavioral and neurological tests were conducted on the mice before sacrifice.

reflexes, the head fur was shaved gently, and the animal was fixed firmly on the stereotaxic device frame. An ophthalmic ointment (Xailin®, Nicox, France) was applied to the eyes to prevent dryness during surgery. The head was disinfected with 70% alcohol and iodopovidine (Betadine); then a midline incision was made on the skin using a surgical scalpel exposing the skull. A craniotomy was made on the somatosensory area between the Bregma and Lambda with standard coordinates +1.0 mm AP, +1.5 mm ML, and −2 mm DV. A unilateral injury was performed as the activated piston vertically above the brain contacted the exposed cortex for 1 s at a velocity of 4 m/s and a depth of 2 mm. The impactor tip has a diameter of 1 mm. Bleeding was controlled following the injury and the skin was sutured. Mice were placed on a warm pad to maintain body temperature at 37 °C. The sham groups underwent the same procedure without injury.

2.4. Nuclear magnetic resonance (NMR)

Mice were weighed and placed in a cylindrical restrainer. The cylindrical restrainer was inserted into the LF110 Minispec Nuclear Magnetic Resonance (NMR) machine (Bruker, MA, USA) (Fig. 2) to measure the percentage body fat, as previously described.[13,27] NMR was performed at 4 weeks (before TBI) and 8 weeks (before sacrifice, i.e., 4 weeks after TBI).

2.5. Noninvasive blood pressure

Noninvasive blood pressure was measured as previously described.[32] Mice were placed in the experimental room for 30 mins to minimize anxiety. They were gently placed in a restrainer that exposes the tail. The restrainer containing mice was then kept on a warm pad for 15 mins. When mice were stable and non-agitated, blood pressure was taken using the CODA® Tail-cuff High Throughput Monitor (Kent Scientific, Torrington, CT). Blood pressure was measured at weeks 0, 4, and 8.

2.6. Echocardiography

Mice were anesthetized by intraperitoneal injection of Ketamine and Xylazine mixture (50 mg/kg Ketamine and 15 mg/kg Xylazine).

Fig. 2. The LF110 minispec (Bruker, MA, USA) Nuclear Magnetic Resonance Machine. Body fat composition was assessed using the LF110 minispec (Bruker, MA, USA) Nuclear Magnetic Resonance (NMR) Machine. This was done at week 4 (before TBI) and week 8 (after TBI).

Chest fur was shaved using a sterile blade. Heart structure and function were assessed using the SonixTouch Q+ ultrasound (BK ultrasound, Peabody, MA), and echocardiography along the parasternal long axis M- and B-mode was done at weeks 4 and 8 as previously described.[33,34]

2.7. Pole climbing

Pole climbing was performed to evaluate motor coordination in mice.[35] The apparatus consists of a rough-surfaced pole 60 cm in length and 1cm in diameter fixed upright in an empty box. Mice

were habituated to the experimental room for 30 min. Each mouse was placed on the pole with its head facing upward and allowed to descend freely.[35] The time needed for the mouse to make a U-turn and the time needed to reach the base of the pole was recorded. Each mouse was trained twice and then three consecutive testing trials were carried out.

2.8. Grip strength

Grip strength was used to evaluate neuromuscular function.[36] The grip strength meter (Cat. No. 47200, Ugo Basile, Gemonio, Italy) consists of a wire gauge and a digital detector that records the maximum grip force of the mouse. After habituation for 30 min, mice were held by the tail and allowed to grip the wire gauge, and then gently pulled. The maximum force and total force were recorded. Three trials were done per mouse and the mean grip strength was normalized to the weight of the mouse.

2.9. Rotarod

To measure motor coordination and motor learning abilities, accelerated rotarod tests (4–40 rpm) were carried out for a maximum of 5 mins. The rotarod device (Ugo Basile, Gemonio, Italy, model 47,600) has a horizontal rod 3 cm in diameter and five lanes, each 5 cm wide.[37] The time required for a mouse to slip off the accelerating rod was measured. Animals were trained twice on the apparatus after which three trials were performed with a 10-min interlude. For analysis, the average of the three trials was calculated for each mouse.

2.10. Morris water maze (MWM)

Spatial learning and memory were assessed using the MWM test similar to previous published studies.[13,30,38,39] Mice were tested for five consecutive days; with three learning trials per day for the first four days, followed by a probe trial (spatial reference memory test) on the fifth day. The water maze pool was filled to two-thirds its depth with water and the temperature was maintained at 25 ± 2 °C. Nontoxic white tempera paint was added to render the water opaque. The pool was virtually divided into four quadrants of equal area (NE, NW, SE, and SW). The escape platform was submerged 1cm below the water level at the center of the NE quadrant and was kept in the same position throughout the learning trials. Three visual cues were placed on the walls around the water maze for mice. A visual cue test was conducted before the learning trials to assess sensorimotor ability and motivation. For this test, a flag was placed on the platform so that mice can locate the platform using a visual stimulus rather than depending on spatial orientation to extra-maze cues. During the learning trials, mice were gently placed tail-first into the pool facing the wall at one of the three quadrants not containing the platform. When mice were placed in the pool, the researcher moved to a constant position to serve as an additional distal visual cue. The ANY-maze software (Stoelting Co., Wood Dale, Illinois, IL, USA) was used to track mice movement simultaneously. Maximum swim time was set at 60 s and each recording ended automatically when the mouse located the platform before the 60 s mark. The mouse was then immediately removed from the pool, dried with a clean towel, and placed on a heated pad for 5 mins to ensure complete dryness before being returned to its home cage. Tests were conducted at approximately the same time each day to minimize variability in performance due to the time of day. During the probe test, the platform was removed from the pool, and the mouse was allowed to swim freely for 1 min. The latency to reach the quadrant housing the platform and time spent in the right quadrant were recorded.

2.11. Sacrifice and brain tissue preparation

Upon completion of all neurobehavioral tests, all animals were sacrificed. Animals were alternated to provide brain tissue for the BrdU assay, fixation and immunofluorescence, or isolation of mitochondria from fresh brain tissue. Brain tissue was extracted after perfusion and fixed for the purpose of sectioning. Mice were anesthetized by intraperitoneal injection of Ketamine and Xylazine mixtures (50 mg/kg Ketamine and 15 mg/kg Xylazine). Blood samples were collected from the heart using a 10mL syringe. To fix the brain tissue, mice were perfused with 30 mL phosphate-buffered saline (PBS). This was followed by tissue fixation using 10 mL of 4% paraformaldehyde (PFA). After fixation, the head was removed, and a mid-line incision was done to expose the skull. The skull was carefully removed using scissors and forceps. The brain was then dissected from the underlying tissue and stored in 4% PFA at 4 °C for 48 hrs. After 48 hrs, the brain was removed from PFA and placed in a 30% sucrose solution for three days before sectioning. Brains were coronally sectioned using the cryo-microtome (Leica Microsystems, USA) at 40μm, and, sections were stored in a 24-well plate containing sodium azide at 4 °C.

2.12. Mitochondria isolation

Mitochondria were isolated from fresh brain tissue similar to previous studies.[40] Fresh brain tissue was extracted from mice and placed in a 50 mL conical tube containing PBS. Brain tissue was maintained on ice throughout the experiment to minimize protein degradation. The injured right hemisphere of the brain was isolated using a blade and weighed. The tissue was chopped into pieces using a surgical blade. The chopped tissue was suspended in 1.2 mL ice-cold extraction medium (250 mM sucrose, 10 mM Tris-HCl, 0.5 mM EDTA, pH adjusted to 7.4). The tissue was homogenized by 8–9 strokes with a precooled potter-Elvehjem Teflon-glass homogenizer. The homogenate was placed in a 1.5 mL conical tube and centrifuged for 6 min at 1000 g at 4 °C. After centrifugation, the supernatant was transferred into a new 1.5 mL tube and centrifuged at 10,000 g for 8 min at 4 °C. The supernatant was discarded and the crude mitochondrial pellet was resuspended in a 50 μL extraction medium. Protein concentration was measured using the Bradford method.

2.13. Assessment of mitochondrial substrate oxidation

Mitochondrial substrate oxidation was assessed polarographically using a Clark oxygen electrode (Hansatech Instruments, Norfolk, England) in a magnetically-stirred chamber maintained at 37 °C. Substrate and inhibitors were added to a respiratory medium consisting of 30 mM K_2HPO_4 (pH 7.2), 0.25 M Sucrose, 15 mM KCl, 1mM EGTA, 5 mM MgCl2.6H2O, and 1 mg/mL fatty acid-free bovine serum albumin, as described below. 250 μL of the respiratory medium was added to the chamber and allowed to stand for a few minutes until a stable oxygen readout was obtained. 10–12 μg protein of the freshly isolated mitochondria were then added. To induce complex I-linked oxidative phosphorylation (OXPHOS), 10 mM of glutamate and 10 mM of malate were added. This was followed by the addition of 1mM of ADP in order to initiate coupled oxygen consumption that was recorded for 3 mins. Afterwards, 15 μM of oligomycin were added for one minute in order to inhibit the activity of ATP synthase. This was followed by the addition of 120 μM of FCCP (uncoupler) to obtain maximum uncoupled oxygen consumption. Finally, 0.3 mM of cyanide (KCN) was added to block mitochondrial-related oxygen consumption. For complex II-associated OXPHOS, 10 mM of succinate was added into the cleaned chamber containing 250 μL of the respiratory medium.

When a steady oxygen consumption rate was reached, 1mM of ADP was added to induce coupled oxygen consumption that was measured for 3 mins. Afterward, 15 μM of oligomycin was added in order to inhibit the activity of ATP synthase. After one minute 120 μM of FCCP (uncoupler) was added to obtain maximum uncoupled oxygen consumption. Finally, 10 mM of malonate was added to inhibit complex II-mediated oxygen consumption. State 3 is defined as the ADP-stimulated oxygen consumption rate and state 4 is the oxygen consumption rate after ADP exhaustion. The respiratory control ratio (RCR) is the ratio of state 3 over state 4 and measures the tightness of mitochondrial coupling.

2.14. Total cholesterol

Plasma samples of mice were assessed for total cholesterol using the vitros 350 Chemistry system (Ortho-Clinical Diagnostics, Raritan, NJ, USA).

2.15. BrdU assay

BrdU assay was performed on free-floating brain sections to assess mature proliferating cells after TBI, as previously described.[13] Three brain sections per animal and three animals per group were used for the experiment. Brain sections were washed in PBS thrice for 5 min on a shaker. Then sections were incubated in 2N HCl for 30 min at 37 °C, followed by incubation in sodium borate for 10 min at room temperature on a shaker. After 10 min, sodium borate was discarded, and sections were washed in PBS thrice for 5 mins. The sections were blocked in 10% FBS containing 0.1 M glycine at 4 °C for 1 hr. After blocking, sections were incubated with 1:500 rat anti-BrdU antibody (Abcam ab6326, Abcam, Cambridge, UK) in PBS overnight at 4 °C. The following day, sections were washed three times in PBS and incubated with 1:500 fluorophore-conjugated goat anti-rat in PBS for 2 hrs in the dark at 4 °C. After 2 hrs, the secondary antibody was discarded, and the sections were incubated in Hoechst dye for 10 min. Then sections were washed three times in PBS for 10 mins on a shaker and mounted on a glass slide using the fluoromount (F4680-25ML, Sigma Aldrich). The slides were viewed using the Zeiss Axio observer microscope (Carl Zeiss, Oberkochen, Germany) with a 20X objective lens.

2.16. Immunofluorescence

Immunofluorescence staining was performed to examine biomarkers and protein expression. Free-floating sections were washed in PBS once and in 0.1 PBST thrice. Antigen retrieval step was performed by incubating sections in citrate buffer for 20 min at 80 °C in a water bath. After the antigen retrieval step, sections were allowed to cool and washed thrice in PBS. Then sections were blocked with 10% PBST containing 0.1 M glycine for 1 hr at room temperature. Following blocking, sections were incubated with primary antibodies diluted in 1% PBST overnight at 4 °C. Primary antibodies used were anti-GFAP (1:1000 dilution; Ab 7260; Abcam, Cambridge, UK), anti-IBA-1 (1:500 dilution; 019-19471, Fujifilm Wako Chemicals, Richmond, Virginia, USA), anti-NeuN (1:1000 dilution; RPCA-FOX3-AP, Encor Biotechnology, Gainsville, FL), anti-SMI 312 (pan axonal, cocktail; 1:500 dilution; Ab 24,574; Abcam, Cambridge, UK). After primary antibodies incubation, sections were washed 3 times for 10 min and conjugated with appropriate secondary antibodies for 1 hr at room temperature. Secondary antibodies used were Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) ab150077, Goat Anti-Mouse IgG H&L (Alexa Fluor® 488) ab150113, Goat Anti-Mouse IgG H&L (Alexa Fluor® 568) ab175473. Three more washes in PBST were done for 10 min each to remove excess antibodies. Sections were incubated with Hoechst for 10 min and washed three times before mounting on slides using fluoromount (F4680-25ML, Sigma Aldrich). Images were taken using the confocal microscope (Carl Zeiss, Oberkochen, Germany) with 40X objective lens.

3. Results

3.1. Effect of WD and TBI on metabolic parameters

Regular assessment of body weight and blood glucose levels confirmed the early nature of metabolic impairment triggered by eight weeks of WD intake in mice. Biweekly examination of body weight showed no significant change among the four groups over the eight-week course of treatment (Fig. 3A). Nevertheless, WD feeding induced a marked body fat accumulation by the eighth week of feeding in both TBI and Sham groups rendering the percentage body fat significantly higher than in their NC-fed counterparts (Fig. 3B). Along the same lines, blood glucose levels measured weekly did not differ statistically among groups (Fig. 3C). To examine potential systemic metabolic alterations induced by WD and TBI, total cholesterol level was measured in plasma. The results showed a significant increase in the total cholesterol levels in the WD groups compared to the NC groups (Fig. 3D). Intriguingly, total cholesterol levels in WD-TBI were slightly, yet significantly, lower than WD SHAM and significantly higher than NC-TBI. The former is consistent with human studies reporting post-injury hypocholesterolemia in TBI subjects and its association with injury severity.[41]

3.2. Effect of WD and TBI on neurological functions

To investigate the impact of WD and/or TBI on neuromuscular function, the grip strength test was performed on mice. Both NC TBI and WD TBI groups performed significantly worse on the test compared to the NC SHAM (Fig. 4A). To further assess motor coordination, rotarod, and pole climbing tests were performed. There was no statistical difference observed among groups in the rotarod test (Fig. 4B); however, in the pole climbing test, significant worsening after TBI was only observed in the WD-fed mice, performing worse than NC- and WD-fed SHAM mice (Fig. 4C).

3.3. WD and TBI impairs spatial learning memory

To assess the effect of WD and/or TBI on spatial learning and memory in mice, MWM test was performed. The result showed that, unlike other groups, WD TBI took significantly more time to locate the hidden platform compared to the NC SHAM group in three out of four days (Fig. 5A). On the probe test day, when the hidden platform was removed, the latency to the platform quadrant and percentage time mice spent in the NE quadrant was measured as a parameter of their spatial memory. As observed in the results and despite the lack of overt metabolic dysfunction, WD feeding impaired spatial memory even prior to TBI and the performance continued to worsen after TBI only in the NC-fed (Fig. 5B). These results were reflected in the spent less time in the NE quadrant (Fig. 5C). This suggests that both injury and diet not only act synergistically but can also impair cognitive function independently.

3.4. Effect of WD and TBI on cardiovascular functions

Indeed, autonomic control of the cardiovascular system is known to deteriorate significantly as a result of a worsening metabolic insult.[42] To investigate the effect of WD and TBI on cardiovascular function, blood pressure (Figs. 6A and B), left ventricular ejection fraction (Fig. 6C) and fractional shortening

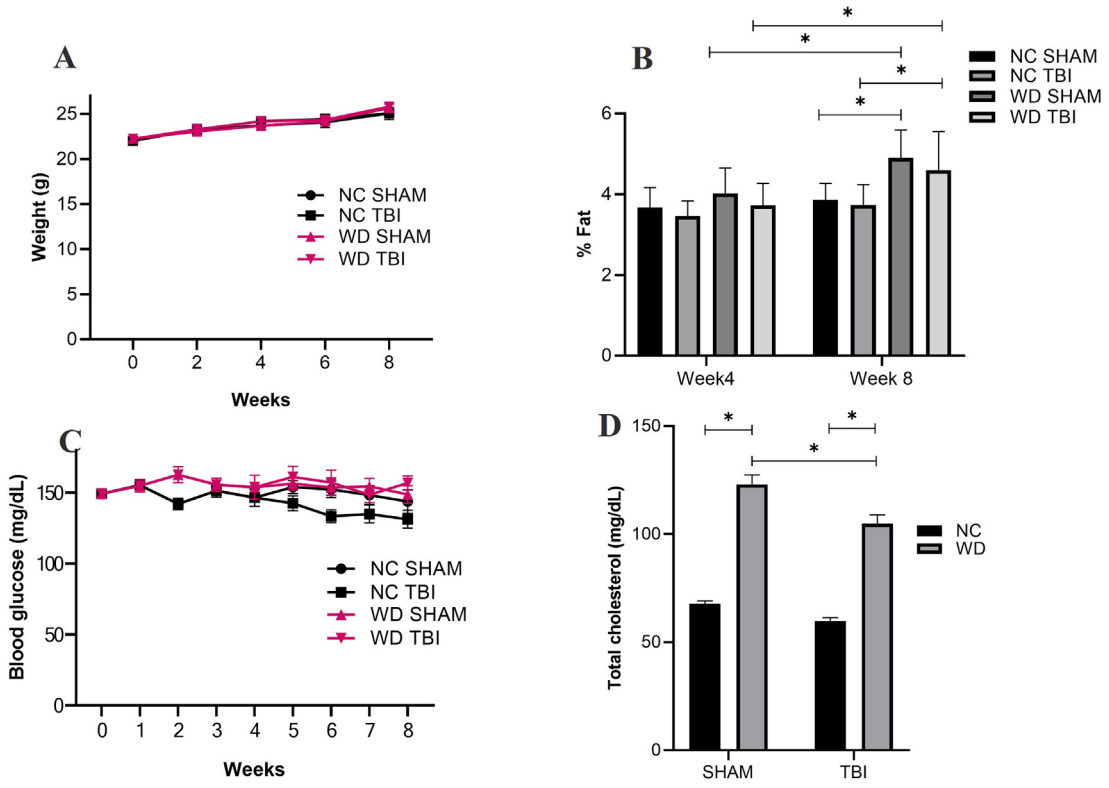

Fig. 3. Western diet (WD) alters some metabolic parameters in mice. **(A)** Body weight changes measured biweekly, **(B)** percentage body fat measured at week 4 and week 8 before sacrifice, **(C)** random blood glucose measured weekly for 8 weeks (n = 9/group), and **(D)** total cholesterol levels (n = 4/group). Statistical significance was determined using three-way ANOVA for **A**–**C** and two-way ANOVA followed by Tukey's multiple comparison test for **D**. Data are presented as mean ± SEM, *$p < 0.05$.

(Fig. 6D) were measured. Confirming the mild nature of metabolic impairment triggered by WD, our results showed no deterioration of the cardiovascular parameters measured in the WD-fed mice. Importantly, no further changes were detected after TBI in either group.

3.5. Effect of WD and TBI on mitochondrial bioenergetics

The brain is extremely reliant on a steady supply of oxygen and glucose to sustain cellular integrity. Mitochondria play a key role in neuronal cell survival and mitochondrial dysfunction is implicated in the early stage of central nervous injury. Mitochondrial dysfunction in TBI was shown to impair glucose utilization in the brain.[43] To examine the effect of WD and TBI on mitochondrial bioenergetics, polarography studies on freshly isolated brain mitochondria were performed. Complex-I and Complex-II linked oxygen consumption was investigated. The results showed no significant difference in both complex I and II induced oxygen consumption between groups after four weeks of TBI (Figs. 7A and B, respectively). The RCR values of glutamate and malate-induced oxygen consumption, although showing variability, indicated well-coupled mitochondria (Fig. 7C).

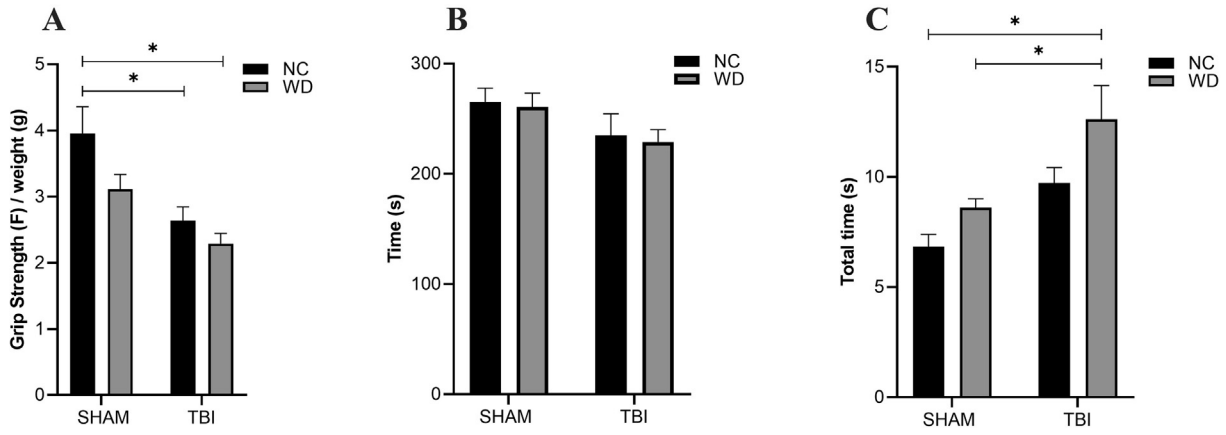

Fig. 4. WD and TBI worsen neuromuscular function in mice. **(A)** Neuromuscular function assessed by grip strength test as maximum grip strength normalized by weight, **(B)** motor coordination test by rotarod measured as latency to fall from the rotarod, **(C)** and pole climbing time taken to reach the bottom of a vertical pole (n = 10). Statistical significance was determined using two-way ANOVA followed by Tukey's multiple comparison test. Data are presented as mean ± SEM, *$p < 0.05$.

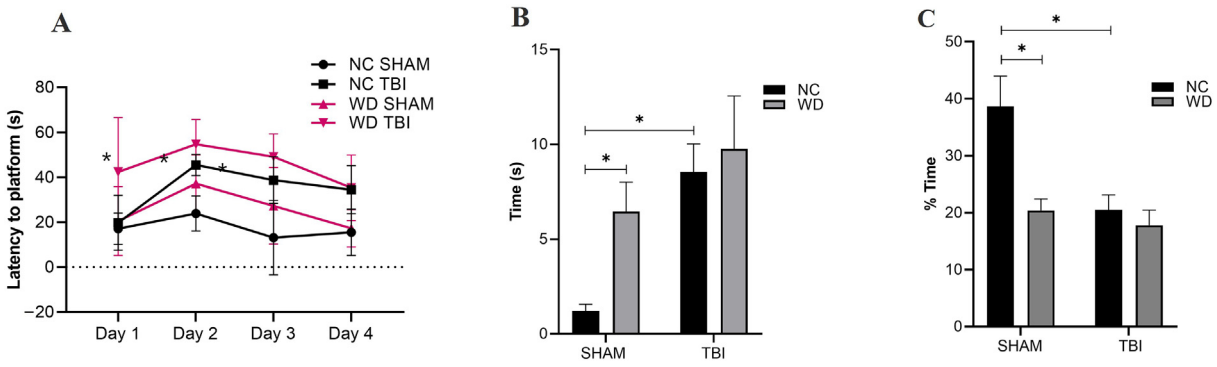

Fig. 5. Impact of WD and TBI on Cognitive Function of spatial learning and memory in MWM. MWM test was done 30 days post-TBI to assess the effect of WD and TBI on spatial learning and memory in mice. Mice were trained for four consecutive days followed by a probe trial on day 5. **(A)** Latency to the hidden platform during the training trials, **(B)** latency to the NE quadrant on the probe day, and **(C)** time spent in the NE quadrant on the probe day. Statistical significance was determined using three-way ANOVA for **A** and two-way ANOVA followed by Tukey's multiple comparison test for **B** and **C**. Data are presented as mean ± SEM, $*p < 0.05$.

3.6. WD and TBI aggravate neuroinflammation

Neuroinflammation is a repair mechanism initiated by the brain in response to TBI. It involves the activation and recruitment of astrocytes, microglia cells, inflammatory mediators, and other immune cells.[44] The impact of WD and TBI on neuroinflammation was assessed using immunolabeling for GFAP (astrocyte marker) and IBA-1 (microglia marker). Results of GFAP immunolabeling revealed that, as expected, TBI significantly increased astrogliosis in hippocampi and cortices of mice on NC or WD, with higher basal activation in the hippocampi of WD SHAM mice (Figs. 8A–C, respectively). On the other hand, results of IBA-1 immunolabeling showed that WD consumption significantly elevated microgliosis in mice hippocampi (Figs. 9A and B) and that WD TBI mice had significantly higher hippocampal and cortical microglial activation compared to NC SHAM (Figs. 9A–C, respectively).

3.7. WD and TBI exacerbated neuronal cell loss

To investigate the effect of WD and TBI on mature neuron cells, neuronal nuclear antigen (NeuN) protein expression was assessed by immunofluorescence staining. There was a significant loss of neurons in the WD TBI group in the hippocampus and cortex compared to NC SHAM (Figs. 10A–C). TBI alone resulted in a significant loss of neurons in the cortex. In the hippocampus, WD along with TBI synergically led to a significant loss of neurons.

3.8. The effect of WD and TBI on axonal degeneration

Neurofilaments (NF) play a role in the growth and stability of axons in both central and peripheral nerves in addition to maintaining mitochondrial stability and microtubule content.[45] To assess axonal degeneration following TBI, immunofluorescence

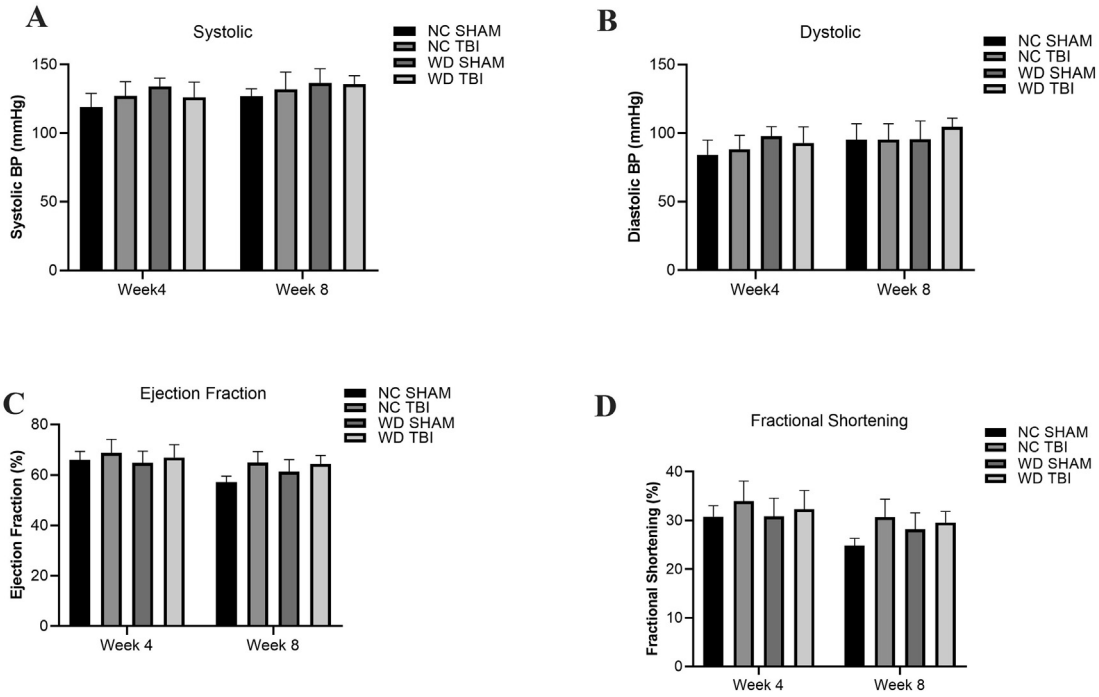

Fig. 6. Effect of WD and TBI on cardiovascular function assessed in mice before- (week 4) and 4 weeks after TBI (week 8). The non-invasive CODA device was used to measure **(A)** systolic blood pressure and **(B)** diastolic blood pressure, while echocardiography was used to measure **(C)** ejection fraction and **(D)** fractional shortening ($n = 9$ per group). Statistical significance was determined using three-way ANOVA. Data are presented as mean ± SEM, $*p < 0.05$.

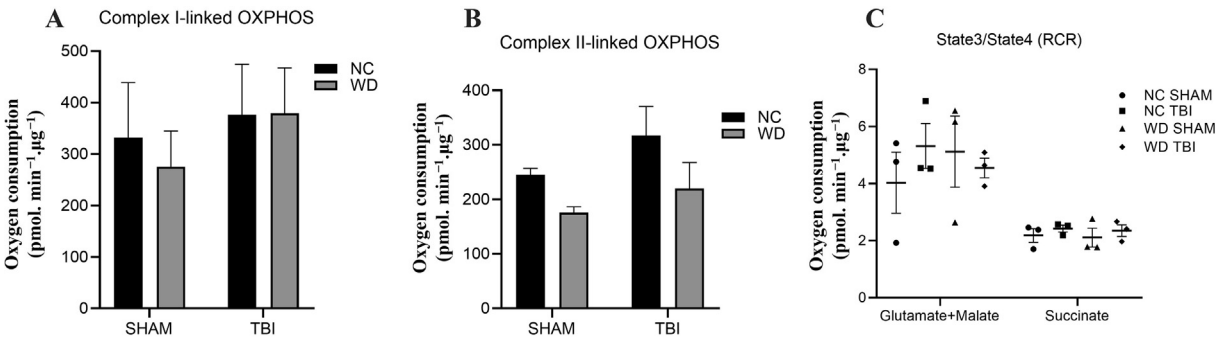

Fig. 7. Mitochondrial oxidative capacities were assessed in isolated mitochondria from the ipsilateral hemisphere in different treatment groups by polarography. (A) Complex I-mediated oxygen consumption is induced by glutamate and malate). **(B)** Complex II-mediated oxygen consumption is induced by succinate. **(C)** Respiratory control ratio (RCR) (state3/state 4) for complex I and complex II-mediated oxygen consumption indicative of the coupling state of extracted mitochondria. Statistical significance was determined using two-way ANOVA followed by Tukey's multiple comparison test. Data are presented as mean ± SEM, $*p < 0.05$.

staining for neurofilaments using a pan axonal NF-cocktail antibody (NF-H, NF-L, and NH-M) was performed using the SMI-312 antibody. The SMI-312 antibody contains a mixture of monoclonal antibodies used to immunolabel complex networks of axons. It labels NF-L, NF-M, and NF-H axonal epitopes that are extensively phosphorylated.[46] Both WD and TBI, alone or together, significantly decreased immunolabeling of SMI-312 in the hippocampus (Figs. 11A and B) but not in the cortex (Figs. 11A and C).

3.9. Effect of WD and TBI on cell proliferation

We assessed the effect of WD and TBI on cell proliferation four weeks post-TBI by immunofluorescence staining for BrdU. Results showed that TBI significantly promoted neuronal cell proliferation as a repair mechanism in the hippocampi of WD-fed mice (Figs. 12A and B). Additionally, WD significantly increased cell proliferation in the hippocampi of TBI mice. WD TBI mice demonstrated significantly higher hippocampal and cortical cell proliferation compared to NC SHAM (Figs. 10A and B and C, respectively).

4. Discussion

Metabolic syndrome (MetS) is a cluster of metabolic derangements characterized by, but not limited to, elevated blood pressure (BP), hyperglycemia, insulin resistance, and dyslipidemia in addition to obesity.[47] Lifestyle choices and diet remain among

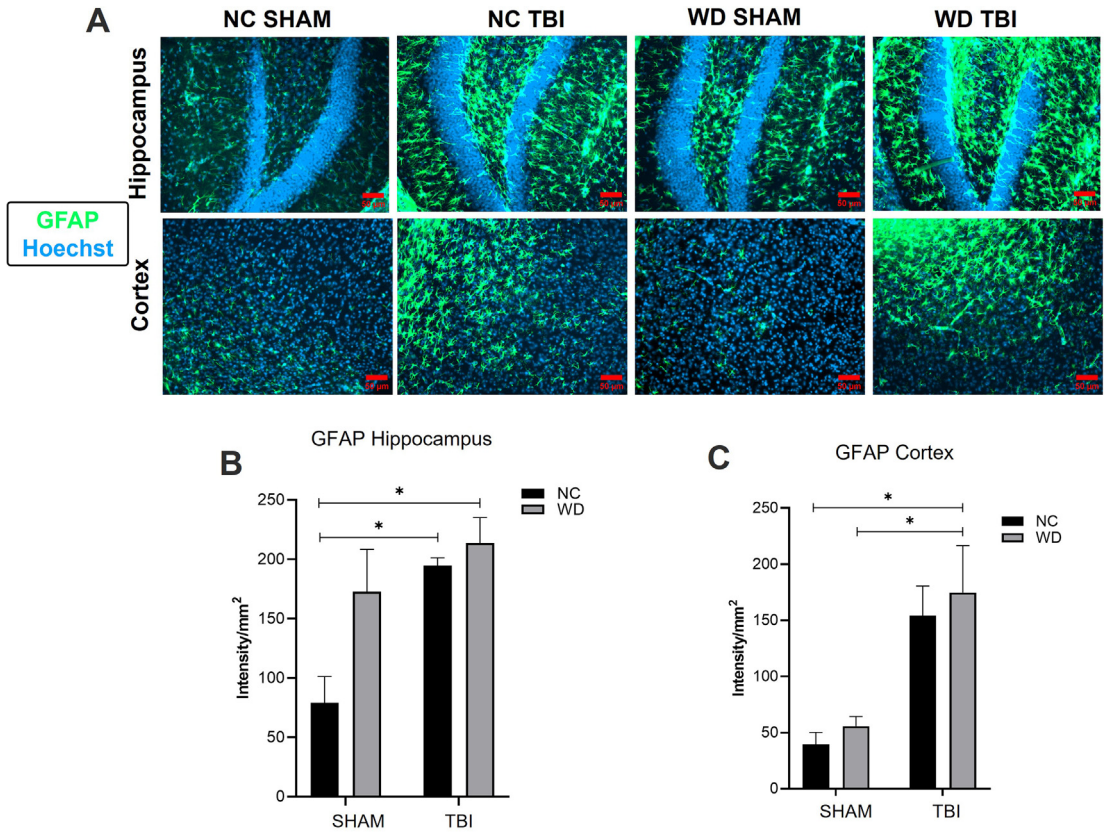

Fig. 8. Glial Fibrillary Acidic Protein (GFAP) expression in the ipsilateral cortex and hippocampus from different treatment groups. (A) Representative immunofluorescence micrographs of GFAP (green) expression in hippocampus and cortex. **(B)** Quantitative analysis of GFAP in hippocampus and **(C)** cortex, $n = 3$ per group (9 sections from 3 mice in each group). All images were taken at 20x objective lens. Statistical significance was determined using two-way ANOVA followed by Tukey's multiple comparison test. Data are presented as mean ± SEM, $*p < 0.05$.

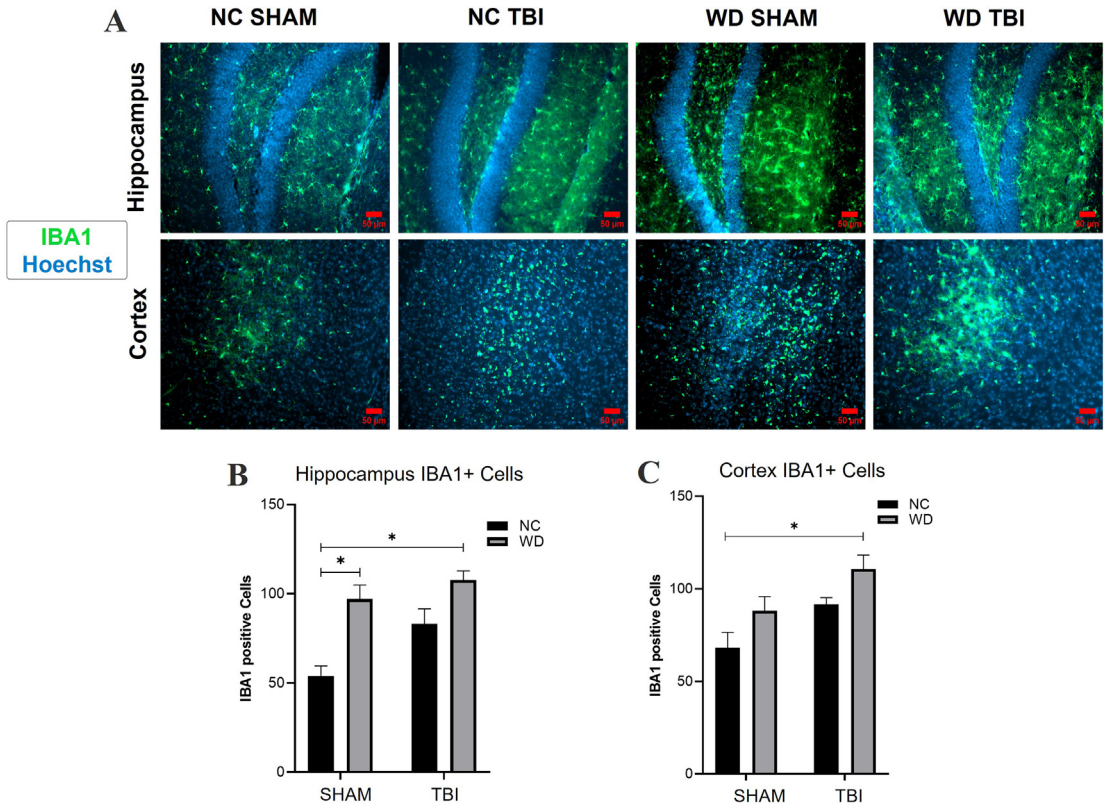

Fig. 9. Ionized calcium-Binding Adapter molecule 1 (IBA1) expression in the ipsilateral cortex and hippocampus from different treatment groups. (A) Representative immunofluorescence micrographs of IBA-1 (green) expression in the hippocampus and cortex. **(B)** Quantitative analysis of IBA-1 in the hippocampus and **(C)** cortex, $n = 3$ per group (9 sections from 3 mice in each group). All images were taken at 20x objective lens. Statistical significance was determined using two-way ANOVA followed by Tukey's multiple comparison test. Data are presented as mean ± SEM, $*p < 0.05$.

the most significant determinants of health outcomes. In fact, the increased shift towards the consumption of a western diet, coupled with a sedentary lifestyle, has led to a significant increase in MetS.[48] The functional deterioration associated with MetS has been reported to induce significant cognitive impairment, predispose to neurodegeneration, and could aggravate the functional deficits associated with several neurological disorders.[14,16] Interestingly, previous studies have reported that obesity is associated with an increased cognitive deficit, worsened injury severity, and a higher risk of mortality post-TBI.[49] On the other hand, TBI is a pathological insult to the brain, that affects the entire body on multiple levels.[43] The functional deficits associated with the secondary brain injury that develops after the original insult can be aggravated in TBI victims, who also suffer from co-morbid conditions like heart disease, diabetes, and/or obesity.[46] Interestingly, WD intake, which is a significant risk factor for MetS, has been reported to induce adverse neurological effects.[50]

With the unavailability of an FDA-approved treatment for TBI, the influence of nutrition and diet on the outcome of this injury has gained significant attention over the past decade. While studies have shown that different forms of diet could ameliorate or exacerbate post-TBI effects,[22,13,50–52] little is known about how unhealthy lifestyle and diets such as WD influences TBI outcomes. This comes at a time when a significant number of people are working from home, a process that has led to an increasingly sedentary lifestyle,[53] thus adversely affecting neurological health. Recent studies from our laboratory demonstrated that mice placed on high-fat diet (HFD) pre- and post-TBI, experienced a worsened functional outcome after the injury,[13] in the context of increased body weight and elevated blood sugar levels characteristic of type 2 diabetes. In the present study, we used a different diet formulation which is more akin to the conventional WD with a composition high in both refined sugar and saturated fat. Similar formulations were shown to induce a slow change in body composition and a mild state of metabolic dysfunction that was also associated with cognitive deterioration.[27] This study aims to understand whether and how WD-induced early stages of MetS influence the outcome of TBI at a chronic time point despite the absence of overt metabolic and cardiovascular complications. Our data showed that WD worsened TBI outcomes by impairing cognitive function, exacerbating neuroinflammation, and neuronal cell loss, which are likely not direct consequences of overt cardiometabolic dysfunction such as that observed in obesity and type 2 diabetes. Such changes occurred independently and synergistically highlighting the detrimental nature of WD even in the absence of an injury as shown in our previous work.[27] Indeed, these observations emphasize the impact of sub-optimal lifestyle and the importance of dietary control in the outcomes of TBI even in seemingly healthy individuals.

Following eight weeks of feeding, WD increased body fat percentage in mice despite the absence of significant differences in body weight and blood glucose levels among groups. Such an observation is not uncommon where similar diets given for 2–3 months had no significant effects on body weight and blood glucose.[5,27,28] However, the metabolic challenge imposed by the WD was demonstrated in an increased body fat composition and hyperlipidemia, both shown to be associated with states of low-grade inflammation and functional deterioration in our previous work.[27,28,54] Although the blood-brain barrier (BBB) is tightly controlled under normal circumstances, metabolic stressors like

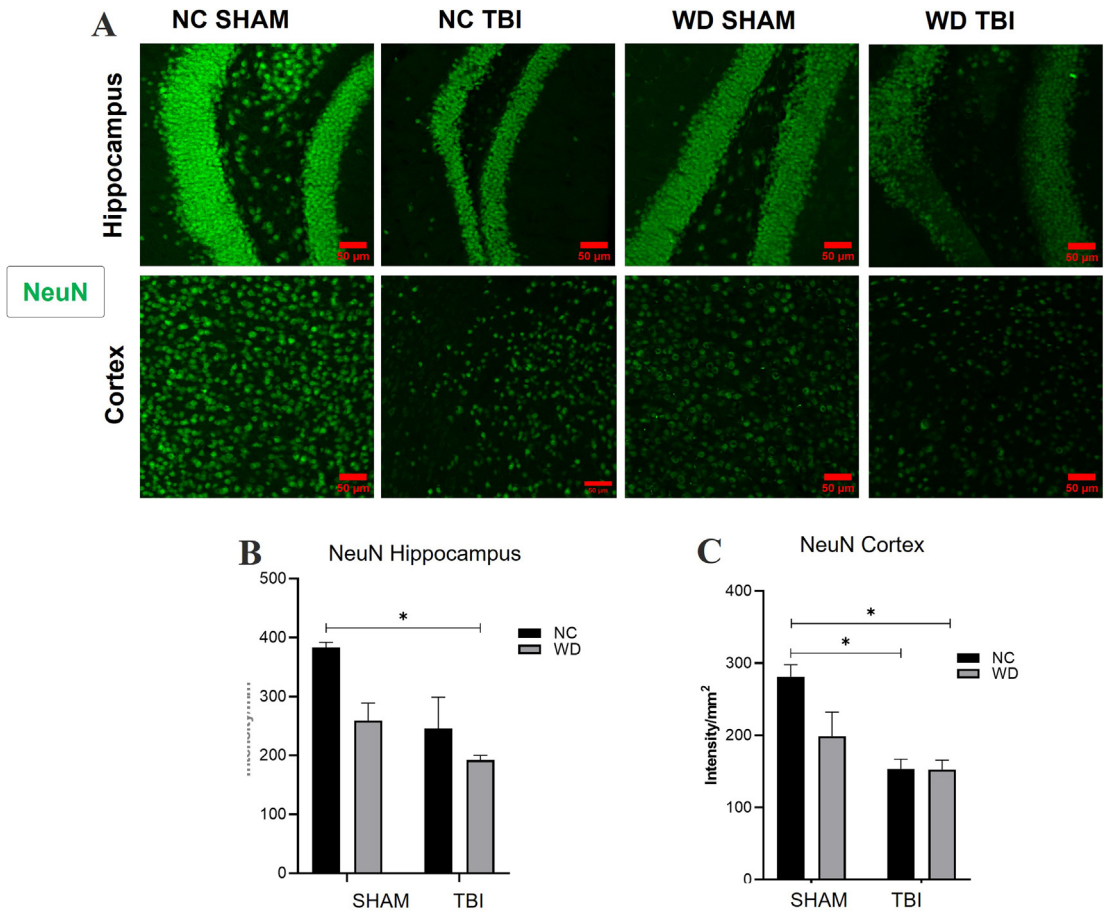

Fig. 10. Assessment of neuronal cell loss in hippocampi and cortices of different treatment groups. (A) Representative immunofluorescence micrographs of Neuronal Nuclear antigen protein (NeuN) (green) expression in the hippocampus and cortex. **(B)** Quantitative analysis of NeuN in the hippocampus and **(C)** cortex, $n = 3$ per group (9 sections from 3 mice in each group). All images were taken with a 20x objective lens. Statistical significance was determined using two-way ANOVA followed by Tukey's multiple comparison test. Data are presented as mean ± SEM, $*p < 0.05$.

hyperlipidemia can impair neurovascular units and increase BBB permeability.[55] Hyperlipidemia results from aberrant systemic lipid metabolism and is defined by increased levels of plasma total cholesterol, total triglycerides, and low-density lipoprotein cholesterol, as well as decreased high-density lipoprotein cholesterol levels.[21] Although total cholesterol was elevated, heart muscle contractility and efficiency, as well as blood pressure levels, were similar among groups indicating the early and subtle nature of the metabolic insult.

We further assessed the impact of WD on functional outcomes following TBI. Our data revealed that WD and TBI synergically impacted neurological and cognitive functions. A significant decrease in neuromuscular function was observed whereby TBI-WD mice performed particularly poorly in the grip strength and pole climbing tests. Moreover, mice fed WD and subjected to TBI showed a deficit in spatial memory as demonstrated in the MWM training phase. Interestingly, the probe trial showed that WD on its own impaired spatial memory performance even in the absence of TBI. Our findings are similar to previous studies that reported that a high fat/sucrose diet worsened mice performance on adhesive removal and working memory tests.[25] Furthermore, the said diet resulted in a significant loss of cortical tissue post-CCI compared to the standard diet-fed group.[21] A previous study reports that WD consumption resulted in cognitive dysfunction secondary to alteration of brain metabolism, activation of brain inflammatory responses, and increases in BBB transport.[56] In comparison to lean mice, obese C57 BL/6 mice fed WD were examined for the effects of subsequent injury following a closed head injury with a single hit (mild TBI). The obesity-induced disruption of the hypothalamic-pituitary-adrenal (HPA) axis was responsible for the obese mice's substantial microglial activation and inflammatory state at a chronic time point (4 weeks).[46] Obese C57 BL/6 mice fed WD displayed a higher level of anxiety than lean mice in an open-field test.[46]

Following TBI, there is an increase in cerebral glucose metabolism as a result of mitochondrial malfunction.[57] The integrity of the mitochondria is primarily compromised as a result of the initial failure of respiratory function resulting from an upsurge in reactive oxygen species.[58] In TBI pathology, mitochondrial dysfunction results in energy depletion and inadequate oxygen supply to the traumatized brain, which subsequently triggers anaerobic metabolism leading to acidosis that poses a detrimental effect on the brain tissue. Cell damage is aggravated by the imbalance between increased energy demand for cell repair and decreased energy production resulting from mitochondrial dysfunction.[43] Mitochondrial damage can trigger both apoptotic and necrotic cell death pathways, which may contribute to neurodegeneration associated with several diseases and disorders including MetS.[27,59] Even after the attainment of adequate cerebral perfusion pressure and energy metabolism substrate restoration, the metabolic crisis persists.[60] Indeed, secondary injury processes were reported to sustain mitochondrial dysfunction.[61] Intriguingly, at week 8, mitochondrial

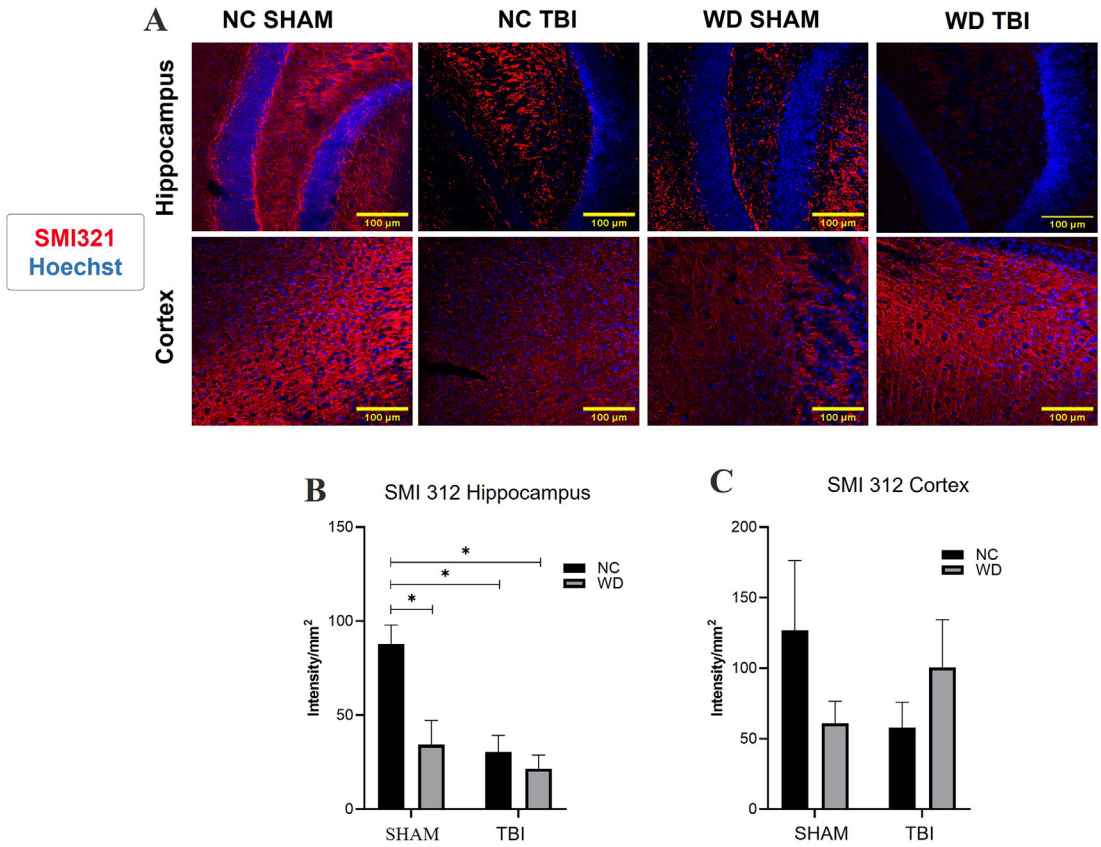

Fig. 11. Assessment of axonal degeneration in hippocampi and cortices of different treatment groups. **(A)** Representative immunofluorescence micrographs showing the expression of phosphorylated neurofilament using SMI 312 antibody (Red) in the hippocampus and Cortex. **(B)** Quantitative analysis of SMI 312 antibody in the hippocampus and **(C)** cortex, n = 3 per group (9 sections from 3 mice in each group). All images were taken with a 40x objective lens. Statistical significance was determined using two-way ANOVA followed by Tukey's multiple comparison test. Data are presented as mean ± SEM, $*p < 0.05$.

integrity was unaffected in all groups. Our data showed no detectable difference in complex I and II linked OXPHOS indicating unaltered mitochondrial function. This may be related to a recovery of mitochondrial oxidative capacities 4 weeks after the initial injury.[62] Indeed, molecular characterization of cell proliferation using BrdU staining at 8 weeks revealed that WD-TBI mice demonstrated particularly upregulated cellular proliferation possibly contributing to cellular metabolic recovery. This finding was particularly intriguing as BrdU staining showed significant differences in TBI groups (NC-TBI and WD-TBI). WD independent of TBI aggravated neuroinflammation in the brain, especially in the hippocampus. The infiltration and activation of immune cells such as the microglial is mediated by Lipocalin-2 (Lcn2). Lcn2 regulates glial activation and inflammatory cytokines expression.[63,64] It is known that high-fat diets induce the proliferation of systemic macrophages[65] which could be recruited into the central nervous system. The increased expression of Lcn2 in the hippocampus of mice fed a high-fat high fructose diet demonstrated the influence of chronic inflammatory peripheral complications on the central nervous system.[66] The elevated BrdU staining in the TBI groups could suggest increased microglial proliferation imposed by WD consumption.

Neuroinflammation is one of the mechanisms through which the brain responds to TBI. This is mediated by the brain-resident immune cells, primarily the microglia and astrocytes.[67,68] Microglial cells act as housekeepers of the brain, surveying the brain microenvironment in order to detect any death signals and initiate inflammatory responses. Upon sensing damage signals during TBI, the brain induces the proliferation of microglia, which is accompanied by subsequent activation and migration of these cells to the injury site.[69] Previous studies from our group have demonstrated that TBI leads to significant microglia activation, which is followed by a propensity of microglia cells to polarize towards the pro-inflammatory phenotype.[70] Our results showed that WD feeding and TBI led to significant microglia activation, which was demonstrated by an increase in IBA1-positive cells. Similarly, GFAP expression, which indicates increased astrocyte activation, was increased with WD and TBI. WD-induced neuroinflammation through microglia and astrocytes activation could be attributed to triggering receptor expressed on myeloid cells 2 (TREM2), which has been previously reported to be activated by WD.[71] TREM2 drives the increased activation and migration of macrophages/microglia toward the injury site in neurological conditions and has been associated with the risk of neurodegenerative diseases.[71,72] Although early microglia and astrocyte activation following TBI is initiated as a neuroprotective process, persistent activation over a chronic time point leads to worsened functional outcomes and could predispose to neurodegeneration.[73] Along the same lines, we observed that TBI in combination with a normal diet or WD led to the degeneration of mature axons, through reduced phosphorylation of neurofilament proteins, resulting in a significant loss of neuronal cells. Previously, WDs have been linked to cognitive impairment and aggravation of neurodegenerative phenotype in Alzheimer's disease.[74,18] Our findings propose that WD and TBI, at a chronic time point, induce significant neuroinflammation, which would ultimately result in axonal degeneration and neuronal cell

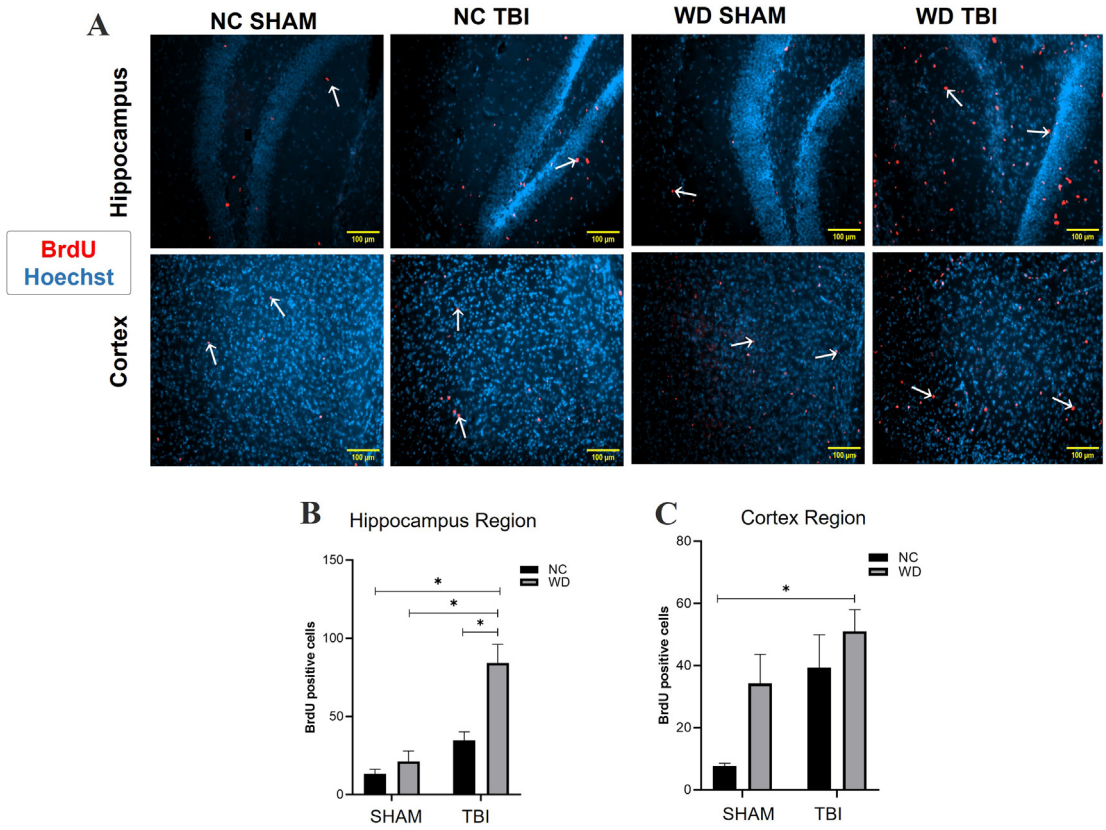

Fig. 12. Cell Proliferation Assessment in hippocampi and cortices of different treatment groups. (A) Representative immunofluorescence micrographs showing BrdU immunolabeling (Red) in the hippocampus and cortex. **(B)** Shown is the quantitative analysis of BrdU labeling in the hippocampus and **(C)** cortex, n = 3 per group (9 sections from 3 mice in each group). All images were taken with a 20x objective lens. Statistical significance was determined using two-way ANOVA followed by Tukey's multiple comparison test. Data are presented as mean ± SEM, $*p < 0.05$.

death. This is corroborated by previous reports in the literature, on how HFD-induced inflammation leads to cognitive and functional decline post-TBI.[12] Importantly, one of the proposed mechanisms through which WD could have led to the worsening of neuropathological processes implicated in neuromuscular as well as cognitive impairment is the elevation of plasma cholesterol in the periphery. Indeed, previous studies have demonstrated the beneficial effect of cholesterol-lowering drugs, i.e. statins, on TBI functional outcomes.[75,76] Some of these effects were attributed to reductions in systemic inflammation. In light of studies reporting compromised the BBB with WD feeding, the latter can incite central inflammatory processes like astrogliosis and microgliosis shown to be elevated in mice where TBI was superimposed with WD feeding.

Metabolic parameters like body mass index, blood glucose levels, as well as serum cholesterol have previously been reported to modify post-injury risk, especially following severe TBI.[41] Different aspects of TBI secondary injury are diet- and time-sensitive. Dietary components and patterns differentially alter the secondary consequences of TBI in a time-dependent manner. In mice consuming WD for 8 weeks, mitochondrial bioenergetics appeared to be unchanged and subsequently not responsible for WD-induced worsening of TBI neurocognitive and neuromuscular insults. Alternatively, the combined effect of TBI superimposed with WD consumption was shown to elicit exaggerated astrogliosis as well as microgliosis possibly bringing about increased neuronal cell loss and axonal degeneration.

The National Institute of Health (NIH) emphasizes the significance of the prevention of TBI as a primary defense because there is presently no approved treatment. Equally significant, though, are factors that could exacerbate injury such as age at the time of the injury and dietary and nutritional elements. A high glycemic diet such as WD is implicated in several diseases and cognitive dysfunction. Here we showed that WD exacerbated neuropathology observed in TBI by increasing neuroinflammation and neuronal cell loss worsening neuromuscular and cognitive outcomes, even in absence of overt metabolic derangement. Systemic alteration by WD may have contributed to exacerbating the secondary injury cascade. This study provides insight into how diet-induced systemic alteration could impact TBI outcomes especially in seemingly healthy individuals with subtle metabolic challenge.

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Authors' contributions

AFE-Y, FK, REK, YM, and JN conceived and designed the study. JN, N-MZB, SM, MAR, and SI collected the data. FK, AFE-Y, REK, JN, N-MZB, LN, AS, and MG contributed to the data analysis. OO, JN, MAR, N-MZB, and MAH performed the experiments. JN, SI, N-MZB, FK, AE, and AFE-Y, drafted the manuscript. All authors reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Declaration of competing interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Data availability statement

All data generated or analyzed during this study are included in this manuscript.

Ethical approval

All the animal experiments in our study were approved by the Institutional Animal Care and Use Committee (IACUC) at the American University of Beirut (IACUC Approval number: 21-09-589, August 2020).

Informed consent

Not applicable.

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