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Original Article

Strength training during pregnancy influences hippocampal plasticity but not body development in neonatal rats

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Abstract

Objective: To describe the effects of strength exercise practice during pregnancy on the offspring's development parameters: growth and motor performance, hippocampal neuroplasticity, and stress levels. **Methods**: Pregnant Wistar rats were divided into two groups: sedentary and exercised rats. Exercised pregnant rats were subjected to a strength training protocol (vertical ladder climbing) throughout the gestational period. Male offspring's body weight, length, and head size were evaluated during the neonatal period (postnatal days [P]2–P21), as well as motor milestones during PO–P8. At P8, a set of male pups were subjected to global hippocampal DNA methylation, hippocampal cell proliferation, and plasma corticosterone concentration. **Results**: Offspring from trained mothers presented a transient change in body morphometric evaluations, no differences in milestone assessments, enhancement of cell proliferation in the dentate gyrus of the hippocampus, and decreased global hippocampal DNA methylation compared with the offspring from sedentary mothers. Furthermore, strength training during pregnancy did not change the corticosterone concentration of exercised mothers and their offspring. **Conclusions**: These data indicate that strength training can protect offspring's development and could impact positively on parameters linked to cognitive function. This study provides a greater understanding of the effects of strength exercise practiced during pregnancy on the offspring's health.

Keywords: Epigenetics, Neural Plasticity, Neurogenesis, Physical Activity

Introduction

The gestational period is considered a critical development window, and environmental influences, such as physical exercise practice and diet, in this period can be important for offspring's health outcomes[1]. However, the influence of progenitor's health habits on descendants is more studied in diseases scenarios[2].

The healthy lifestyle of parents could influence offspring by programming somatic cells in utero, influencing the development of tissues and organs, such as the brain[3]. One of the mechanisms involved in the programming of somatic cells is epigenetic, which can be defined as changes to gene expression that are independent of sequence modifications and that are mitotically stable and, in some cases, heritable[3,4]. One of the epigenetic marks is DNA methylation. This modification leads to a conversion of cytosine to 5-methylcytosine, with reduced accessibility of the DNA to transcription factors. Inhibition of DNA methyltransferases

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Table 1. Maternal resistance exercise variables in experimental group.

Group	Set	Repetitions	Intensity / Maximum lead loaded	Frequency	Rest
SS	-	-	-	-	-
SE	4 – 9 climbs per day of training	8 – 12 repetitions per climb	35 – 75% MLC/ 439.81 ± 43.81	3 times per week / total of 8 - 9 days of training	120 sec

MLC, Maximum load capacity test. n=8 - 9 per group. Data are expressed as mean ± standard error of the mean.

(enzymes that catalyze the transfer of a methyl group to DNA), for example, prejudices long-term potentiation in the hippocampus and alters the methylation of the promoter regions of *Reelin* and *Bdnf*, two hippocampal genes that are directly involved in synaptic plasticity[5,6].

Basic and clinical research has studied the effects of maternal aerobic exercise on progeny health. Animal studies demonstrate that maternal aerobic exercise during pregnancy improves offspring's spatial learning by increasing neuroplasticity factors[7,8]; in turn, clinical studies revealed positives influences of pregnancy aerobic exercise on language and intelligence scores of the descendants[9,10]. However, other modalities such as strength exercise, acrobatic exercise, and functional exercise are not widely explored.

Strength training during the gestation period has been avoided by pregnant women, due to concerns that the practice of this modality could cause harm to mother and child[11,12]. However, clinical trials[13,14] concluded that this modality of exercise practiced during pregnancy does not cause any harmful effects to mothers and children. Studies investigating the effects of strength exercise, specifically in adult rats, demonstrated improvement in cognitive performance and enhancement in hippocampal neurogenesis[15,16]. Cassilhas et al.[16], for example, showed that a strength training protocol yields positive results on learning and spatial memory and increases the concentration of some neuroplasticity markers (synapsin, synaptophysin, and IGF-1) in the hippocampus. Until now, there are no studies exploring the effects of strength exercise during pregnancy on offspring brain.

Thus, the present study describes the effects of strength training during the gestational period on physical and motor development, as well as on the offspring's hippocampal neuroplasticity and corticosterone (CORT) concentration in the neonatal period.

Material and Methods

Animals and Groups

Twenty-four females and eight males Wistar rats (80 days old) from a local breeding colony (Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Brazil) were used for obtaining male progeny. To avoid the effects of hormonal variation in females – that could influence the results of the behavioral and molecular outcomes studied here – only male offspring were used for the study. The estrous cycle of the primiparous females was monitored daily and in the proestrus phase, the rats were housed overnight with a sedentary male to mate. If the spermatozoa were detected in the vaginal smear in the next morning to the mate, that day was considered as the fertilization day or day 0 of gestation (G0); after the mating period, male progenitor was discarded from the present study. Pregnant rats were divided into sedentary and exercised. After birth, two experimental groups were composed of male offspring (n=72) from sixteen different litters, [1] offspring from sedentary mothers and [2] offspring from exercised mothers. Animals were kept in standard living boxes (300 mm x 190 mm x 130 mm), – one animal per box, with your litter (n=7–8), in a controlled temperature environment (20±2°C), dark/light cycle of 12 hours (lights on at 7:00 a.m.), with water and food *ad libitum*. Procedures performed in this study were approved by the Ethical Committee at the Universidade Federal do Rio Grande do Sul (#29840).

Physical Exercise Protocol

The physical exercise protocol consisted of pregnant rats climbing a vertical ladder (1,1 x 0,8 m), with an inclination of 85°, with lead weights fixed to animal tail with adhesive tape.

Two weeks before the pregnancy, female rats were familiarized with the apparatus by climbing it three consecutive days. On the first day, 3 climbs without weight fixed in their bodies. On the second and third days, 3 climbs with 30 g of weight fixed in their bodies.

One week before the pregnancy, a maximum load capacity test (MLC) was performed individually in the future exercised mothers, which consisted of 4 to 8 climbs while carrying progressively heavier loads. The starting load in the MLC test corresponded to 50% of the animal's body weight. Once the rat reached the top of the stairs, the animal could rest for 120 seconds. After successfully completing climbing with the starting load, 30g of lead was added. This process was repeated until the animals were no longer able to complete the full climb. The highest load successfully carried in the complete length of the vertical ladder was considered the MLC of the animal and was used for the first four training days.

In the firsts 4 training days (after pregnancy confirmation) animals had to climb a vertical ladder 4 to 9 times, with 30%, 45%, 55% and 75% of their MLC load. Before the next training day (training day five) another MLC test

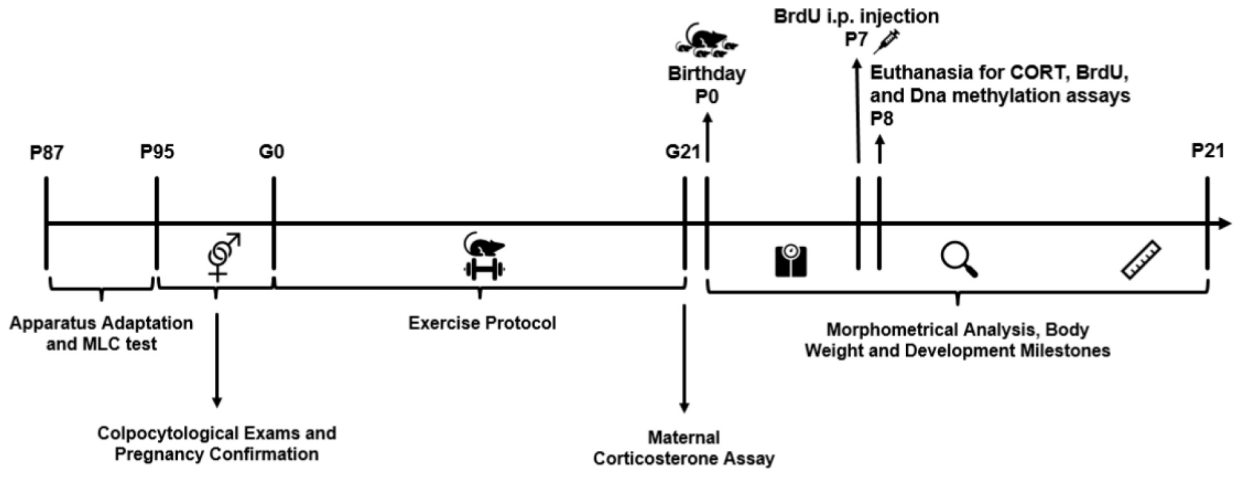

Figure 1. Timeline of key experimental procedures. MLC (maximum load capacity test), CORT (corticosterone), G (Gestational day), P (postnatal day).

was performed to define the load for the next four or five sessions. This exercise protocol was done three times a week, beginning at GO and finishing at G21, with 48 h or 72 h intervals between training days. In all periods of training, rats that declined to climb the ladder voluntarily were encouraged by gently tapping on their backs. If they persisted in refusing to climb the ladder, they were discarded from the study. In this study, one rat refused to perform the exercise protocol.

A total of 8-9 days of training were performed by exercised mothers. A summary of information about the training variables per group is shown in Table 1. The present exercise protocol was adapted[17], according to the needs imposed by our study.

A timeline with the key events of the experiment and exercise protocol is shown in Figure 1.

Stress Evaluation

After the period of exercise protocol, a set of mothers of both groups and theirs litters with 8 days old, were euthanized. Trunk blood was collected between 9:00 a.m. and 11:00 a.m. in heparinized tubes, centrifuged at 10.000 rpm for 15 min. to attain the plasma and then stored at -80°C until the assay. Total CORT concentration was measured with a commercial ELISA kit (Enzo ADI-900-097 Enzo Life Sciences), according to the manufacturer's specifications.

Physical Evaluation and Neonatal Development

Body weight and length, and cephalic measurements were evaluated at P2, P7, P14 and P21, using an analytic balance and a digital pachymeter. Additionally, at P1 to P8, the following development milestones were examined: surface righting, cliff aversion, negative geotaxis, and proprioceptive posterior limb placement. The day of milestone emergence was when the pup performed the task in less than thirty seconds[18]. All neonatal physical evaluations were performed in the light cycle of animals.

Immunohistochemistry for BrdU

At P7, a set of the pups received one BrdU injection (5-Bromo-2'-deoxyuridine; Sigma, 100 mg/kg, intraperitoneally, dissolved in 0.1 M NH_4OH, 20 mg/mL). At P8, male offspring were euthanized by decapitation and the entire brain was dissected and transferred to cryoprotected by immersion in 15% and 30% sucrose solution (Synth, Brazil) at 4°C. Then, brains were post-fixed in the 4% paraformaldehyde solution for 24 h. After, they were frozen in isopentane, cooled in liquid nitrogen and stored at -80°C until processing.

Serial coronal sections from the dorsal hippocampus of 20 µm thickness were obtained utilizing a cryostat (CM1850, Leica, Germany) at -20°C. Five slices of coronal sections per animal were obtained, with a range of 30 µm between slices. The sections corresponded to approximately 2.8 to 4.3 mm posterior to the bregma[19]. Slices were washed in xylol for 5 min. (2 times) and in decreasing concentrations of ethanol. Antigenic retrieval was obtained at 92°C with citrate buffer for 20 min. Then, tissues were washed with distilled water and the endogen peroxidase was inactivated (H_2O_2 5% dissolved in methanol). Next, sections were washed in Triton-X dissolved in PBS (PBS-tx), incubated with anti-5'-2'-deoxyuridine monoclonal antibody 1:500 (General Health) for 2 hours at ambient room temperature and overnight at 4°C. Slices were then washed again in PBS-tx and incubated with the secondary antibody IgG Peroxidase anti-mouse rabbit (1:100; Sigma-Aldrich) for 1 hour at room temperature. The immunohistochemical reaction was revealed by

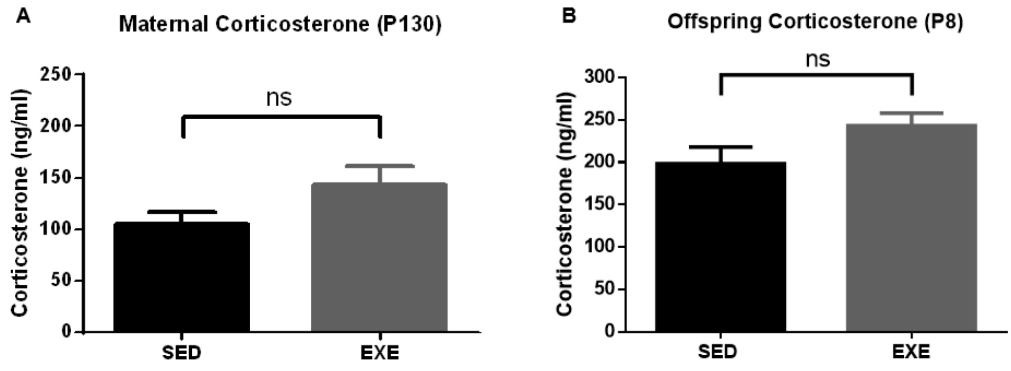

Figure 2. Plasma concentration of corticosterone in mothers exercised or sedentary (A) and in their offspring (B). SED (sedentary); EXE (exercised); ns (non-significant). n=4 - 5. Values are expressed in mean ± SEM.

0.06% 3,3-diaminobenzidine (DAB) in PBS. After being rinsed in distilled water, sections were counterstained with hematoxylin, dehydrated in ethanol and mounted on slides using Entellan®. The negative control was performed by switching the primary antibody with PBS[20].

Quantification of BrdU labeled cells

All immunoreactive cells in the medial end of the dorsal section of the dentate gyrus of the hippocampus (DG) were captured with an Olympus BX40 microscope and were carefully counted. Cell counts were restricted to the granular cell layer (GCL) subgranular zone (SGZ) and the hills of the DG. BrdU-immunoreactive cells in the DG were counted with respect to the different layers using the Image-Pro Plus 7.0 software.

Epigenetic assay

DNA isolation

Another set of animals was destined for epigenetic assay at P8. Pups of both groups were euthanized by decapitation and the hippocampi were quickly dissected. On the day of assay, the male offspring's hippocampus DNA was isolated using a commercial Tissue Section DNA isolation Kit (TRIzol™ Reagent catalog #15596026, Thermo Fisher Scientific) according to the manufacturer's instructions. Hippocampus DNA yield was quantified by absorbance using NanoDrop™ 2000 Spectrophotometer.

Global DNA methylation

The pup's hippocampal global DNA methylation was assessed utilizing a commercial kit MethylFlash™ Global DNA Methylation (5-mC) ELISA Easy Kit (Epigentek™) (Base catalog # P-1030). Briefly, 100 ng of DNA per well was added to the plates and incubated with 28 µL of DNA ligation buffer for 60 min. at 37°C.

Thereafter, 150 µL of blocking buffer was added and incubated for 50 min. at room temperature followed by washed five times with washing buffer. Developer solution (100 µL/well) was then added and incubated with 1–5 min. in a dark place. Absorbance at 450 nm was measured using an automatic microplate reader (Anthos Zenyth® – 200rt).

Statistical Analysis

Initially, data were analyzed by Shapiro-Wilk normality test, and homogeneity of variance was calculated with the Levene's test. Differences between sedentary and exercised groups were determined using the Student's unpaired t-test and Mann-Whitney test. Statistical analysis was performed using IBM SPSS Statistics 24.00, and statistical significance was considered when $p<0.05$. Data are expressed as mean ± standard error of the mean (SEM).

Results

Corticosterone concentration levels

Plasma concentration of CORT was observed in the mothers and their litters. No significant difference in plasma concentration of CORT was observed between exercised and sedentary mothers ($t(8)= -1.788$, $p=0.112$), nor between the different groups of pups ($t(6)= -1.822$, $p=0.118$) (Figures. 2A and 2B).

Physical evaluation – weight body and length, and motor milestones

Offspring body weights of each group at P2, P7, P14, and P21 are summarized in Table 2. Student's unpaired t-test indicated that the male offspring from exercised mothers had lower body weight at P7 ($t(18,2)=2.58$, $p=0.015$) and P14 ($t(21,7)=3.52$, $p=0.002$). No differences were observed for the other days (Table 2).

At P2 ($t(20)= -2.73$, $p=0.013$) and P21 ($t(20)= -2.92$, $p=0.008$), male offspring from mothers that exercised had an anteroposterior skull size that was larger than that of the descendants from sedentary mothers. Lengths of skull, body,

Table 2. Body weight, pachymetry and motor milestones in male offspring of sedentary and exercise mothers.

		SED		EXE	
	N	Mean ± SEM	n	Mean ± SEM	p
WEIGHT					
P2	16	9.35 ± 0.11	16	9.15 ± 0.11	0.207
P7	16	18.94 ± 0.12	16	17.93 ± 0.36	0.019*
P14	16	36.35 ± 0.21	16	34.64 ± 0.43	0.002*
P21	16	55.84 ± 0.57	16	54.68 ± 0.71	0.217
PACHYMETRY (P2)	12		10		
Anteroposterior axis of skull		17.16 ± 0.21 mm		17.97 ± 0.19 mm	0.013*
Latero-lateral axis of skull		13.48 ± 0.15 mm		13.01 ± 0.16 mm	0.050*
Longitudinal axis		56.42 ± 0.56 mm		56.10 ± 0.07 mm	0.726
Length of tail		17.89 ± 0.23 mm		18.72 ± 0.46 mm	0.136
P21	12		10		
Anteroposterior axis of skull		34.23 ± 0.60 mm		36.59 ± 0.51 mm	0.008*
Latero-lateral axis of skull		24.16 ± 0.52 mm		25.22 ± 0.41 mm	0.143
Longitudinal axis		119.29 ± 0.79 mm		120.46 ± 0.75 mm	0.304
Length of tail		76.19 ± 0.36 mm		75.98 ± 0.58 mm	0.931
MOTOR MILESTONES	15		7		
Surface Righting		1.06 ± 0.06 d		1.00 ± 0.00 d	0.495[a]
Posterior Limb Placement		5.80 ± 0.31 d		6.28 ± 0.42 d	0.164
Negative Geotaxis		5.80 ± 0.41 d		4.7 ± 0.64 d	0.379
Cliff Aversion		1.80 ± 0.22 d		1.28 ± 0.28 d	0.099[a]

Values are means ± SEM. Abbreviations: SED, sedentary; EXE, exercise; P, postnatal day; d, day of appearance. Unpaired Student T-test and [a] Mann-Whitney test, and utilized (p<0.05).*

and tail at days P7 and P14 were not different and are not presented in Table 2.

No differences were observed between the groups in motor milestone evaluations (Table 2).

Hippocampal cells proliferation

Male offspring from exercised mothers presented a higher number of cells in the hilus of the dentate gyrus of the hippocampus compared with the offspring from sedentary mothers (t (15)= -2.25, p=0.040) (Figure 3A). Representative images of sedentary and exercise groups are exposed in Figures 3C and 3D, respectively.

Hippocampal global DNA methylation

Male offspring from exercised mothers presented a lower global DNA methylation in the hippocampus when compared with the offspring from sedentary mothers (t (6)=2.77, p=0.032) (Figure 3B).

Discussion

This study showed that a strength exercise protocol during pregnancy did not influence the CORT levels in the mothers or the descendants, or cause modifications in the male offspring's physical development. However, the development of the hippocampus was influenced by the mothers' training. An increase in the number of cells in the hilus of the DG within the hippocampus and a decrease in global hippocampal DNA methylation were observed.

High-intensity exercise protocols and the use of involuntary apparatuses in the gestational period may abruptly raise CORT levels, causing deleterious effects to offspring. Wasinski et al.[21] exploring the effects of forced swimming during pregnancy, for example, observed an increase in maternal CORT and a decrease in neuroplasticity markers in the progeny at neonatal life. Similar results were also observed by Jang et al.[22]. Investigating a protocol of involuntary swimming exercise during pregnancy, the authors reported that exercised mothers presented a severe reduction in the number of embryos implanted and amniotic fluid with a high concentration of cortisol and a low concentration of growth factors (NGF and VEGF). Due to possible deleterious effects caused by high-intensity protocols during pregnancy, here we adopted a low to moderate intensity exercise protocol. In fact, this training protocol did not increase the CORT concentration levels in mothers or offspring. A similar result to that of our study was observed by Rosa

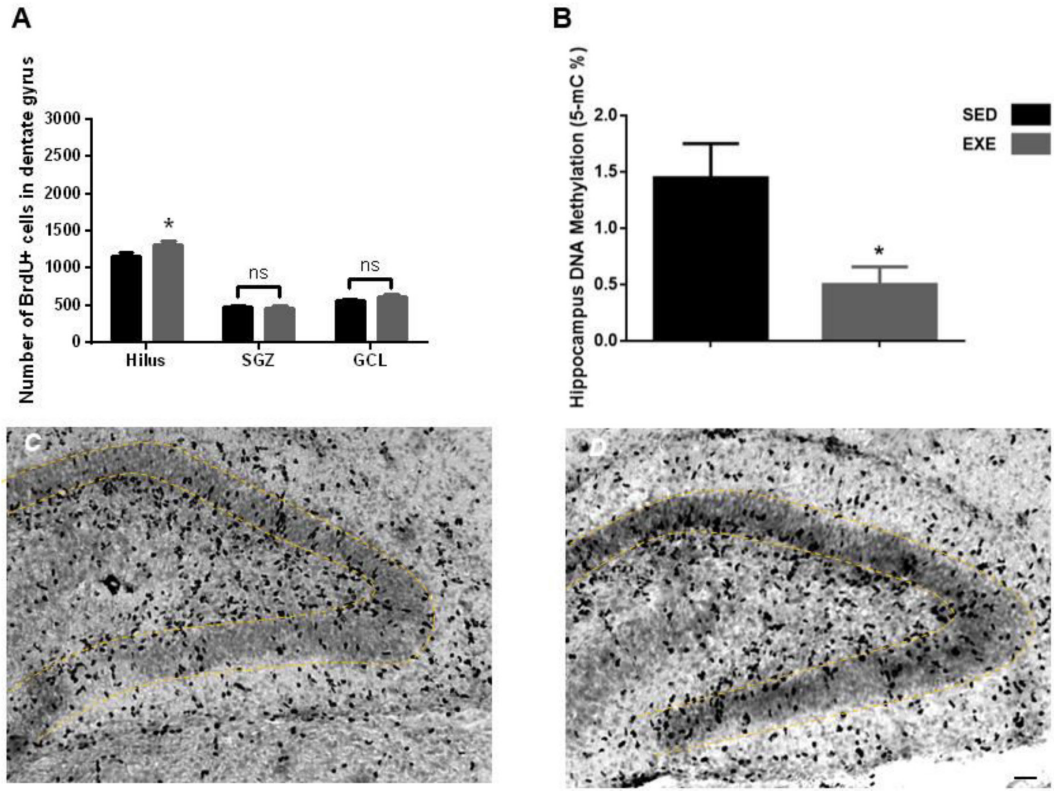

Figure 3. (A) Number of BrdU positive cells in hippocampus dentate gyrus of male offspring (n=8 - 9); (p=0.040). (B) Global hippocampal DNA methylation of the male offspring (n=4); (p=0.032). (C-D) Digitalized image of the DG hematoxylin counterstaining of SED and EXE groups (captured at 40x). SED, sedentary group; EXE, exercised group; DG, dentate gyrus; SGZ, Subgranular zone; GCL, Granule cell layer; ns (non-significant). Unpaired Student's t-test. Values are expressed in mean ± SEM and percentage of mean, respectively. Scale bar = 80μm.

et al.[23], using two different strength exercise protocols, tower climbing and squat exercise, during the gestational period. The authors reported that exercised mothers had a high concentration of fecal CORT, but this increment was not significant. Another similarity between our data and those of Rosa et al.[23] is the model of exercise adopted–voluntary exercise. Protocols of involuntary exercises, such as forced swimming, are related to high levels of CORT concentration, while voluntary exercise, such as vertical ladder climbing and wheel running, is considered less stressful for the animals[21,24].

High maternal levels of stress, restriction diet, and high-intensity exercise could cause a delay in the acquisition of neurodevelopmental milestones and important negative changes in body morphometric measures in the offspring[21,25–27]. Here, descendants' physical evaluation revealed that the strength exercise protocol did not alter the physical development of male offspring but just transiently changed some morphometric parameters studied. This is in accordance with clinical and basic research, which shows that physical exercise during pregnancy could cause changes in the weight of some organs, fat, and various structures, but these changes are transitory and do not cause any functional change or have clinical relevance[2,28]. Thus, strength exercise does not seem to significantly alter the body's morphometric development, and this outcome could be considered a positive result, because it indicates that this modality of exercise practiced by mothers is safe for the development of male offspring's body.

Two potential parameters involved in the influences of maternal strength exercise on the cognitive processes of the descendants were studied: cell proliferation and global DNA methylation in the hippocampus. Both were found to be modified in the offspring from mothers submitted to strength training. Several studies conclusively demonstrated that physical exercise can affect the expression of genes related to cognitive processes, memory behavior tests, and synaptic plasticity in the brain by epigenetic mechanisms[6]. One of these epigenetic mechanisms is the methylation of DNA, which is associated with the silencing of genes, by an increase in chromatin compaction; it is also related to the enhancement in the expression of plasticity-promoting genes, such as the *Bdnf* and *reelin* genes. Furthermore, the actions of DNMT1 and DNMT3A are directly involved in

neurogenesis[29,30]. Although it is not possible to make a direct comparison of our results with those of others, in one of the few studies reporting modifications in epigenetic marks in the offspring from exercised mothers, Xu et al.[31] observed a lower level of global DNA methylation in the embryos of exercised mothers. With methods more similar to ours, Segabinazi et al.[32] showed that the offspring from exercised mothers during the pregestational period presents a decrease in global hippocampal DNA methylation associated with a high expression of hippocampal neuroplasticity factors in adult life.

The hippocampal hilus is considered an important neurogenic niche, and it is the area from which granule cells are derived in late development[33]. The enhancement in hippocampal cells in the male offspring from exercised mothers, besides demonstrating a possible positive influence of strength training. Exploring the effects of aerobic training during pregnancy, Gomes da Silva et al.[7] also observed high hippocampal cell proliferation, associated with an overexpression of the hippocampal BDNF level. This is the most described biological mechanism for the increase in cellular proliferation caused by aerobic exercise in this and other studies. Nevertheless, other neuroplasticity markers could also explain the enhancement in hippocampal cell proliferation in the descendants. Studies using strength exercise protocols in adult rats reported that hippocampal neurogenesis is associated with an enhancement in IGF-1 and synapsin levels in the hippocampus, together with better performance in memory and learning tests[15,16]. Thus, as observed in maternal aerobic exercise, strength training during pregnancy also seems to have a positive influence on the offspring's hippocampal neuroplasticity factors.

This study had as objective to investigate the influence of maternal strength training on male offspring's development and brain plasticity in order to advance knowledge in this area. Some issues that were not explored here, and were limitations of this study, should be investigated in future researches: [1] the expression of other neuroplasticity markers and epigenetic mechanisms, [2] the effects of maternal strength training in different periods of offspring's life, and [3] the influence of maternal strength training in female offspring.

The present data agree with current clinical research evidence on the safety of this modality of exercise for mothers and offspring. Furthermore, as with maternal aerobic exercise, strength training during pregnancy had a positive impact on markers linked to descendants' cognitive function. These initial results open a range of possibilities for further investigations about the influence of this physical exercise modality on the offspring's cognitive function and other aspects related to their development.

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