Sex-specific pathological response to Traumatic Brain Injury

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Abstract

Traumatic brain injury (TBI) is one of leading causes of death in the world; survivors commonly exhibit behavioural changes and neurocognitive defects in the long-term. Following TBI, rapid changes in the neurovascular unit (NVU) occur as the permeability of the blood brain barrier (BBB) increases and cerebral blood flow (CBF) becomes dysregulated. An integral component of the NVU are pericytes which help maintain the integrity of the BBB, which are lost following mTBI resulting in BBB permeability and consequential leakage of blood plasma proteins into the brain parenchyma. After sustaining a TBI, females are more likely to develop persistent symptoms and show greater cognitive impairment than males. Importantly, the underlying causes for the differences in recovery following TBI are not well understood. Here, we investigate sex-specific differences following experimental TBI, by targeting capillary CD31 and Podocalyxin and pericyte PDGFrβ in the lateral cortex of mice with immunohistochemical and immunofluorescent techniques. Using an *in vivo* central fluid percussion injury (cFPI) model, male and female mice were analysed at day 2, 7 and 30 post-injury and were compared with controls. Our findings show that male capillaries and pericytes do not change following cFPI, whereas females showed a response in both capillaries and pericytes. Female mice exhibited reduced capillary width 30 days after cFPI as well as early phase response in pericytes. Our data suggests that sex-specific differences occur in capillaries and in pericytes following cFPI. Further research is required to determine the underlying mechanisms driving sex-specific responses, which may potentially lead to the development of sex-specific therapeutic interventions for TBI.

Introduction

Traumatic brain injury (TBI) is a major cause of death worldwide and can have severe lifestyle repercussions later in life (Maas et al., 2017; Scholten et al., 2016). TBI results from traumatic impact to the head, or energy transmitted to head from an impact elsewhere on the body by an external force or acceleration-deceleration forces, resulting in an intracranial injury. Injury varies in severity, ranging from mild TBI (mTBI) to severe TBI. A concussion, a term mainly used in the context of sports, is defined as an mTBI. Patients suffering mTBI typically make a full neurological recovery, though a short-lived display of symptoms such as headaches, dizziness, imbalance, fatigue, sleep disruption and impaired cognition are common (Katz et al., 2015). However 15-30% of patients experiencing sports related concussion exhibit prolonged neurocognitive and behavioural changes (Mckee & Daneshvar, 2015). Whereas, severe TBI has immediate detrimental effects on motor skills and may lead to death (Mckee & Daneshvar, 2015). TBI pathology is characterised as a biphasic response. The primary injuries are produced by direct mechanical forces impacting brain structure and normal brain function. The initial insult physically damages brain architecture in a focal and diffuse pattern, as well as triggering the secondary cascade (Mckee & Daneshvar, 2015). Within hours the pathological process of the secondary insult induces harmful changes in cellular and molecular responses producing further neurological damage and morbidity (Mckee & Daneshvar, 2015). Critically, TBI is known to elevate the risk of developing progressive neurodegenerative disease long after the primary injury. It is predicted that TBI contributes to 5-15% of all dementia cases worldwide (Shively et al., 2012). The primary injury in TBI doesn't offer an opportunity for therapeutic strategies considering physical damage occurs upon impact, and the primary injury can only be targeted by preventive measures. However, the secondary injury offers a wide range and potentially critical therapeutic targets which may prevent long-term diseases.

The cerebrovasculature is fastidiously regulated by the neurovascular unit (NVU). The NVU comprises of astrocytes, glial cells, neurons, endothelial cells, pericytes and vascular smooth muscle cells acting in concert to form and maintain the blood brain barrier (BBB), as well as regulate cerebral blood flow (Bell et al., 2010). Critical to the NVU are pericytes. Brain pericytes have been intensively researched in the last 10-15 years due to mounting evidence indicating their importance to NVU function. Pericytes are found at the interface of endothelial cells and astrocytes and are entirely enveloped by basal lamina (Brown et al., 2019). Pericytes have a distinguishable bulbous cell body and envelop the abluminal surface of endothelial cells with an intricate network of arborizing processes, establishing effective capillary coverage (Dalkara et al., 2011). Pericyte morphology depends on capillary localization. At the arteriolar end of capillaries, pericytes exhibit circumferential processes that envelop the capillary and are predicted to regulate cerebral blood flow (CBF) and alter capillary stiffness (Attwell et al., 2016). Pericytes positioned centrally on the capillary express

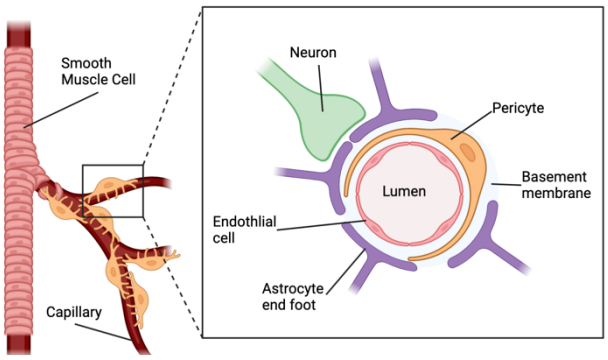

Figure 1: Cellular components of the neurovascular unit. Created with BioRender.com.

longitudinal processes are predicted to maintain the BBB (Attwell et al., 2016). Finally, venular pericytes have a stellate composition and may be involved in regulating immune cell infiltration, however their role remains debated (Attwell et al., 2016). Furthermore, brain pericytes are regarded as heterogenous as expression of prominent protein markers vary depending on their localization (Uemura et al., 2020). The markers PDGFrβ, NG2, CD13 and vimentin are commonly expressed by all pericytes. Arteriolar pericytes express the highest levels of αSMA as well as desmin and transgelin (Uemura et al., 2020) whereas mid-capillary pericytes express ABCC9, express lower levels of αSMA and hardly express transgelin or desmin and venule-capillaries strongly express αSMA and ABCC9 (Uemura et al., 2020).

Pericyte blood vessel recruitment and survival of pericytes is sustained through PDGFB signalling from endothelial cells (Bhowmick et al., 2019). During angiogenesis PDGFB secretion recruits pericytes to neocapillaries as well as stimulating pericyte proliferation (Uemura et al., 2020). The importance of pericytes is further highlighted as reduced CBF and capillary perfusion were observed in pericyte-deficient mice (Bell et al., 2010). Secondary injuries such as ischemia, hypoxia and vasogenic edema in the brain are enhanced by increased BBB permeation (Bell et al., 2010; Zehendner et al., 2015). Robert D Bell et al. observed vascular damage due to pericyte loss following ablation of PDGFRb signalling which preceded vascular-mediated irreversible neuronal injury (Bell et al., 2010). The pericyte-endothelium signalling is impaired following TBI resulting in the loss of pericyte interaction. In the early phase of TBI, rapid pericyte loss is known to occur followed by pericyte proliferation up to two weeks after injury (Chen et al., 2003; Zehendner et al., 2015). Significantly, the loss of pericytes results in BBB dysregulation and increased BBB permeability into the brain parenchyma (Bhowmick et al., 2019).

TBI can cause extensive vascular damage, potentially further triggering secondary injury mechanisms. During TBI pathogenesis brain haemorrhages , edema formation, BBB disruption and changes in CBF are known to occur (Salehi et al., 2017). Decreases in CBF occur within hours of sustaining TBI and its recovery to normal level within 3 weeks is reported to be critical for better neurological outcome (Jullienne et al., 2016). Capillary loss following TBI is also documented with neovascularisation and repair being observed up to 48 hours after injury (Salehi et al., 2017). Vascular damage is heavily implicated in in neurodegenerative disease. In Alzheimer's disease, BBB breakdown and reduced CBF are observed in patients before AD biomarkers are detected (Nation et al., 2019; Ruitenberg et al., 2005).

Recently, a concerted effort into understanding any sex-dependent disparities exist since animal and human studies indicate biological sex differences after TBI. Females are at greater risk of developing persisting post-concussion symptoms following mTBI and show greater cognitive impairment following moderate-severe TBI, though the number of males sustaining TBI is higher(Gupte et al., 2019). Post TBI recovery in females is reported to possibly take longer and may require more treatment than in males(Rabinowitz et al., 2015). Conversely, 1 year after severe TBI females report greater cognitive performance, though sex wasn't reported to affect perceived life satisfaction (Saban et al., 2011). It's likely multiple factors have a compounding effect on sex-specific differences on the pathological outcomes of TBI. Crucially, the underlying causes determining a worse clinical outcome after TBI in women haven't been specifically determined and require further research. The mechanisms driving such disparities are not well understood. Traditionally, pre-clinical TBI models has

predominantly been conducted using male animals or results weren't stratified based on sex (Haynes & Goodwin, 2021). Previously, weaker neck musculature in female mTBI patients was suggested to increase head rotation upon impact, potentially exacerbating white matter and cerebrovascular injury (Vedung et al., 2022). Sex-specific research has largely focused on the effects of hormones in pathological mechanisms; however, this has produced inconsistent results. However, little research surrounding differential response mechanisms at the molecular level have been conducted. Characterising different pathophysiological mechanisms between males and females following TBI could be crucial in developing future therapies. We hypothesise that the deficits between the sexes seen in recovery following TBI, in part stem from differential responses at the vascular level. Here we investigate the development of vasculature and pericyte pathology following experimental TBI using the central fluid percussion injury model in male and female mice.

Methods

Animals

Adult male and female mice C57BL/6 mice (8-12 weeks old; 25-30gr; Taconic, Denmark) were housed with free access to food and water for a minimum of seven days prior to surgery. The study was approved by the Ethical Committee at Lund University (Dnr: M4789-17) and followed the regulations of the Swedish Animal Welfare Agency.

Central Fluid Percussion Model (cFPI) of diffuse traumatic brain injury

The surgical procedure for central fluid percussion injury (C57BL/6 mice cFPI) has been previously described in detail (Flygt et al., 2018). Briefly, the mouse was placed in a ventilated Plexiglas chamber with 4% isoflurane in air, and anaesthesia was applied by isoflurane 1.2% and N_2O/O_2 70/30% delivered through a nosecone. A 3.0 mm diameter craniotomy was made over the midline by keeping the dura mater and the superior sagittal sinus intact. Then, a plastic cap placed on craniotomy was filled with isotonic saline at room temperature and attached to the Luer-Lock on the fluid percussion device (VCU Biomedical Engineering Facility, Richmond, VA). In order to induce a diffuse TBI characterized by widespread white matter injury, the fluid percussion pendulum was released to create a pressure wave subsequently transmitted into the cranial cavity. The injury-induced apnea and immediate post-injury seizures were recorded. Post-injury apnea, observed in all cFPI-injured mice, was 25 ± 15 sec (range 10–40 sec). Twenty cFPI animals died at time of impact resulting in injury related mortality rate of approximately 17%. Sham-injured animals (C57BL/6 mice Sham, n=34) were subjected to anaesthesia and surgery, but the pendulum was not released. The cap was removed following surgery, the bone flap replaced, and the skin sutured using resorbable sutures.

The mice were sacrificed by i.p.-injection of sodium pentobarbital (Pentobarbital natrium 400mg/ml, VET ATL, Apoteket, Sweden; 200 mg/kg) at 0, 2, 7, or 30 dpi and subsequently transcranially perfused using 4% formaldehyde (formaldehyde 4% Fosfatbuffrad, HistoLab Products AB, Gothenburg). The brains were post-fixed overnight at 4°C and placed in 30% sucrose solution for 48 hours. Brains were cut in the coronal plane at 30-40µm thickness using a Vibrotome (Leica SM 200R) and kept in anti-freezing solution (30% glycerin, 30% ethylene glycol, and 40% 1x phosphate buffered saline [PBS]) at −20°C until further tissue processing. For immunohistochemical staining, female mice (N=17) and male mice (N=23) were sacrificed and for immunofluorescence staining, female (N=29) and male (N=26) mice were sacrificed Sections from bregma -1.46 to -2.30 were selected for staining's.

Immunohistochemistry

Brain coronal sections were washed three times in phosphate buffer saline (PBS) at pH 7.4 and quenched in a solution of Methanol, Hydrogen peroxide and distilled H2O (1:1:8) for 15 minutes. Sections were blocked in 3% normal donkey serum (NDS) (AB_2337258; Jackson ImmunoResearch) in 1X PBS supplemented with 0.5% Triton X-100 (PBTX) for one hour at room temperature. The coronal sections were incubated overnight with rat anti-CD31 (550274; 1:400; BD Pharmingen) in PBST with 3% NDS at room temperature. Afterwards, the

sections were washed three times in PBTX for 5 minutes. Anti-rat biotinylated secondary antibodies at 1:200 were incubated with PBTX for one hour at room temperature. After incubation with secondary antibodies, the sections were washed 3 times in PBS for 5 minutes. The ABC kit (PK-6100; Vector Laboratories) was used to enhance the signal through an hour incubation with the coronally sectioned samples, followed by three 5 minutes wash steps with PBS with no potassium ($PBSK^-$). The samples were then immersed in 3,3-diaminobenzidine-tetrahydrochloride (DAB) to highlight CD31 antigens (timing was based on self-observation). The coronal sections were then washed three times in $PBSK^-$ for 5 minutes. The sections were then dehydrated in a stepwise procedure of ethanol 70%, ethanol 95% and 100% ethanol, followed by xylene and were mounted using Pertex. Each step was repeated twice apart for 70% ethanol for 2 minutes.

Image analysis for $CD31^+$ capillaries

The DAB-stained capillaries were imaged with Olympus BX53 light microscope. Using the Olympus cellSense software, three 20x images per hemisphere of the lateral cortex were obtained at 20x for each animal (Figure 2). Region of interest (ROI) was 735µm x 552µm. Images were saved as TIF files. Area, width, and branch point analysis for $CD31^+$ capillaries was performed using NHI ImageJ2 version *2.3.0/1.53q.*

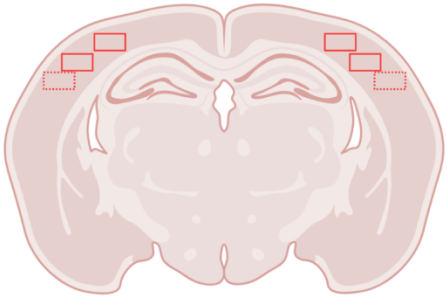

Figure 2: ROI in cortex imaged for data analysis. Solid squares represent ROI for immunofluorescence. Solid and dashed squares represent ROI for immunohistochemistry. Created with BioRender.com.

Area analysis

To determine the area of $CD31^+$ capillaries, the TIF images were processed into a binary image and $CD31^+$ capillaries were converted into a mask (Particle Size 50 - infinite). $CD31^+$ capillaries with a diameter larger than 6µm were removed from the image. The capillary area is expressed as percentage of total area.

Width analysis

The width of $CD31^+$ capillaries were measured using unprocessed TIF images. Capillary width in µm was manually determined by measuring the outermost point of each vessel wall and measured perpendicular to the vessel wall. Measurements were performed with the straight-line function tool and the length of each line was measured. Up to three discrete measurements were taken per capillary. Measurements near branch points were avoided.

Branch Point Analysis

The branch points of $CD31^+$ capillaries were counted using unprocessed TIF images. A branch point was defined as a node connected to three of more vessel segments (Edgar et al., 2014). The individual branch points were manually counted using the multi-point tool.

Immunofluorescence staining

Brain coronal sections were washed three times for 5 minutes in PBS pH7.6. The sections were then incubated for 30 minutes at 90°C in 2ml of citrate buffer pH6 to improve sample antigenicity. Afterwards, the coronal sections were rested at room temperature for 10 minutes. Next, the coronal sections were treated with primary antibodies in 3% NDS supplemented with 0.5% PBST and were incubated overnight at 4°C. The primary antibodies used were rabbit anti-platelet-derived growth factor receptor beta (PDGFrβ) (3169S; 1:100; Cell Signalling Technologies) and goat anti-podocalyxin (PDX) (AF1556; 1:400; R&D Systems). Following primary antibody incubation, the coronal sections were washed three times for 5 minutes in PBS. Then the coronal sections were incubated with the secondary antibodies in 3% NDS supplemented with 0.5% PBST for one hour at room temperature. The secondary antibodies used were green Dylight 488 anti-goat (705-485-147; 1:125; Jackson ImmunoResearch) and Biotinylated Ab anti-rabbit (711-065-152; 1:100; Cell Signalling Technologies). The samples were then washed in 0.5% PBST for 5 minutes, followed by an hour incubation at room temperature with Streptavidin Cy3 (S32355; 1:2000; Invitrogen). Afterwards, the coronal sections were washed three times for 5 minutes in PBST and then PBS once. DAPI (32670; 1:1000; Sigma) was used to stain the DNA for 10 minutes at room temperature. A final five-minute wash step with PBS was performed. The coronal sections were mounted using DABCO (D2522; Sigma) in 90% glycerol and 10% 1xPBS.

Image analysis for PDX$^+$ capillaries and PDGFrβ$^+$ pericytes

Imaging was performed using a Zeiss 780 confocal microscope for fixed tissue analyses. Using the Zen black edition software, two 20x images per hemisphere of the lateral cortex were taking for each animal with a 20µm Z-stack (Figure 2). The ROI size was 1024x 1024. The 488µm line from an argon laser was selected to excite green Dylight 488 using a main beam splitter (MBS) set at 488/561/633 and the emissions were detected through 490µm-562µm bypass filter. The 561µm line from a DPSS laser was selected to excite Cy3 using a MBS set at 488/561/633 and the emissions were detected through 579µm-652µm bypass filter. The 405µm line from an argon laser was selected to excite DAPI using a MBS set at 405 and the emissions were detected through 410µm-488µm bypass filter. Images were subsequently subjected to Maximum Projections and filtering functions. The processed images were saved as TIF files.

Area analysis

Area quantification was performed using NHI ImageJ2 version *2.3.0/1.53q.* The TIF images were split into single monochromatic RGB channels. Following, a separate binary image of the red channel (PDGFrβ$^+$ Pericytes) and the green channel (PDX$^+$ Capillaries) were produced using an intensity threshold. The eraser tool was used to remove background from the binary image. Then, the total percentage area of PDX$^+$ capillary or PDGFrβ$^+$ pericyte from the binary image was measured. The mean percentage of total area of field of view for PDX$^+$ capillaries and PDGFrβ$^+$ pericytes for each animal was determined.

Pericyte coverage analysis

Pericyte coverage was defined as total pericyte area divided by total capillary area. Determination of the total percentage areas of pericytes and capillaries were described above. Mean pericyte coverage per animal was then determined.

Pericyte number analysis

The total number of pericytes were counted using the binary image previously described. Using the multi-point function, the total number of individual pericytes were counted. The mean pericyte number for each animal was calculated.

Data analysis

Data were analysed using GraphPad Prizm software version 9.3.0. Data distribution was checked for normality by Shapiro-Wilk test. If normal, One-Way ANOVA with Tukeys multiple comparison was performed. If data was not normally distributed, Kruskal Wallis test with Dunn's multiple comparison was performed. Significance was set at $p < 0.05$.

Results

Sex-specific differences following mTBI have focused largely on clinical response to treatment rather the pathological mechanisms driving the secondary injuries. Here, we investigated the sex-specific effects on cortical capillaries and pericytes using a cFPI model in mice.

No changes in CD31⁺ capillaries of the lateral cortex of male mice following Traumatic Brain Injury

Male mice vasculature was visualised immunohistochemical staining of CD31 at day 2-, 7- and 30-dpi. Total area, width and branching of CD31⁺ capillaries were then compared to controls.

Figure 3

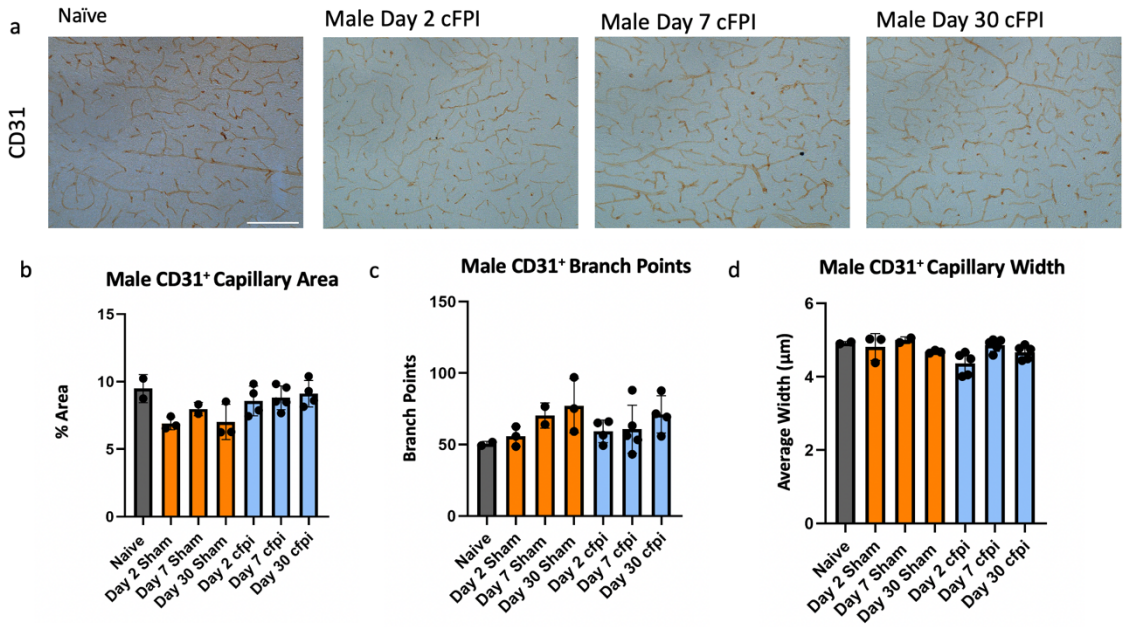

Figure 3: Visualising CD31⁺ capillaries of the lateral cortex of male mice following cFPI. Male mice (Naïve n= 2; Sham Day 2 n= 3; Sham Day 7 n = 2; Sham Day 30 n= 3; cFPI Day 2 n= 4; cFPI Day 7= 5; cFPI Day 30= 4) vasculature was analysed at 2-, 7- and 30-dpi using 3, 3'-diaminobenzidine (DAB) staining to target CD31. **a** Representative images of CD31⁺ cortical capillaries of brain- injured male mice**.** **b** Area quantification of CD31⁺ capillaries in male mice following mTBI. Data expressed as percentage capillary area. **c** Quantification of capillary branching. **d** Quantification of CD31-positive capillary width of male mice. Scale bar = 150µm. Statistical analysis using Kruskal-Wallis, Dunn's multiple comparison's test.

Following cFPI, the total area of CD31-positive capillaries in male mice did not change over 30 days post-injury when compared to sham or naïve animals (Figure 3a, b). The number of capillary branches in male mice did not change over 30 days post-injury when compared to sham or naïve animals (Figure 3a, c). No change in male CD31⁺ capillary width was observed following cFPI when compared with controls (Figure 3a, d). Our findings suggest that cortical capillaries in the cortex of male do not undergo change following cFPI.

Pericytes of male mice do not exhibit a pathological response to cFPI

Cortical capillaries and pericytes of male mice were imaged with the confocal microscope following co-staining of podocalyxin (PDX) and platelet derived growth factor beta receptor (PDGFrβ). Mice were analysed 2-, 7- and 30 dpi.

Figure 4

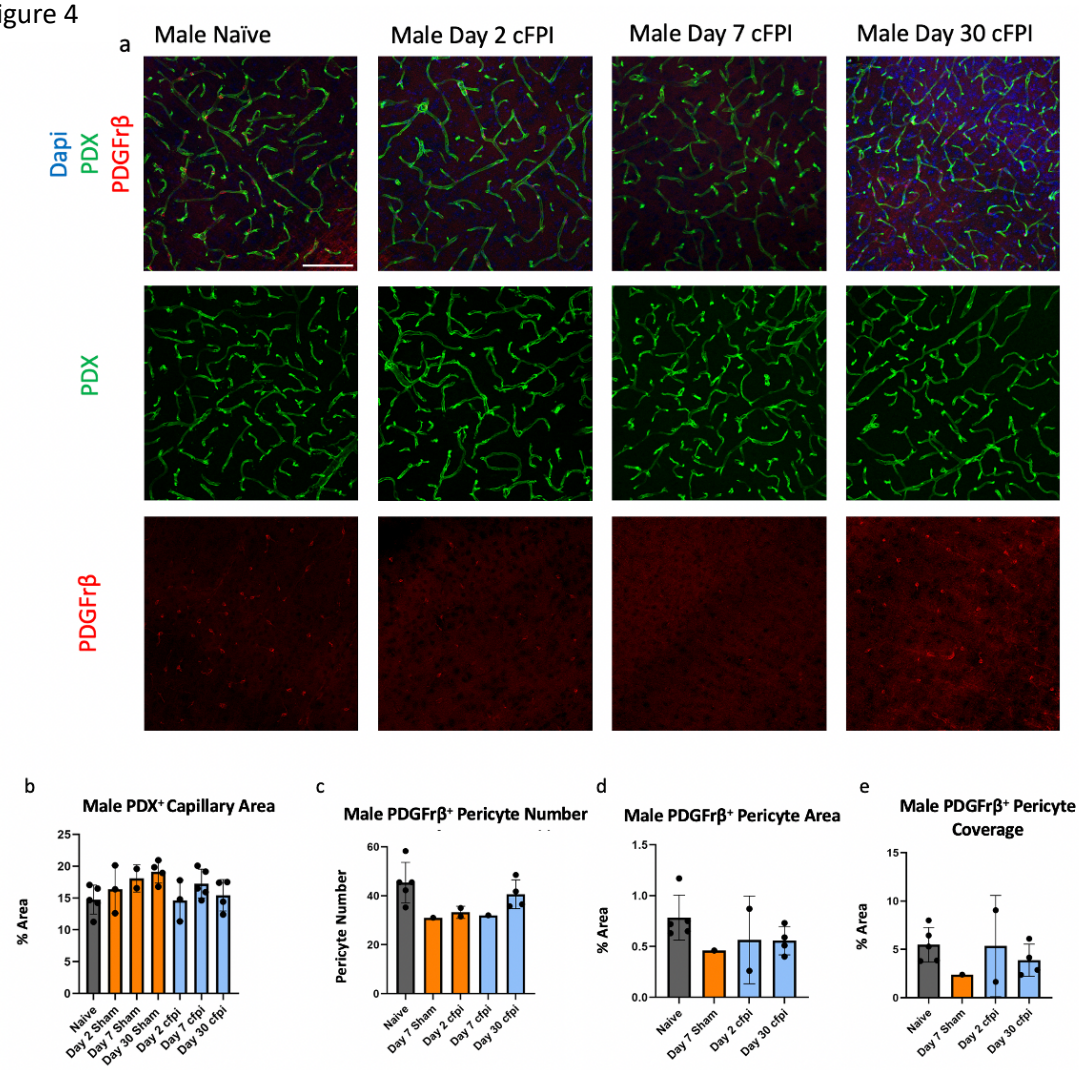

Figure 4: Experimental TBI does not induce significant changes in pericytes or capillaries of male mice. Capillaries (PDX, green) and pericytes (PDGFrβ, red) of male mice (Naïve n=5; Sham Day 2 n= 3; Sham Day 7 n= 2; Sham Day 30 n= 4; cFPI Day 2 n= 3; cFPIDay 7= 5; cFPI Day 30= 4) were investigated following cFPI and compared with controls using confocal microscopy. **a** Representative panels of PDX$^+$ capillaries and PDGFrβ$^+$ pericytes response to cFPI in male mice over 30 days. For figures c-e only pericyte data of naïve animals and day 30 cFPI animals could be acquired. **b** Area quantification of PDX$^+$ capillary area in male mice following mTBI. Data was presented as percentage of capillary area. **c** Quantification of PDGFrβ$^+$ pericyte number. **d** Area quantification of PDGFrβ$^+$ pericytes. Data expressed as percentage of pericyte area. **e** Quantification of PDGFrβ$^+$ pericyte coverage of PDX$^+$ capillaries analysed by one-way ANOVA. Statistical analyses for Figures 5b, 5c and 5d by Kruskal-Wallis, Dunn's multiple comparison's test. Data expressed as mean and individual data. Scale bar= 100µm.

Male mice did not exhibit significant changes in the PDX$^+$ capillary area when compared to naïve or sham controls at any time point after cFPI (Figure 4a, b). Similarly, there were no significant changes in neither PDGFrβ$^+$ pericyte area nor PDGFrβ$^+$ pericytes coverage of PDX$^+$ capillaries in the lateral cortex at any time points after the injury. Our findings suggest that the capillary area and pericytes do not exhibit a significant response after cFPI in male mice.

Changes in CD31$^+$ cortical capillaries of female mice following traumatic brain injury

To investigate female mice capillaries, we used the same methodology as for male mice (figure 2). The CD31$^+$ capillary total area, branching and width of female mice were analysed and compared to controls at 7- and 30 dpi.

Figure 5

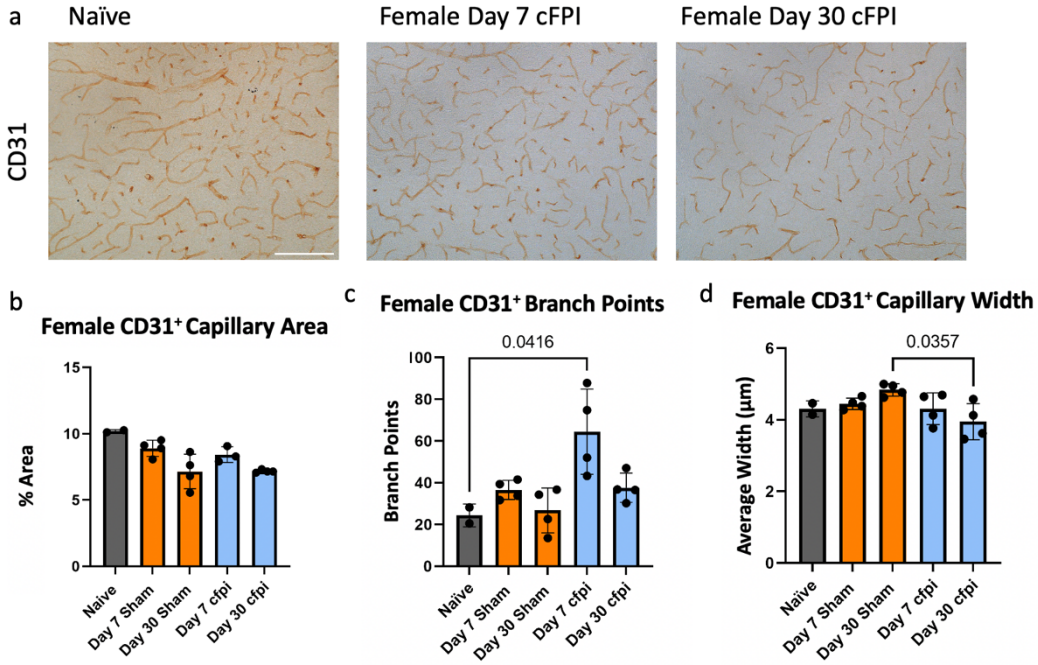

Figure 5: Alterations of CD31$^+$ capillaries in the lateral cortex of female mice over 30 days following cFPI. Female mice (Naïve; n= 2; Sham Day 7 n= 4; Sham Day 30 n= 4; cFPI Day 7 n= 3 ; cFPI Day 30 n= 4) capillaries were analysed at 7- and 30 dpi compared to controls using DAB staining targeting CD31. **a** Representative panels of CD31$^+$ capillaries in injured female mice. **b** Quantification of CD31$^+$ capillary area in female mice following cFPI. Data was expressed as percentage of capillary area. **c** Quantification of capillary branching per unit. **d** Quantification of CD31$^+$ capillary width in female mice. Scale bar = 150 µm. Statistical analysis using Kruskal-Wallis, Dunn's multiple comparison's test.

Following cFPI, no significant reduction in CD31$^+$capillary area was found in female mice at 7 and 30dpi (Figure 5a, b). The number of capillary branches increased at day 7 in brain-injured mice when compared to sham and naïve female mice, though the increase was only significant in comparison to naïve female mice (Figure 5a, c). At 30dpi no difference in branching in CD31$^+$ capillaries was observed in injured females. No difference in CD31$^+$ capillary width was observed at 7dpi; however a significant decrease was observed at 30dpi in female mice when compared to sham animals. Our results show that the capillary area does not significantly

change at one week or 30 days after TBI in female mice, although the capillaries in the lateral cortex exhibit decreased width at 30 days post-injury.

Pericytes in brain injured female mice exhibit an early response to diffuse mTBI

To investigate capillaries and pericytes of female mice we used the same methodology as in figure 2. Female mice PDX$^+$ capillary area and PDGFrβ$^+$ pericyte number, area and coverage were analysed and compared to controls at 2-, 7- and 30 dpi.

Figure 6

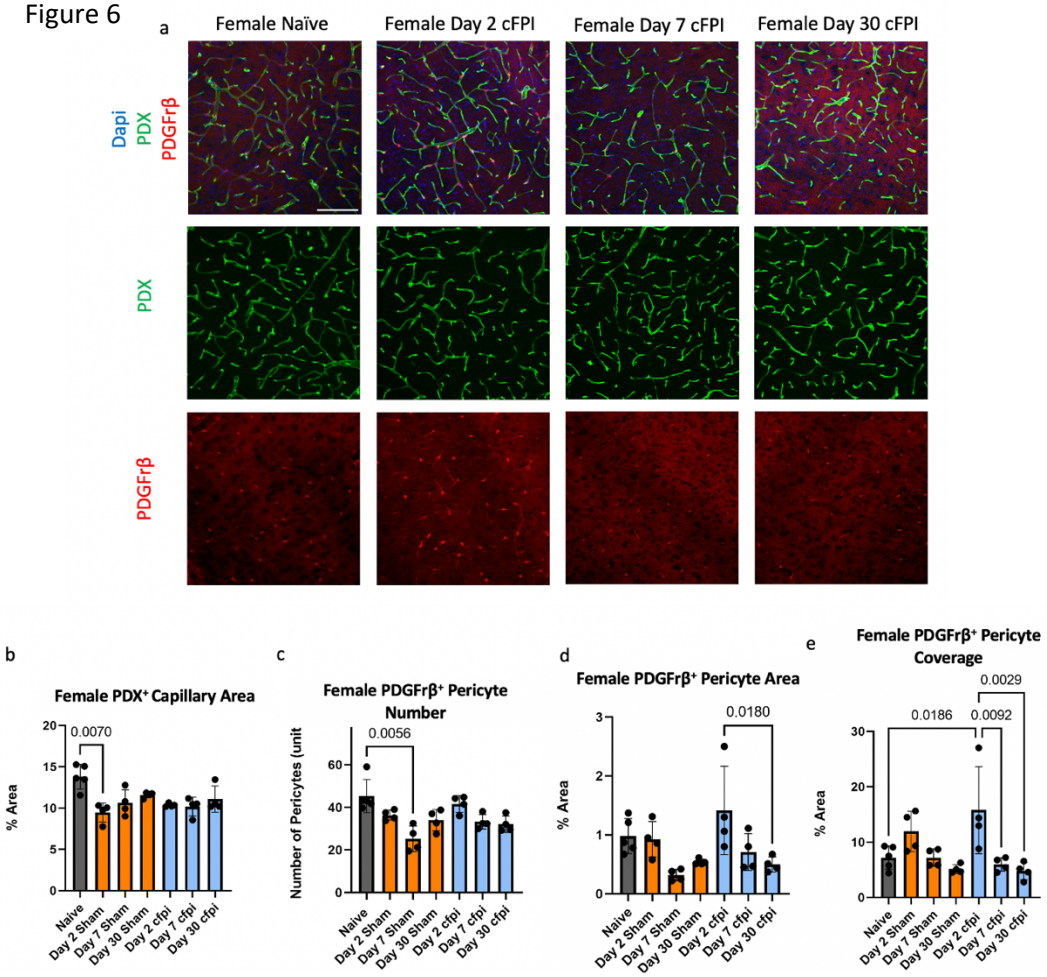

Figure 6: Cortical pericytes exhibit an early response following TBI in female mice. The female mice (Naïve n= 5; Sham Day 2 n= 4; Sham Day 7 n= 4; Sham Day 30 n= 4; cFPI Day 2 n= 4; cFPI Day 7 n= 4; cFPI Day 30 n= 4)) capillaries (PDX, green) and pericytes (PDGFrβ, red) was investigated following injury and compared to controls using confocal microscopy. **a** Representative panels of PDX$^+$ capillaries and PDGFrβ$^+$ pericytes response to TBI in female mice over 30 days. **b** Quantification of PDX$^+$ capillary area in female mice following TBI. **c** Quantification of PDGFrβ$^+$ pericyte number. **c** Quantification of PDGFrβ$^+$ pericyte area. Data was expressed as percentage area of pericytes. **E** Quantification of PDGFrβ$^+$ pericyte coverage of PDX$^+$ capillaries. Statistical analyses for Figures 6b, 6c and 6d by Kruskal-Wallis, Dunn's multiple comparison's test. Data expressed as percentage of capillaries covered by pericytes and analysed by one-way ANOVA. Data expressed as mean and individual data. Scale bar= 100µm.

No difference was observed in the total area of PDX$^+$ capillaries between sham- and brain-injured female mice (Figure 6a, b). All sham- and brain-injured mice groups showed lower

area of PDX-positive capillaries when compared to naïve mice, though only sham 2dpi was found to be significant (Figure 6a, c). There was no significant difference in the number of PDGFrβ$^+$ pericytes between brain-injured female mice and the female control groups (Figure 6a, c). Pericyte numbers fluctuated in sham animals throughout the 30 days and were found to be significantly reduced at 7 days when compared to the naïve control group. Following cFPI, a significant decrease in PDGFrβ+ pericyte area in female mice was observed from 2 to 30 dpi (Figure 6d, d). However, when compared with naïve or sham animals no significant difference in pericyte area was found at either 2dpi or 30dpi. cFPI female mice exhibited a significant increase in PDGFrβ$^+$ pericyte coverage at 2 days when compared tonaïve mice (Figure 6a, e). We then observed a significant decrease in PDGFrβ$^+$ pericyte coverage from 2 to 7dpi following cFPI, which was maintained up to 30dpi. PDGFrβ$^+$ pericyte coverage at 7 and 30 dpi was normal when compared with controls. However, when compared with sham controls, the increase in PDGFrβ$^+$ pericyte coverage in female mice in the early phase was not significant. Our findings indicate that PDGFrβ$^+$ pericytes in the lateral cortex elicit a response in the early stage of TBI in females before normalization.

Discussion

Sex-specific differences in TBI recovery have been observed, however the underlying pathological mechanisms driving such differences have not yet been elucidated. Cerebral vasculature in TBI has been well documented to exhibit several pathological states; CBF dysregulation, edema, hypoxia, and increased BBB permeability. Importantly, such disease states are associated with neurological dysfunction. In this study, we used the cFPI model to investigate sex-specific effects of experimental TBI. In our investigation, we observed sex-specific responses, in which female mice showed reduced capillary diameter and an early response in pericytes, in contrast to males.

In the original experimental set-up, a female day 2 group was due to be included in the final study, however due to COVID related issues we couldn't receive the female mice in time to perform immunohistochemistry. Certainly, a 2dpi group could provide invaluable insights into the early response of capillary area, branching and diameter, considering that early effects of TBI can be observed within hours. Additionally, data concerning male pericytes are lacking from our figures. The samples from 2dpi, 7dpi, day 2 sham, day 7 sham and day 30 sham were successfully stained, however due to issues of non-specific staining with PDGFrβ^{+}that we could not resolve despite repeating the experiment and modulation of the immunohistochemical protocol we could not identify pericytes to extract data. It should be noted that the non-specific staining of PDGFrβ^{+} in our samples may indicate shedding and release of soluble PDGFrβ^{+} which may be a biomarker of brain pericyte injury (Sagare et al., 2015).

To our knowledge, no previous study has investigated sex-specific effects of TBI on capillary width. We observed a significant decrease in capillary width in female mice at 30dpi, whereas no change was observed in males following cFPI. Previously, juvenile male rats exhibited a prolonged decrease in capillary width at 2 and 6 months, without changes in microvascular density, following TBI using the controlled cortical impact (CCI) model (Jullienne et al., 2014). The CCI model may inflict a more severe cortical injury than our cFPI model does. These findings suggest female capillaries may be more sensitive than male capillaries and elicit a response to lower grade head impacts. Furthermore, a decreased capillary width was observed in male mice after 24hrs following moderate FPI, therefore whether an early response in females capillaries occurs in the acute phase in our diffuse TBI model remains to be investigated (Park et al., 2008). Interestingly, decreased capillary width is observed in Alzheimer's disease (Burke et al., 2014). Therefore, further study of changes in capillary width following TBI is of clinical relevance, considering the association of TBI and neurodegenerative diseases. Additionally, it has also been suggested that a decrease in vessel diameter is likely to induce reduced CBF (Jullienne et al., 2014). Notably, it was reported that in repeated sports-related concussions (rSRC) females had a chronically decreased CBF whereas males did not (Vedung et al., 2022). Taken together, reduced CBF observed in females following rSRC may be caused by a decrease in capillary width.

To the best of our knowledge, we are the first to investigate sex-specific responses of cortical pericytes following TBI, and we observed sex-specific responses in pericyte coverage. Females exhibited significantly increased pericyte coverage of capillaries at 2 days post injury (dpi) which was normalized by 7 dpi, whereas male pericytes did not show a response to the brain injury. Increased pericyte coverage of blood vessels is associated with increased PDGF-

B/PDGFR-β signalling and suppressed VEGF signalling (Berthiaume et al., 2018). This may indicate a tendency for pericytes to reinforce BBB integrity during the early phase following TBI in females. In a previous report using an impact acceleration model of diffuse TBI, an increased BBB permeability was observed in males but not in females (O'Connor et al., 2006). Thus, an unaltered BBB permeability in the early post-injury phase in females may be in part due to increased pericyte coverage.

In our study, capillary area was investigated by targeting two different endothelium antigens, CD31 and PDX. In both experiments, neither male nor female mice exhibited a change in capillary area throughout the 30 days period following cFPI. In a study using the CCI model, an early global reduction in cerebral vasculature was observed in male mice at 1dpi (Obenaus et al., 2017). Considering the higher severity of cortical injury observed in the CCI applied in their investigation compared to the cFPI model used in our present report, it may suggest that focal cortical TBI induces a global reduction in cerebrovasculature whereas diffuse TBI as used by us does not induce such losses. Though we did not observe a change in capillary area in either male or female mice, it cannot be ruled out that other vasculature pathologies such as reduced CBF, hypoxia or edema occur in TBI. Decreased cortical CBF observed a decreased cortical CBF, whilst there was no change in blood vessel density following lateral FPI(Hayward et al., 2011; Long et al., 2015). Potentially the state of CBF may not be entirely dependent on the injury state of the vasculature architecture. Thus, taking our data into account as well as previous research, sex-specific differences in CBF could be clinically relevant following TBI.

Whilst there was no change in capillary area, morphological changes in capillaries in female mice were still observed. At 7dpi, a significant increase in the number of branch points was observed in females when compared to naïve animals (Figure 4 b). Previously, a decrease in branch points was observed only at 1 dpi in male rats using a moderate FPI model (Obenaus et al., 2017). In our investigation, increased capillary branching occurred at seven days, however we're missing a 2dpi data set, thus whether a response in capillary branching in the early phase occurs in females following mTBI should be investigated. An increase in capillary branching is associated with angiogenesis, however no increase in capillary area was observed in females at 7dpi. Additionally, angiogenesis is repressed by pericyte-endothelium interaction and requires pericyte dissociation from the endothelium for angiogenesis to occur (Bodnar et al., 2013). In our study we didn't observe a decrease in pericyte number at 7dpi. Taken together, an increase in capillary branching may not necessarily be a unique angiogenic response. It should also be considered that the increase in branching may be an artifact of the DAB staining procedure or the method of branching counting as it may not differentiate between the kurtosis of the capillary and a capillary junction. Therefore, a repetition of our study using another set of brain-injured mice would be suited to validate our results.

The methods used in our investigation are well established in TBI research. Though a number of alternative models of TBI exist, the cFPI model is suited for investigating diffuse TBI (Witcher et al., 2020). Additionally, the immunohistochemistry and immunofluorescence techniques in our study are also commonly used. 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) dye can be used as an alternative to immunostaining capillaries by targeting antigens like CD31 and PDX (Hughes et al., 2014; Obenaus et al., 2017). This technique globally stains brain vasculature, which allows for analyses of global and regional vasculature following sectioning in the coronal plane,

however whether the technique is more sensitive to DAB staining is yet to be determined (Hughes et al., 2014). The measurement of capillary width was performed manually in our study, and in future studies it may be worthwhile to analyse capillary width using the pluggin software VasoMetrics with ImageJ. This would remove potential user error, reduce bias, increase reproducibility of the measurements and reduce the time collecting data (McDowell et al., 2021). Furthermore, our study is limited to a 30-day period in which the progression of TBI pathogenesis is investigated. However it is well established that TBI symptoms can persist for many months to years post-injury (Maas et al., 2017). In the future, studies characterising sex-specific responses to TBI should include a 3- and 6-month time point to study the long-term effects of injury.

Our study did not consider the effect of sex hormones. Literature detailing sex-specific responses to TBI has focused on the influence of female sex hormones, though the neuroprotective effect of progesterone and oestrogen observed experimentally did not translate into a beneficial effect when explored in human trials (Rubin & Lipton, 2019). In our study, the ovulation stage of female mice was not tracked, critically the cyclical hormonal changes may enhance or disguise responses to TBI (McCarthy et al., 2012). Thus, characterising the differences that were previously discussed, should be interpreted with caution due to the possible influence of hormones. Furthermore, a repeat study using gonadectomized mice may determine whether hormones affect the response to TBI. If sex differences still occur then disparities may stem from the developmental stage or due to the sex-linked genes found on the sex chromosomes (McCarthy et al., 2012). Importantly, anatomical disparities in the brain exist between males and females, where females have greater cortical density but have lower grey matter volume, whilst sex differences in cortical thickness are regional (Kaczkurkin et al., 2019). Possibly such anatomical differences between males and females partly account for the disparities in the response to TBI. It has also been observed that females have a higher CBF than males in the frontal and temporal lobes (Aanerud et al., 2017). Therefore, whether sex-specific differences in TBI arise due to differences in CBF should be investigated.

In our study, our sham-injured control animals exhibited a response to the surgery similar to cFPI mice. This was particularly highlighted in the significantly reduced PDX^+ capillary area at 2dpi and significantly reduced $PDGFr\beta^+$ pericyte number at 7dpi (Figure 6b and c). Considering that minimal differences should ideally exist between controls within the sham groups and naïve group, the significant differences that we have observed in our sham groups suggest that a response to the surgical preparation has occurred. Ultimately, if sham models exhibit similar pathologies to cFPI animals, then any significant differences would not be found. Therefore, further refining the surgical procedure of the craniotomy to effectively reduce the cortical response observed in sham animals may be needed for optimal use of the cFPI model.

Taken together, the potential lifelong impacts of TBI, further research is vital for developing effective strategies to target pathological mechanisms seen in TBI. There are sex-specific differences in TBI recovery; critically the mechanisms influencing such differential responses have not yet been established. Here we demonstrate differentiating responses in pericytes and capillary diameter between males and females following TBI. However future studies are required to determine whether the differences observed in our study carry pathological

implications in TBI. Thus, future therapies may have to consider the impact of a patient's sex in the treatment of TBI.

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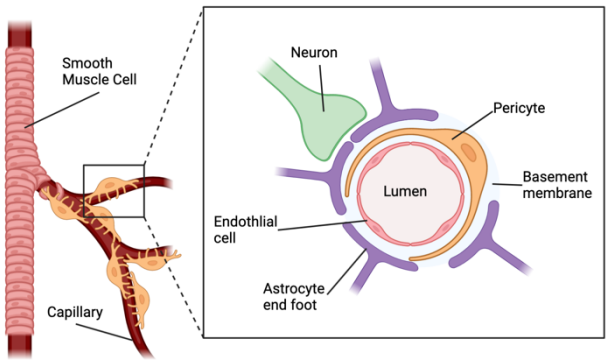
Popularised Summary

Christopher Fry

Sex-specific pathological response to Traumatic Brain Injury

Traumatic brain injury (TBI) is one of the leading causes of death in the world. Harsh insults to the head can have long lasting impacts on physical and mental health. TBI isn't solely a physical injury; intricate mechanisms at the molecular level are triggered potentially causing further harm which only become visible much later in life. Currently TBI is being more frequently linked to a class of neurodegenerative diseases which include Alzheimer's and Dementia.

The blood vessel network in the brain is a highly complex interplay of different cell types to make up the blood brain barrier (BBB). One of the cell types that are critical to the BBB are pericytes. Pericytes directly interact with the blood vessels in the brain and are critical for maintaining the BBB and cerebral blood flow (CBF). In the early stage of TBI, the density of the blood vessel network is decreased and pericyte interaction with the blood vessel network is reduced, leading to BBB leakage into the brain and loss of CBF control.



Recently, research has focused on whether sex-differences exist in TBI. Findings suggest that differences in response to TBI and recovery between the sexes do exist. However, it is not well known why such disparities occur. Therefore, determining the mechanisms driving sex-specific differences in TBI is critical to improve clinical prognosis and patient outcome. We hypothesised that the sex specific differences in TBI exist at the BBB level. Therefore, in our study, we investigated the sex-specific responses of blood vessels and pericytes using a mild TBI mouse model.

Using microscopy techniques, we imaged the blood vessels and pericytes of male and female mice 2, 7 and 30 days after TBI. We found that females exhibited a response in both the blood vessels and pericytes. In female mice, the diameter of blood vessels was found to be significantly decreased at 30 days, whilst the number of vessel branches increased one week after injury. Pericytes in female mice were found to exhibit increased capillary coverage in the early stages of mild TBI, which then returned to a normal state. Unlike in female mice, males did not show a response in blood vessels or pericytes following mild TBI over 30 days.

Our findings support that sex-specific differences exist following mTBI and differential responses occur in blood vessels as well as pericytes. Further research is required to determine which mechanisms at the molecular level are driving such differences.

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