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# Prenatal Hypoxia–Ischemia in Rats Causes Sex-Dependent Effects on the Corpus Callosum Neuroglia and Physical Training During Pregnancy Prevents These Changes

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## Abstract

Background/objectives: Perinatal hypoxia–ischemia (HI) is a major cause of brain injury and is associated with long-term neurological impairments, including white matter injury, astrogliosis, microgliosis, and hypomyelination. Maternal exercise has emerged as a potential non-pharmacological strategy to improve fetal development and increase resilience to prenatal insults. This study investigated whether maternal exercise during pregnancy protects against neuroglial alterations in the corpus callosum of offspring subjected to prenatal HI. Methods: Adult female Wistar rats were assigned to sedentary or exercise groups. The exercise protocol consisted of daily swimming sessions with progressive durations (3–30 min/day) over 10 days. Blood glucose levels and oxidative stress markers (GSH, TBARS, and DPPH) were evaluated to characterize the physiological effects of the protocol. Prenatal HI was induced on gestational day 18 by transient occlusion of the uterine arteries for 45 min, whereas sham-operated dams underwent identical surgical procedures without arterial occlusion. Offspring were euthanized on postnatal days (P) 16 and 23, and brain sections were processed for immunohistochemistry using antibodies against glial fibrillary acidic protein (GFAP) and myelin basic protein (MBP). Images were quantitatively analyzed using Image-Pro Plus, and statistical analyses were performed using SPSS software. Results: The exercise protocol did not show oxidative stress, hypoglycemia, or exhaustion. Female offspring from HI group exhibited astrogliosis at P16, which persisted until P23; however, maternal exercise prevented this response at P23. No changes in MBP immunoreactivity were detected in females. Male offspring did not develop astrogliosis but displayed hypomyelination at P23, which was prevented by maternal exercise. Conclusions: These findings demonstrate that maternal exercise during pregnancy attenuates sex-dependent neuroglial alterations induced by prenatal HI, supporting its potential as a preventive strategy to reduce white matter injury and improve neurodevelopmental outcomes.

**Keywords:** hypoxic–ischemic encephalopathy; gestational exercise; astrogliosis; oligodendrocytes; sexual dimorphism

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## 1. Introduction

Perinatal brain injury remains a major cause of neonatal mortality and long-term neurodevelopmental disability. Among its clinical manifestations, hypoxic–ischemic encephalopathy (HIE) is particularly relevant because it represents a common final pathway of brain injury in term and preterm neonates. Hypoxia–ischemia (HI) induces diffuse damage to both gray and white matter, affecting especially the most vulnerable regions of the developing brain, including the hippocampus, basal ganglia, and major white matter tracts such as the corpus callosum [1,2]. In experimental rodent models, the corpus callosum has emerged as a particularly informative structure for studying neonatal HI because of its high metabolic demand, ongoing oligodendrocyte maturation, and critical role in interhemispheric communication.

From a pathophysiological perspective, reduced cerebral blood flow triggers a cascade of biochemical events that culminate in neuronal and glial injury. ATP depletion caused by primary energy failure compromises ion pumps, leading to membrane depolarization, intracellular calcium overload, and excessive release of excitatory neurotransmitters, especially glutamate. This excessive glutamatergic stimulation activates AMPA and NMDA receptors, amplifying excitotoxicity and accelerating cellular dysfunction [3–6].

Excitotoxicity further promotes oxidative stress, the activation of inflammatory pathways, and recruitment of immune cells, resulting in cerebral edema, blood–brain barrier disruption, and progressive cell death through both apoptotic and necrotic mechanisms [4,6]. Following this acute phase, a latent period of approximately six hours is observed, during which partial reperfusion and transient metabolic recovery occur. This interval constitutes a critical therapeutic window for neuroprotective interventions such as therapeutic hypothermia [4,7]. However, if untreated, secondary energy failure develops between 6 and 48 h after the insult and is characterized by persistent mitochondrial dysfunction, inflammatory amplification, continued excitotoxicity, and progressive cell death in vulnerable regions [3,4]. In some cases, prolonged pathological mechanisms lead to tertiary energy failure, marked by sustained impairments in neurogenesis, axonal growth, synaptogenesis, and myelination, which may persist for months or even years after the initial insult [4,6].

Within this pathophysiological cascade, glial cells play a central role, particularly in the developing white matter. The neonatal corpus callosum is especially vulnerable because oligodendrocyte precursor cells are highly sensitive to oxidative stress, glutamate excitotoxicity, and inflammatory mediators. Injury to these immature oligodendrocytes compromises myelin development and axonal conductivity, contributing to long-term cognitive and sensorimotor deficits [1,8]. Astrocytes are rapidly activated after HI and initially contribute to ionic buffering, glutamate uptake, and metabolic support. However, excessive astroglial activation can become maladaptive, favoring glial scar formation and perpetuation of local inflammation [9].

Biological variables, particularly sex, influence vulnerability to HI. Clinical and experimental evidence consistently show that male neonates exhibit greater susceptibility to hypoxic–ischemic injury and poorer neurological outcomes [10,11]. This sexual dimorphism cannot be explained solely by postnatal hormonal differences but reflects intrinsic molecular mechanisms that regulate cell death pathways.

These sex-dependent differences are relevant because they may also shape glial responses. Emerging evidence suggests that microglial activation profiles, cytokine expression, and the susceptibility of oligodendrocyte lineage cells may differ between males and females following neonatal HI, potentially contributing to distinct patterns of corpus callosum vulnerability and repair [1,10]. Thus, sex should be considered a critical biological variable when investigating mechanisms of white matter injury and testing neuroprotective strategies.

Despite advances in understanding the mechanisms of neonatal HI, effective therapies are limited. Therapeutic hypothermia remains the standard of care for moderate HIE, reducing mortality and improving neurological outcomes when initiated within six hours after birth, but its efficacy is incomplete, particularly in severe cases and preterm infants [4,6,12]. Consequently, there is increasing interest in preventive strategies capable of enhancing endogenous resilience before the insult occurs.

In this context, maternal physical training has emerged as a promising non-pharmacological intervention. Regular physical activity during pregnancy provides maternal cardiovascular and metabolic benefits without adverse fetal effects and is associated with reduced oxidative stress, improved endothelial function, and a more favorable anti-inflammatory systemic profile [13–16]. These maternal adaptations may directly influence the intrauterine environment and fetal brain development.

Animal studies further suggest that maternal exercise promotes important neurobiological adaptations in the offspring, including increased expression of brain-derived neurotrophic factor (BDNF), enhanced hippocampal neurogenesis, improved synaptic plasticity, and increased mitochondrial biogenesis [17,18]. Importantly, maternal exercise may also influence glial programming. Enhanced antioxidant capacity and reduced basal inflammatory tone in the offspring could attenuate excessive astroglial and microglial activation after HI, thereby protecting oligodendrocyte maturation and preserving corpus callosum integrity.

Current evidence suggest that perinatal HI injury should be understood as a dynamic interaction among bioenergetic failure, excitotoxicity, oxidative stress, inflammation, and glial dysregulation [2] that may be better evaluated using systemic HI models like the one used in our previous studies [19–23]. Understanding whether maternal physical training can modulate these glial responses and reduce white matter vulnerability may open new avenues for neuroprotection.

Therefore, the present study aims to investigate whether maternal physical training can attenuate HI injury in the offspring, with particular emphasis on glial responses and corpus callosum integrity in rodent models. For this, we standardized a physical training protocol for pregnant rats and verified its effects on the offspring submitted to a prenatal HI model. By integrating the cellular mechanisms of prenatal HI with the potential conditioning effects of maternal exercise, this approach may contribute to the development of more precise preventive strategies for perinatal brain injury.

## 2. Materials and Methods

### *2.1. Animals and Experimental Design*

Nulliparous female Wistar rats (250–300 g) were obtained from the institutional animal facility and housed under controlled temperatures and a 12 h light/dark cycle (lights on at 06:00 am). They had free access to standard commercial chow (Nuvilab®- Quimtia, Quatro Barras, PR, Brazil) and water. All procedures were approved by the Ethics Committee of the Universidade do Estado do Rio de Janeiro (UERJ/CEA/027/2021).

Mating was performed overnight, and pregnancy was confirmed by vaginal smear (gestational day 1, G1). Physical training began on gestational day 4 (G4). After protocol standardization, animals were randomly allocated into four groups: sedentary sham (SDSH), trained sham (TRSH), sedentary hypoxia—ischemia (SDHI), and trained hypoxia–ischemia (TRHI).

### *2.2. Physical Training Protocol*

A pilot study was conducted to establish a physical training protocol capable of inducing beneficial physiological adaptations without excessive stress. Animals were

trained in a tank filled with tap water (depth: 30 cm; temperature: ~32 °C) equipped with a turbulence system to ensure continuous swimming, adapted from Lancha et al. [24]. Training sessions were performed in the evening.

The training protocol consisted of two blocks of five consecutive training days, that in pregnant rats corresponds to gestational days four to eight (G4–G8) and eleven to fifteen (G11–G15), separated by a 2-day rest period. Exercise duration started at 3 min on the first day and increased progressively by 3 min per day, reaching a maximum of 30 min on the final day. During the exercise protocol, 1–2 females were maintained in each division of the tank and the status of each animal was continuously observed.

Experimental groups for protocol validation included: trained pregnant rats (TRP, $n$ = 12); trained non-pregnant rats (TRNP, $n$ = 12); sedentary pregnant rats (SDP, $n$ = 8); sedentary non-pregnant rats (SDNP, $n$ = 12). The sample size for each group was based on a previous study [24].

To evaluate the effects of acute physical effort in blood glucose levels, a separate group of sedentary non-pregnant rats ($n$ = 6) underwent a single 30 min swimming session. After each session, animals were dried and placed under warming lamps to prevent hypothermia.

### *2.3. Biochemical Analyses*

#### 2.3.1. Blood Glucose Levels

Glycemia was measured using a glucometer (Accu-Chek Performa—Roche Diabetes Care GmbH, Mannheim, Germany, distributed by Roche Diabetes Care Brasil Ltda., São Paulo, Brazil) from tail blood samples collected before and after exercise on training day 10 (30 min) in TRNP, TRP, and SDNP groups subjected to acute physical effort. Additionally, blood samples were also collected on training days 1 (3 min) and 5 (15 min) in the TRP group to evaluate possible fluctuations in blood glucose levels along the period of training.

#### 2.3.2. Oxidative Markers Levels

Female rats from the experimental groups were euthanized with a thiopental overdose i.p. (over 60 mg/Kg), and blood samples were collected by cardiac puncture one hour after the final training session. Serum was separated by centrifugation at 5000× $g$ for 15 min at room temperature and stored at −20 °C until analysis.

- Thiobarbituric Acid Reactive Substances (TBARS)

Lipid peroxidation was assessed as previously described [25]. Plasma samples (100 µL) were incubated with trichloroacetic acid (TCA—Sigma-Aldrich, St. Louis, MO, USA) and Tris-HCl (hydroxymethyl)aminomethane hydrochloride, (Sigma-Aldrich, St. Louis, MO, USA), followed by thiobarbituric acid (Sigma-Aldrich, St. Louis, MO, USA) reaction at 95 °C. Absorbance was measured at 530 nm.

- Total Antioxidant Capacity (DPPH Assay)

Total antioxidant capacity was evaluated using the DPPH radical scavenging method [26]. Plasma samples (100 µL) were incubated with DPPH solution, and absorbance was measured at 520 nm.

- Reduced Glutathione (GSH)

GSH levels were determined according to Tietze [27]. Plasma samples (100 µL) were precipitated with 10% TCA, centrifuged, and the supernatant reacted with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB Sigma-Aldrich, St. Louis, MO, USA). Absorbance was measured at 412 nm using a microplate reader (EZ READ 400; Biochrom, Holliston, MA, USA).

### *2.4. Induction of Hypoxia–Ischemia*

As described in Savignon et al. [19], on gestational day 18 (G18), pregnant rats were anesthetized with isoflurane (4% induction, 1.5–2% maintenance). Following laparotomy, uterine horns were exposed. HI was induced by occlusion of the four uterine arteries, using aneurysm clips for 45 min (HI group). In sham-operated animals, uterine horns were exposed without vascular occlusion.

After clip removal, uterine horns were repositioned, and the abdominal wall was sutured. Lidocaine was applied locally before recovery. Postoperative analgesia was provided with oral dipyrone (10 mg/100 g body weight) for three days.

After training protocol and HI induction surgery, pregnant rats were assigned to four groups: sedentary (non-trained) sham (SDSH); trained sham (TRSH); sedentary hypoxia–ischemia (SDHI); trained hypoxia–ischemia (TRHI).

Pregnancy proceeded to term (E22), and litter was standardized to eight pups within 24 h of birth. Litters with fewer than six pups were excluded to avoid overnutrition. A total of 12 litters from each experimental group was used in this study.

Across all groups, no gross differences were observed in the number of fetuses present in the uterine horns between the day of surgery and the day of birth, indicating that the surgical procedure did not decrease the survival rate. Furthermore, during lactation, water and food intake was comparable to that of dams that did not undergo surgery. The females recovered well, and the incision site was completely healed within a few days. Figure 1 represents the timeline of experiments.

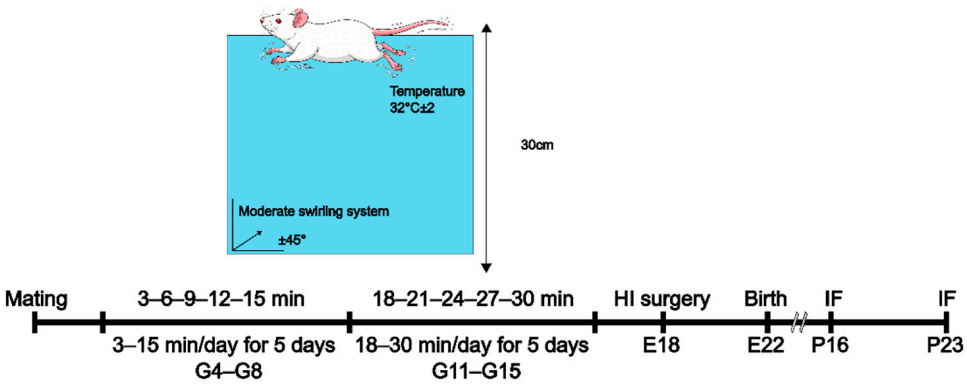



**Figure 1.** Experimental design, including the gestational exercise protocol. Pregnant female rats began the swimming training protocol on gestational day (G) 4, four days after mating, with an initial exercise duration of 3 min that was progressively increased by 3 min each day, reaching 15 min by the end of the first training week. A two-day rest period was observed between G9 and G10. During the second week, training resumed following the same progressive increase of 3 min per day. On G18 (corresponding to embryonic day 18 [E18] in the offspring), surgery was performed to generate the SHAM and HI groups. Pups were delivered naturally on E22 without any experimental intervention during parturition. At postnatal days (P) 16 and P23, pups were submitted to histological procedure and immunofluorescence labeling.

### *2.5. Histological Processing*

Male and female offspring at postnatal days 16 and 23 (P16 and P23; $n$ = 4–6 per group, age, and sex, based on previous studies using the same model [19–23]) were anesthetized with thiopental i.p. (50 mg/Kg) and transcardially perfused with saline, followed by 4% paraformaldehyde and paraformaldehyde with 10% sucrose. To avoid a litter effect, no more than two female or two male animals of the same age from each litter were included in each experimental group. Brains were post-fixed, cryoprotected in 20% and 30% sucrose solutions, embedded in OCT, and sectioned coronally (20 µm) using a cryotome.

Sections were collected according to stereotaxic coordinates 1.6 to 3.5 mm anterior to Bregma based on the developing rat brain atlas [28] and stored at −20 °C until processing.

### *2.6. Immunohistochemistry*

Sections were washed in phosphate-buffered saline (PBS—all reagents from Proquimios, Rio de Janeiro, Brazil) with 0.3% Triton X-100 (Neon Comercial (Suzano, SP, Brazil)) and blocked with 5% bovine serum albumin (BSA—Sigma-Aldrich Brasil LTDA, São Paulo, Brazil). Primary antibodies were incubated overnight at 4 °C. The following primary antibodies against neuroglial markers were used: to astrocytes, anti-glial fibrillary acidic protein (anti-GFAP–G9269—Sigma Aldrich, St. Louis, MO, USA—dilution 1:600), and to mature oligodendrocytes/myelin, anti-myelin basic protein (anti-MBP-MAB386—Millipore, Billerica, MA, USA—dilution 1:100).

Appropriate fluorescent secondary antibodies (Alexa Fluor 488 and 555 conjugated—Thermo Fischer/Invitrogen, Waltham, MA, USA) were applied for 1–2 h at room temperature. Sections were mounted with n-propyl gallate (Sigma-Aldrich Brasil LTDA, São Paulo, Brazil) and analyzed using a fluorescence microscope (Olympus BX40—Olympus Corporation, Tokyo, Japan) coupled to a digital camera (DP71—Olympus Life Science, Waltham, MA, USA).

Qualitative and quantitative analyses were performed in the corpus callosum, focusing on the trunk region, to assess glial reactivity and myelination patterns.

### *2.7. Quantification*

For the analysis of GFAP- and MBP-immunostained images, one image per section was captured at the midline of the corpus callosum, totaling 6 to 8 images per group. Quantitative image analysis was performed by an investigator blinded to the experimental group allocation. Image segmentation and quantitative analysis were performed using Image-Pro Plus version 6.0.0.260 for Windows (Media Cybernetics, Silver Spring, MD, USA). Images were first converted to grayscale, and the anatomical region of interest (ROI) was defined using standardized anatomical landmarks and a predefined graticule (200 × 400 µm). The pixel intensity corresponding to the immunolabeled signal was selected, and a threshold was applied to distinguish labeled pixels from background. The selected area was then masked, and a histogram was generated to determine the pixel-density distribution within the ROI. Quantification was based on the density and area of labeled pixels within the predefined ROI, rather than on optical density alone. The same image-processing and analysis criteria were applied to all experimental groups.

### *2.8. Statistical Analysis*

GraphPad Prism version 8.0.2 for Windows (GraphPad Software, San Diego, CA, USA) was used for graph generation, and statistical analysis was performed using IBM SPSS Statistics for Windows, Version 25.0 (IBM Corp., Armonk, NY, USA). The Kolmogorov–Smirnov test was used to evaluate data normality, and the Brown–Forsythe test was used to assess the homogeneity of variance. Differences were considered statistically significant at $p < 0.05$. In blood glucose analysis, a paired Student's *t*-test was conducted to compare the same sample across different time points, and an independent Student's *t*-test was used for pairwise comparisons between two independent groups. Immunofluorescence samples were compared considering three independent factors: sex (female and male), treatment (SD and TR), and surgical procedure (SH and HI). Three-way (sex, treatment, and procedure) or two-way (treatment and pregnancy) univariate analyses of variance (ANOVA) were performed, followed by Šídák's post hoc test for pairwise comparisons where significant differences were found.

## 3. Results

### *3.1. Physical Exercise Protocol Validation*

#### 3.1.1. Glycemic Profile

To validate the physical exercise protocol, blood glucose levels were measured in non-pregnant and pregnant rats before and after training sessions. Physical exercise protocol was established through a progressive swimming training, consisting of daily exercise with incremental increases of 3 min per day until reaching a maximum duration of 30 min. To characterize the acute physical effort profile, non-pregnant sedentary rats (SD) were submitted to a single session at maximal load (30 min).

Figure 2A shows the blood glucose levels in non-pregnant rats. Paired Student's *t*-tests demonstrated that SD exhibited a significant reduction in blood glucose levels after the maximal load training session (before: 92.17 ± 4.070 mg/dL; after: 37.33 ± 5.64 mg/dL; $p = 0.00125$, observed power = 0.997), consistent with an acute hypoglycemic response. In contrast, no significant changes in glycemic levels were observed in the trained non-pregnant (TR) group following 30 min of exercise (before: 107.80 ± 7.07 mg/dL; after: 117.20 ± 7.66 mg/dL; $p = 0.307$). Independent *t*-tests indicated that pre-training glucose levels were comparable between groups, whereas post-training glucose levels differed significantly ($p < 0.001$, observed power = 0.999).

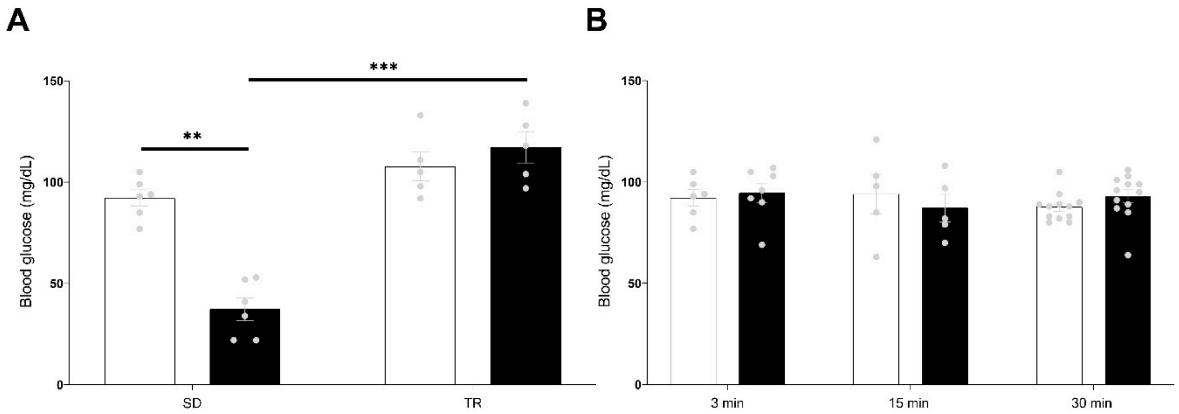



**Figure 2.** Blood glucose levels in non-pregnant (**A**) or pregnant (**B**) rats; white bars, before the exercise, and black bars, after the exercise. In (**A**), observe that after 30 min of exercise, SD group showed a significant decrease in glycemic level (**, $p < 0.01$) compared to the baseline level, whereas no difference was observed in the TR group. Post-training blood glucose level in TR group was significantly increased when compared to SD (***, $p < 0.001$). In (**B**), notice that pregnant rats did not show any difference in glucose levels before and after the exercise along different times of training.

In pregnant trained rats (TRP), no significant differences in blood glucose levels were observed at 3 (before: 95.33 ± 5.75 mg/dL; after: 94.57 ± 4.92 mg/dL; $p = 0.627$), 15 (before: 94 ± 9.66 mg/dL; after: 87.2 ± 6.78 mg/dL; $p = 0.465$) or 30 min of exercise (before: 87.58 ± 2.028 mg/dL; after: 92.91 ± 3.232 mg/dL; $p = 0.1161$) (Figure 2B).

These findings indicate that the maximal effort protocol induced a distinct metabolic response characterized by acute hypoglycemia, whereas the trained groups demonstrated metabolic adaptation to the exercise regimen.

#### 3.1.2. Serum Oxidative Stress Markers and Antioxidant Capacity (TBARS, GSH, and DPPH)

Serum oxidative stress markers, including TBARS, GSH, and DPPH radical scavenging activity, were evaluated in both pregnant and non-pregnant animals.

Analysis of lipid peroxidation via TBARS (Figure 3A) revealed a statistically significant interaction between pregnancy and physical training (F = 16.755, $p = 0.0004$, observed

power = 0.976). SDP, TRNP and TRP groups showed significantly decreased TBARS levels compared to SDNP (SDNP: 24.919 ± 6.528 µM; TRNP: 10.568 ± 8.514 µM-SDNP-TRNP observed power = 0.997; SDP: 3.848 ± 1.325 µM-SDNP-SDP observed power = 1; and TRP: 8.441 ± 3.508 µM, $p < 0.001$).

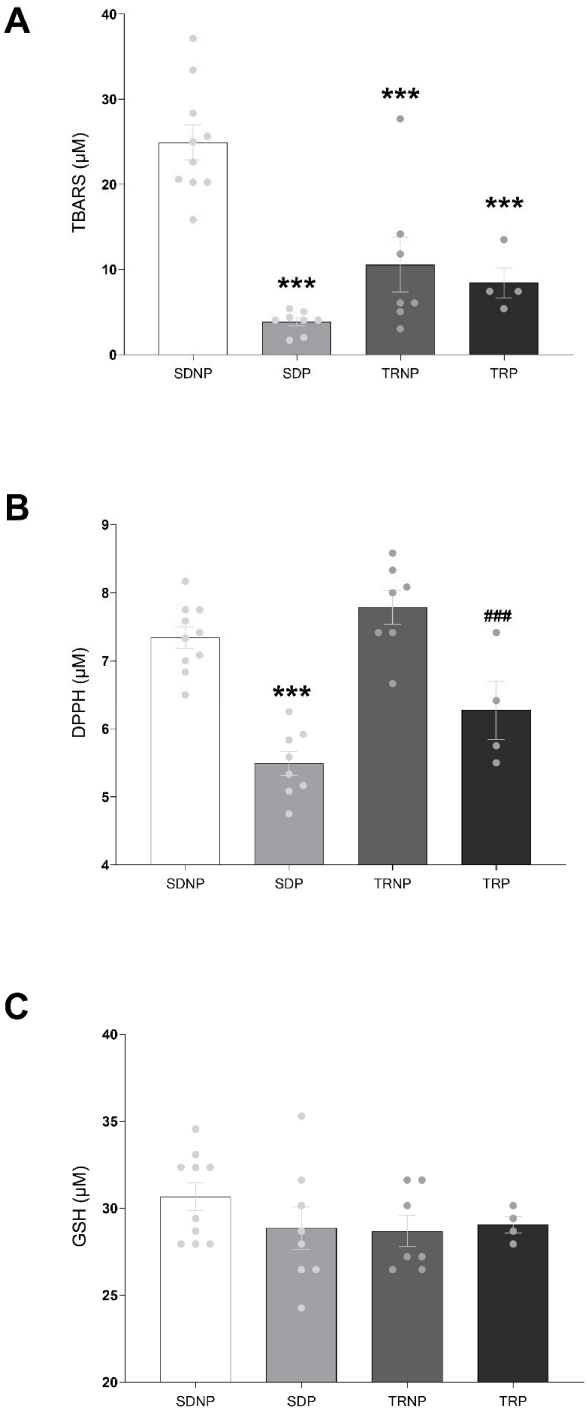



**Figure 3.** TBARS (**A**), DPPH (**B**) and GSH (**C**) blood levels in the experimental groups. In (**A**), either pregnancy or training significantly decreased the TBARS levels. In (**B**), observe that the DPPH levels were significantly decreased both in SD or TR pregnant groups in comparison to SDNP and TRNP, respectively. In (**C**), GSH levels did not show any difference between the experimental groups. *** vs. SDNP; ### vs. TRNP; $p < 0.001$.

For DPPH radical scavenging activity, significant main effects were found for pregnancy (F = 52.410, $p < 0.001$, observed power = 1) and physical training (F = 6.945, $p = 0.0142$,

observed power = 0.717). Pregnancy decreased DPPH levels either in SD or in TR groups (SDNP: 7.34 ± 0.497 µM; TRNP: 7.785 ± 0.657 µM; SDP: 5.48 ± 0.496 µM-SDNP-SDP observed power = 1; and TRP: 6.27 ± 0.86 µM-SDNP-TRNP observed power = 0.975, $p < 0.001$) (Figure 3B).

Regarding GSH, no statistically significant differences were observed in any of the analyses (ANOVA interactions: pregnancy $p = 0.500036$; treatment $p = 0.398139$; pregnancy and treatment $p = 0.310607$) (Figure 3C), suggesting that neither exercise nor pregnancy affected this parameter.

Despite the reduction in DPPH radical scavenging activity, gestational physical training did not appear to exert deleterious effects on redox homeostasis, as lipid peroxidation was reduced and GSH levels remained unchanged.

### *3.2. GFAP Immunohistochemistry in the Corpus Callosum*

To evaluate the distribution and morphological profile of astrocytes in the corpus callosum, immunolabeling with anti-GFAP antibody was performed in brains of the offspring of the experimental groups at P16 and P23. In P16 animals, GFAP-positive cells exhibited heterogeneous morphologies, as illustrated in Figure 4. Some astrocytes displayed thin and short processes, whereas others showed thicker and longer processes with more intense labeling, occupying a larger tissue area (arrows and inserts). Qualitative analysis revealed a higher prevalence of hypertrophic astrocytes, characterized by thicker processes and stronger GFAP immunoreactivity in the HI group. No evident effect of physical training on astrocyte morphology was observed at this age in both sexes.

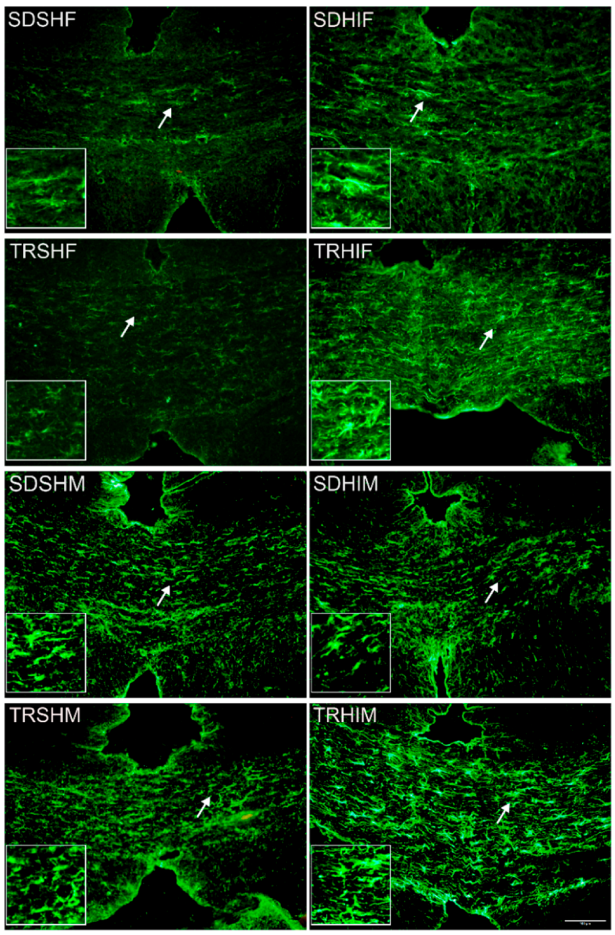



**Figure 4.** Immunofluorescence with anti-GFAP antibody in P16 animals. Immunostained astrocytes showed different morphologies and thicker processes, with evidence of astrogliosis in HI groups (arrows).

The inserts in each figure correspond to the region assigned by the arrow. Gestational exercise or sex did not appear to affect astroglial morphology. SDSH = sedentary surgical control group; SDHI = sedentary hypoxia–ischemia group; TRSH = trained surgical control group; TRHI = trained hypoxia–ischemia group. F = female; M = male. Calibration bar = 100 μm.

In P23 animals, qualitative analysis showed an increased astrocyte population in the corpus callosum of sedentary females subjected to HI (Figure 5-SDHIF). This group also exhibited hypertrophic astrocytes with thicker processes and more intense GFAP immunoreactivity, consistent with astroglial activation (astrogliosis). In contrast, the remaining groups displayed smaller astrocytes with thinner processes and less intense labeling (arrows and inserts).

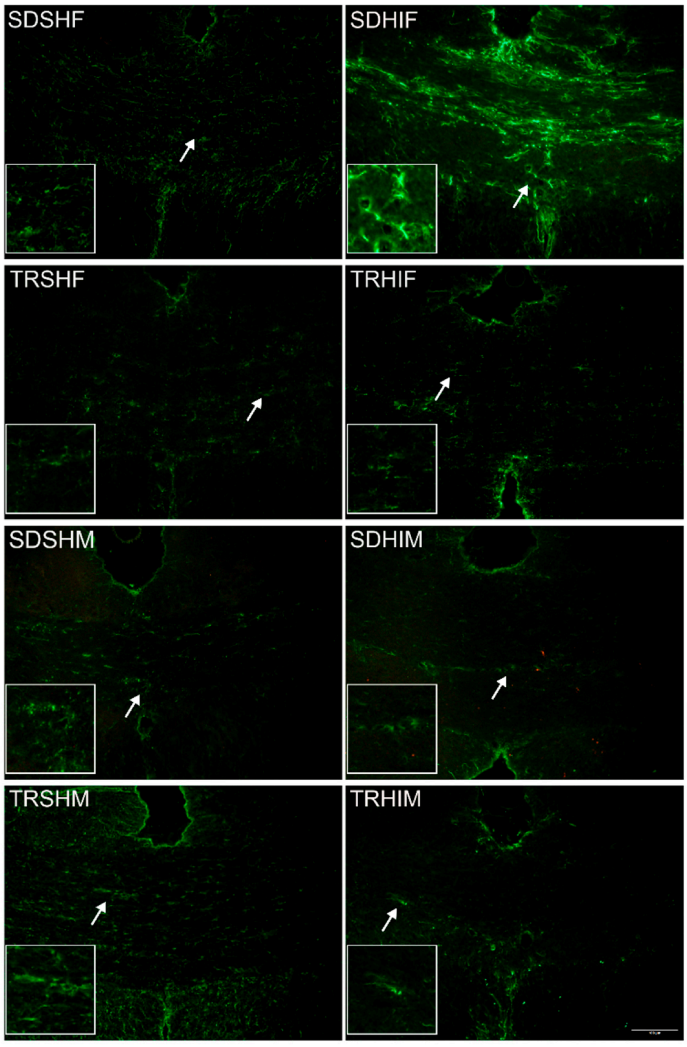



**Figure 5.** Immunofluorescence with anti-GFAP antibody in P23 animals. Arrows point to immunostained astrocytes shown in a higher zoom in the inserts. SDHIF group showed intense immunolabeling in thicker astroglial processes. SDHIM and the remaining groups (SDSH, TRSH and TRHI) exhibited faint staining and small astrocytes with thin processes. SDSH = sedentary surgical control group; SDHI = sedentary hypoxia–ischemia group; TRSH = trained surgical control group; TRHI = trained hypoxia–ischemia group. F = female; M = male. Calibration bar = 100 μm.

Quantitative analysis of GFAP immunostaining showed that, in P16 animals, differences were found solely in relation to sex and surgical procedure ($F = 8.468$, $p = 0.008$, observed power = 0.792), allowing data to be collapsed across conditioning groups (Figure 6A), HIF: 65,351.47 ± 10,298.99; HIM: 30,985.20 ± 10,298.99 (HIF-HIM observed

power = 0.614), SHF: 23,436.58 ± 11,124.18 (SHF-HIF observed power = 0.751) and SHM: 52,646.73 ± 11,124.18. Post hoc analysis demonstrated that the GFAP immunolabeling was significantly increased in the HIF group compared to SHF group ($p = 0.012$) and that the HIF group was also significantly different from the HIM group ($p = 0.028$), These findings indicate that HI increases astroglial reactivity in the corpus callosum in a sex-dependent manner at P16.

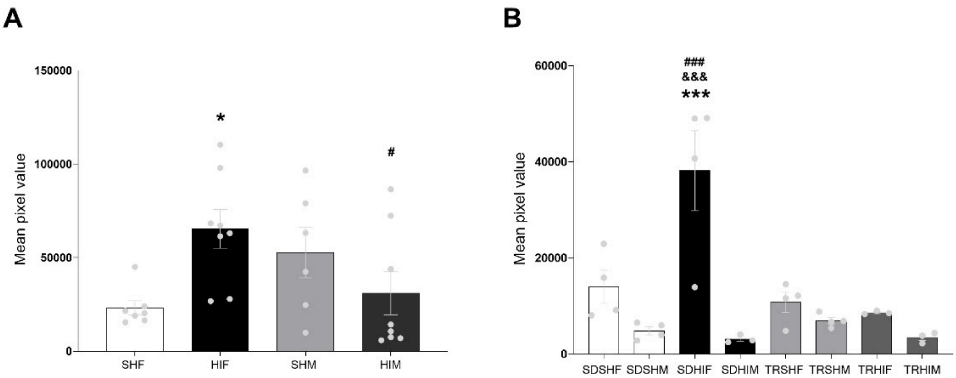



**Figure 6.** Quantitative analysis of GFAP immunolabeling at P16 (**A**) and P23 (**B**). At P16, HIF and HIM groups showed increased GFAP immunofluorescence in relation to SHF and SHM, respectively, and there was no effect of physical conditioning. At P23, the effect was restricted to females, as SDHIF exhibited more intense staining in comparison to SDSHF. GFAP immunolabeling was also higher in SDHIF when compared to SDHIM. Gestational training induced a decrease in GFAP observed in the comparison between SDHIF and TRHIF. *** vs. SDSHF, ### vs. SDHIM, &&& vs. TRHIF, $p < 0.001$; * vs. SHF, # vs. HIM, $p < 0.05$.

At P23, quantitative analysis revealed a significant interaction among sex, treatment, and surgical procedure (F = 5.387, $p = 0.03$, observed power = 0.600), as seen in Figure 6B. The sedentary female HI group (SDHIF) exhibited a marked and statistically significant elevation in the GFAP-immunoreactive area compared to all other experimental groups SDHIF: 38,177.35 ± 3527.27; SDHIM: 3128.26 ± 4072.94 (SDHIF-SDHIM observed power = 1); SDSHF: 14,007.42 ± 3527.27 (SDHIF-SDSHF observed power = 0.996); SDSHM: 4828.99 ± 3527.27; TRHIF: 8616.57 ± 4072.94 (SDHIF-TRHIF observed power = 0.999); TRHIM: 3465.57 ± 4072.94; TRSHF: 10,778.10 ± 3527.27; TRSHM: 6938.42 ± 3527.27; $p < 0.0001$ for all comparisons.

These findings indicate that physical training during gestation successfully prevents the abnormal increase in astroglial reactivity induced by HI in sedentary females.

### *3.3. MBP Immunohistochemistry in the Corpus Callosum*

MBP immunoreactivity, used to identify processes of mature oligodendrocytes and compact myelin sheaths surrounding axons, was analyzed in the corpus callosum at P16 and P23. No differences were observed among experimental groups in either male or female offspring at P16 (Figure 7), indicating that HI and physical conditioning did not affect MBP labeling at this developmental stage.

Qualitative analysis of MBP immunolabeling at P23 revealed increased staining intensity compared to P16, consistent with the higher density of myelinated fibers at this developmental stage (Figure 8). Notably, a reduction in MBP immunoreactivity was observed in the SDHIM group when compared to all other groups, suggesting impaired myelination specifically in sedentary males subjected to HI insult.

Quantitative analysis of MBP at P16 did not show statistically significant differences in any of the experimental analyses (Figure 9A). In contrast, quantitative analysis of MBP immunoreactivity at P23 (Figure 9B) revealed a significant triple interaction among sex,

physical conditioning, and surgical procedure (F = 4.369, $p$ = 0.049, observed power = 0.514). The mean values were: SDSHF: 85,641.34 ± 16,508.18; SDSHM: 131,455.73 ± 19,062.00 (SDHIM-SDSHM observed power = 0.777); SDHIF: 115,898.40 ± 16,508.18 (SDHIF-SDHIM observed power = 0.636); SDHIM: 59,444.71 ± 16,508.18 (SDHIM-TRHIM observed power = 0.876); TRSHF: 77,164.01 ± 16,508.18 (TRSHF-TRSHM observed power = 0.573); TRSHM: 133,802.79 ± 19,062.00; TRHIF: 83,978.30 ± 16,508.18; (TRHIF-TRHIM observed power = 0.591); and TRHIM: 141,863.42 ± 19,062.00).

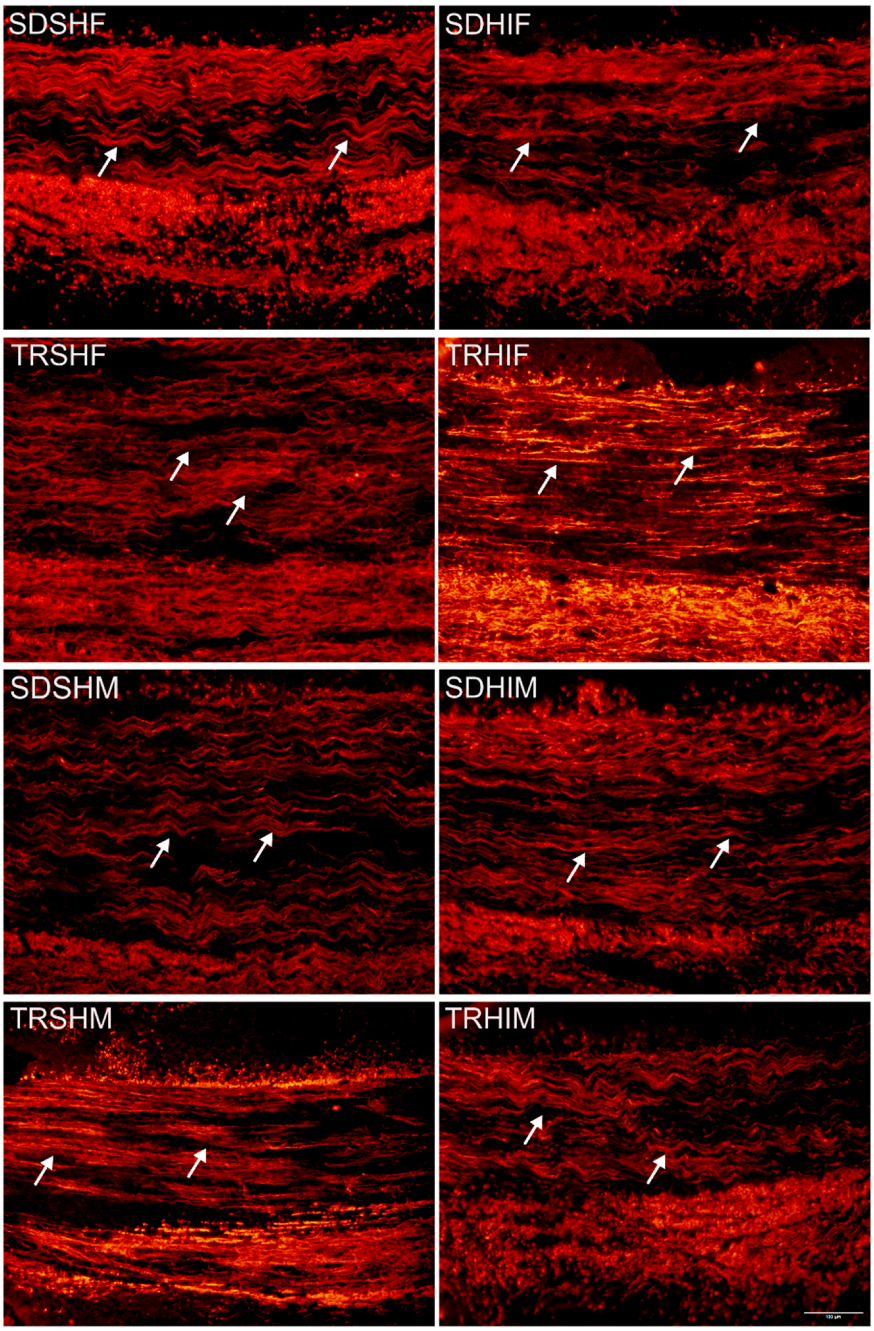



**Figure 7.** Immunofluorescence using anti-MBP antibody in P16 animals. Arrows indicate immunostained myelinated axons. Prenatal HI or gestational exercise did not affect offspring myelination at P16 as no gross alterations were found in MBP immunofluorescence among the groups. SDSH = sedentary surgical control group; SDHI = sedentary hypoxia–ischemia group; TRSH = trained surgical control group; TRHI = trained hypoxia–ischemia group. F = female; M = male. Calibration bar = 100 μm.

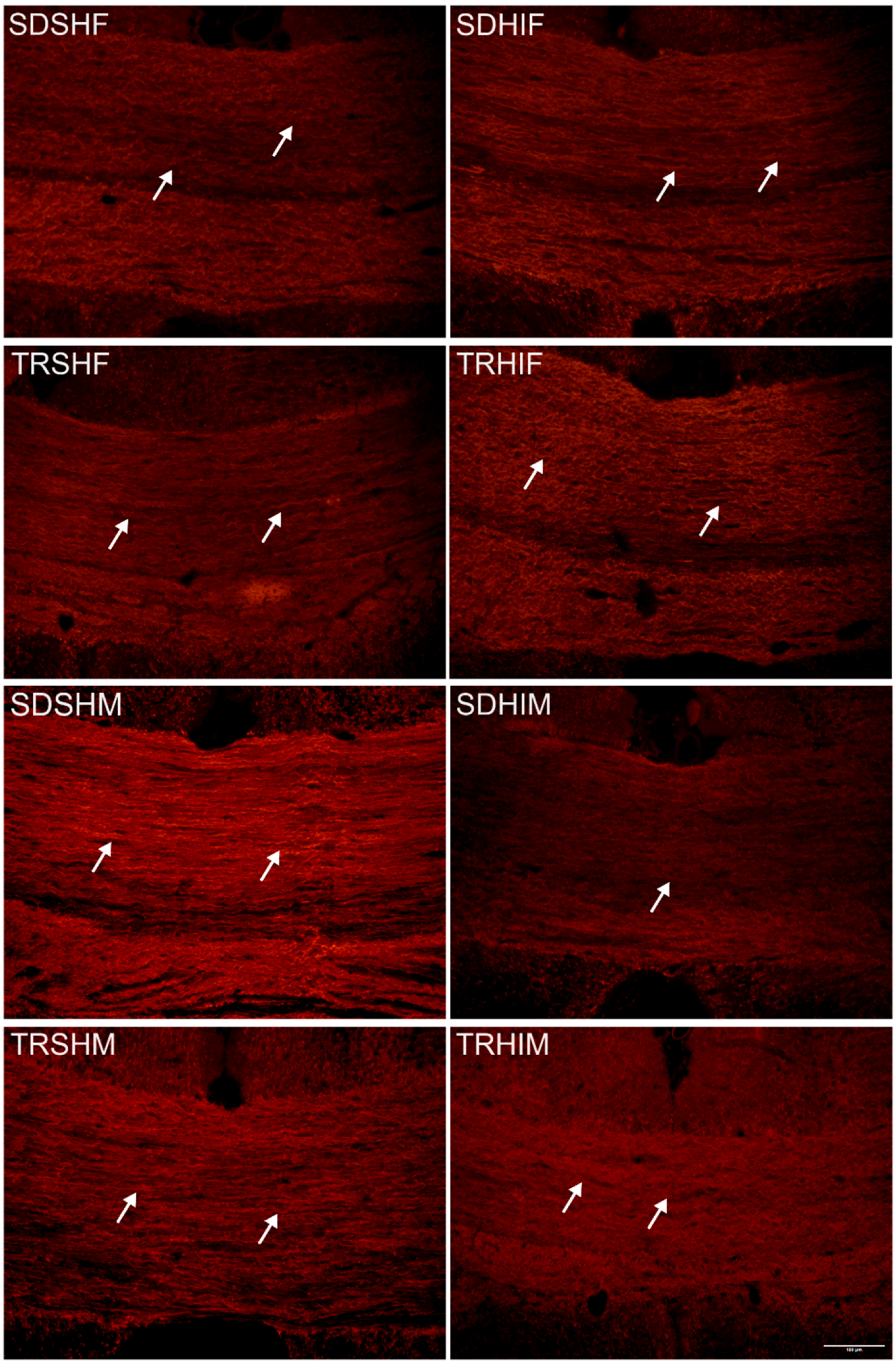



**Figure 8.** Immunofluorescence using anti-MBP antibody in P23 animals. Arrows indicate immunostained myelinated axons. Male offspring exhibited impaired myelination followed by prenatal HI, as differences in MBP immunoidentification were visualized exclusively in SDHIM. SDSH = sedentary surgical control group; SDHI = sedentary hypoxia–ischemia group; TRSH = trained surgical control group; TRHI = trained hypoxia–ischemia group. F = female; M = male. Calibration bar = 100 µm.

Sidak's post hoc comparisons demonstrated that the SDHIM showed a significant reduction compared to its respective sham control (SDSHM; $p = 0.009$), indicating that HI impaired myelination specifically in sedentary males. SDHIM also exhibited statistically lower levels of MBP immunoreactivity in relation to SDHIF ($p = 0.025$), reinforcing this sex-dependent effect. Interestingly, the TRHI M group presented significantly higher immunostaining levels when compared to SDHIM ($p = 0.004$), which suggests that gestational physical conditioning was able to prevent myelin deficits in HI males.

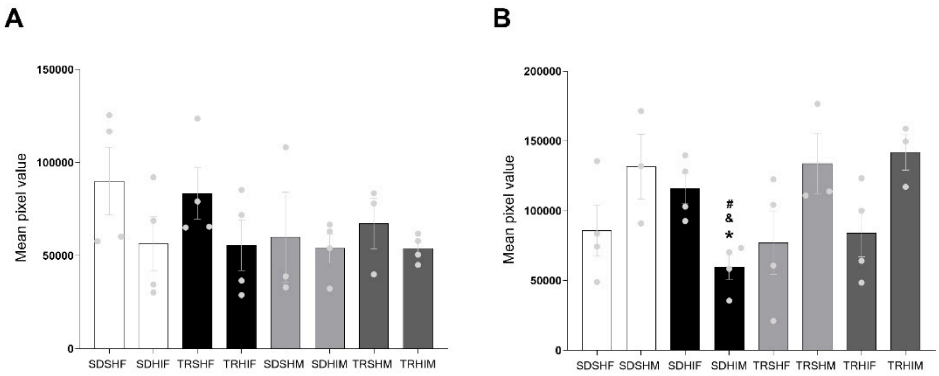



**Figure 9.** Quantitative analysis of MBP immunolabeling at P16 (**A**) and P23 (**B**). MBP immunoreactivity levels did not differ across groups at P16. At P23, however, male offspring showed a significant effect of HI, which was prevented by maternal conditioning: MBP immunolabeling was lower in SDHIM in comparison to SDSHM, SDHIF, and TRHIM. * vs. SDSHM, # vs. SDHIF, & vs. TRHIM, $p < 0.05$. Differences between sexes were found in the TRSHF and TRSHM ($p = 0.036$) and the TRHIF and TRHIM ($p = 0.032$).

Other significant differences identified were related to sex-specific comparisons, showing that maternal physical exercise may exert its effects primarily on male individuals, as TRSHM and TRHIM presented higher myelination than TRSHF ($p = 0.036$) and TRHIF ($p = 0.032$), respectively.

## 4. Discussion

Prenatal HI produced distinct neuroglial alterations in the corpus callosum according to sex and developmental stage. Female offspring exhibited an early increase in astrocytic reactivity that persisted until P23, whereas males primarily developed impaired myelination during the later stages of white matter maturation. Maternal physical training selectively prevented these alterations, attenuating persistent astrogliosis in females and preserving MBP immunoreactivity in males. Rather than exerting a generalized effect on glial responses, exercise appeared to modulate the pathological processes that predominated in each sex.

Before examining the effects of prenatal HI on the offspring, it was important to confirm that the swimming protocol represented moderate physical training instead of excessive physiological stress. Sedentary animals exposed to maximal effort swimming developed marked hypoglycemia, whereas trained animals, both pregnant and non-pregnant, maintained stable glycemic levels after exercise. Pregnant rats did not show significant glycemic fluctuations along the training protocol which contributes to maintaining metabolic homeostasis.

The oxidative stress analyses also supported this interpretation. Sedentary non-pregnant animals showed the highest TBARS levels, while pregnancy was associated with lower lipid peroxidation regardless of exercise. Although GSH concentrations remained unchanged, DPPH analysis revealed significant effects of both pregnancy and physical training on antioxidant capacity. Altogether, these results indicate that the protocol induced physiological adaptation without causing metabolic exhaustion or oxidative imbalance. This is particularly relevant because oxidative stress plays a central role in hypoxic–ischemic brain injury and contributes directly to glial dysfunction and white matter damage [4]. Moreover, moderate exercise during pregnancy is known to improve maternal metabolic regulation without compromising fetal development [14–16].

Astrocytic activation was the earliest glial alteration detected after prenatal HI. At P16, increased GFAP immunoreactivity was observed exclusively in females, regardless of

maternal exercise. This response is in accordance with the well-established role of astrocytes as one of the first cell populations activated after hypoxic–ischemic injury. In the acute phase, reactive astrocytes contribute to maintaining extracellular homeostasis through glutamate uptake, potassium buffering and metabolic support to neural tissue [9,19]. Therefore, the absence of an effect of maternal exercise at this stage suggests that prenatal conditioning did not interfere with this initial adaptive response.

A different pattern emerged at P23. Astroglial activation remained elevated only in the female offspring of sedentary dams, whereas the female offspring of trained dams exhibited GFAP levels comparable to those of sham animals. Notably, the sedentary HI female group differed from all other experimental groups, indicating that persistent astrogliosis represented the most prominent glial alteration identified in the present study. This finding suggests that maternal exercise did not prevent the initial activation of astrocytes but limited its persistence over time. Such an interpretation is consistent with current evidence indicating that prolonged astrocyte activation shifts from a protective response to a maladaptive state characterized by sustained inflammatory signaling, extracellular matrix remodeling and impaired tissue repair [2,9]. Limiting persistent astroglial activation may therefore represent one of the mechanisms through which maternal exercise preserves white matter homeostasis after prenatal HI.

The clear sexual dimorphism observed in astrocytic activation deserves particular attention. Although male neonates generally show greater susceptibility to hypoxic–ischemic brain injury, accumulating evidence indicates that males and females activate different cellular programs after neonatal insults [1,10]. In the present study, females exhibited a stronger astroglial response, whereas males were more vulnerable to alterations in myelination. These findings reinforce the concept that biological sex influences not only the severity of injury but also the cellular pathways involved in tissue remodeling and recovery.

In contrast to GFAP, MBP immunoreactivity was unchanged at P16. Since myelin formation in the rat corpus callosum becomes more pronounced during the third and fourth postnatal weeks, structural alterations in mature myelin proteins would be expected to become detectable only later in development [29,30]. Thus, the absence of MBP differences at P16 likely reflects the developmental timing of myelination rather than resistance to the hypoxic–ischemic insult.

By P23, however, a distinct pattern became evident. Prenatal HI reduced MBP immunoreactivity exclusively in SD-males, while TR-males maintained values comparable to those of sham controls. Females, in contrast, showed no detectable changes in MBP despite presenting persistent astroglial activation. These observations indicate that astrocytic activation and impaired myelination did not occur in parallel but instead represented different consequences of prenatal HI depending on sex.

The greater susceptibility of males to hypomyelination is in line with the current knowledge regarding white matter injury after neonatal HI. Oligodendrocyte lineage cells are highly vulnerable during development because they have limited antioxidant capacity and are particularly sensitive to excitotoxicity, oxidative stress and inflammatory mediators [2,11]. Damage to these immature cells compromises myelin formation and may ultimately impair interhemispheric connectivity and neurological function. Our findings extend this concept by showing that these alterations were effectively prevented by maternal exercise.

Myelin sheaths not only insulate axons, but are also essential for neuron survival. Thus, hypomyelination has been extensively associated with decreased lifespan in transgenic mouse models [31], with more severe hypomyelination leading to shorter survival [32]. Myelin deficits are also responsible for several functional neurobehavioral deficits such

as the onset of ataxia and tremors [32], negative impacts on motor performance [33] and impairments in learning and memory [34,35].

Although the long-term consequences of reduced myelination remain unclear, evidence from neonatal rat models of HI injury suggests that impaired oligodendrocyte maturation and myelin integrity may drive persistent behavioral and neurological deficits in adulthood [36,37]. Conversely, promoting oligodendrocyte maturation and myelination correlates with improved long-term neurological outcomes following neonatal HI [38]. Thus, early disruptions in myelin development likely serve as a key substrate for heightened long-term neurological vulnerability.

Importantly, previous studies using the same HI model have demonstrated long-term behavioral consequences, including impairments in learning and memory [21] and depressive-like behavior [23] at P45. These behavioral alterations are particularly relevant in the context of corpus callosum pathology, as both cognitive and affective functions have been associated with the integrity and myelination of interhemispheric white matter tracts. Therefore, alterations in corpus callosum myelination following perinatal HI may contribute to the long-term neurobehavioral abnormalities observed in this model.

Relatively few studies have specifically investigated the effects of maternal exercise in prenatal HI models, but evidence from closely related experimental paradigms supports the protective effects observed in the present study. Maternal exercise performed under conditions of prenatal hypoxia, gestational stress, or prenatal inflammation has been shown to attenuate behavioral impairments in the offspring, including improvements in emotional, cognitive, and motivational outcomes, as well as partial preservation of cortical and limbic brain structures [39–42]. The practice of swimming exercise during pregnancy mitigated mitochondrial dysfunction and oxidative stress, while improving long-term cognitive outcomes after neonatal HI in a sex-dependent manner [43]. Collectively, these findings suggest that maternal exercise acts as a systemic modulator of fetal programming, enhancing neural resilience and increasing the offspring's capacity to withstand a broad range of perinatal insults.

Although the mechanisms responsible for this protection were not directly investigated, several pathways may explain the observed effects. Maternal exercise has been associated with improved placental function, enhanced uteroplacental blood flow, lower systemic inflammation and increased availability of neurotrophic factors that support fetal brain development [44,45]. Improvements in the cerebral enzymatic antioxidant system and the consequent modulation of oxidative damage may delay or even prevent redox alterations associated with HIE. These effects can be achieved not only through the upregulation of the antioxidant defense system, which reduces reactive oxygen species (ROS) levels and oxidative stress severity, but also because long-term exercise-induced adaptations may directly decrease mitochondrial ROS production [45–47].

Molecular chaperones such as 70 kDa heat shock proteins (HSP70) have a vital role in protein homeostasis and apoptotic pathway inhibition, while hypoxia-inducible factors (HIFs) coordinate metabolic reprogramming and adaptation to oxygen deprivation [48]. Recent investigations reveal that HSP70 could directly support glutathione homeostasis. Administration or induction of HSP70 helps restore cytosolic and mitochondrial GSH pools during severe metabolic stress, preventing lipid peroxidation, maintaining cellular thiol–disulfide balance, and curbing excitotoxicity [49]. Thus, these factors can play a relevant role in endogenous neuroprotective mechanisms in the face of insults such as HI and oxidative stress, notably through the integrated action of heat shock proteins and cellular antioxidants [50]. Together, these adaptations may create a more favorable intrauterine environment, increasing fetal tolerance to hypoxic–ischemic injury and preserving white matter development.

An interesting aspect of the present findings is that maternal exercise did not uniformly affect all glial parameters. Instead, its effects were restricted to the pathological alterations established in each sex. Persistent astrogliosis was prevented in females, whereas hypomyelination was prevented in males. This selective profile suggests that exercise modulates the progression of injury rather than suppressing physiological glial responses. Such an interpretation agrees with the current concepts of neuroprotection, which emphasize preserving endogenous repair mechanisms while limiting chronic neuroinflammation and white matter degeneration [2,11].

Some limitations should be considered. The study focused exclusively on GFAP and MBP immunoreactivity, preventing a more comprehensive characterization of microglial activation, oligodendrocyte precursor dynamics and inflammatory signaling. In addition, behavioral outcomes were not evaluated at the ages analyzed here, precluding direct associations between the histological findings and functional performance. Future studies combining molecular, cellular, and behavioral approaches will be important to clarify the mechanisms underlying the sex-dependent effects of maternal exercise observed in this model.

It must be considered that exercise prescriptions for pregnant women should be individualized based on their metabolic status, baseline fitness level, and hormonal profile [13]. While highly fit pregnant women may maintain moderate-to-vigorous training, those with lower fitness levels should aim to exercise at intensities appropriate for their current physical condition. In this regard, physical exercise can stimulate oxytocin levels in pregnant women, a factor that also appears to be associated with improved labor progression [51,52]. Further studies are needed to better determine the optimal exercise dosages and loads required to elicit this effect.

In summary, prenatal HI produced distinct patterns of corpus callosum injury in male and female offspring. Persistent astroglial activation predominated in females, whereas impaired myelination was the principal alteration observed in males. Moderate maternal physical training effectively prevented both responses without interfering with normal glial development, supporting gestational exercise as a promising non-pharmacological strategy to increase fetal resilience to prenatal hypoxic–ischemic injury. These findings also reinforce the importance of considering biological sex as a key variable in studies investigating the mechanisms of perinatal white matter injury and the development of preventive neuroprotective strategies.

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Animals and approved by the Rio de Janeiro State University (UERJ) Ethics Committee for Animal Experimentation (protocol codes UERJ/CEA/027/2021, approval date 3 August 2021).

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**Data Availability Statement:** The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

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