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Impact of maternal stress on metabolism and penile morphology in young offspring rats

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Summary. Exposure to prolonged stress in pregnancy and/or lactation can lead to the future development of diseases. We aimed to study the effects of maternal stress on the biometry, metabolism, and penile morphology of young Wistar rats. Animals were divided into two experimental groups: Control Group (C) - pups from control mothers, without any intervention (n=5); and Chronic Stress Group (S) - pups from mothers who suffered variable stress in the third week of pregnancy (14th to 21st day; n=5). Food intake and body mass of the pups (n=10, in the C group and n=9 in the S group) were checked; at euthanasia (three months old), fat deposits and penis were removed. At birth and weaning, S animals were lighter than C animals, [-33.72% (*p*=0.0422) and -17.07% (*p*=0.0018)], respectively. However, the final body mass and body mass delta showed no differences. Food intake and fat deposits also did not differ. However, the S group was hyperglycemic at 30 and 60 days of life [+20.59% (*p*=0.0042) and +14.56% (*p*=0.0079), respectively], despite the glycemia measured at 90 days showing no difference between groups. Penile areas and surface densities of the corpora cavernosa components were similar between groups. The results indicate that maternal stress is an important metabolic programmer, which generates low birth weight and accelerated recovery of body mass after birth (catch-up). However, in an early analysis (90 days of life), exposure to gestational stress did not change the morphology of the offspring's penis in adulthood.

Key words: Fetal programming, Prenatal stress, Stress, Penis

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Introduction

Stress has become one of the most common conditions of modern life, affecting about 90% of the world's population (Tomiyama, 2019). By definition, it is considered an adaptive response of the organism to a certain stressor stimulus (Galluzzi et al., 2018). Some studies showed that prolonged exposure to stressful stimuli during adulthood can cause hyperglycemia and insulin resistance, attention deficit, redistribution of body fat, decrease in immunity, nausea, and erectile dysfunction (Carroll et al., 2017; Roy et al., 2021).

When pregnant women are stressed, there is a higher susceptibility to infections, pre-eclampsia, hyperglycemia, spontaneous abortion, and premature birth; all of these conditions compromise the mother-child binomial (Gotlieb et al., 2022). The fetal programming hypothesis, first described by David Barker in 1986 (Barker and Osmond, 1986), affirms that stimuli generated during critical periods of development (gestation and/or lactation) cause morphological changes in organs such as heart (Barker and Osmond, 1986), liver (Keshavjee, 2022), kidneys (Barker, 1998), pancreas (Salimi et al., 2022), and testes (Pallarés et al., 2013), which can become permanent throughout the animal's life. Among these insults, maternal stress is thought to be an important form of fetal programming (Bolten et al., 2013).

The consequences of exposure to stress in the prenatal period vary according to the gestational period, sex, type of stress, and duration (Hamada and Matthews, 2019). Human studies show that stress experienced in early pregnancy can contribute to the development of preeclampsia in the third trimester of pregnancy (Hamada and Matthews, 2019). In contrast, studies in murine models show that stress in the second and third weeks of pregnancy promotes a reduction in litters

(Gotlieb et al., 2022), low birth weight, and a decrease in the anogenital distance in male offspring (Pallarés et al., 2013).

Although the literature contains relevant data on the impact of gestational stress on the testes (Pallarés et al., 2013; Garcia-Vargas et al., 2019), its effects on the penis are still unknown. In humans, the penis begins to develop from the genital tubercle, between the 8th and 9th week of gestation, and this process ends between the 12th and 13th gestational week (Baskin et al., 2018). The penis of rats also develops from the same embryonic structure, through the action of testosterone; however, its development begins approximately on the 16th day of gestation and completes development around the 10th postnatal day (Hyuga et al., 2019). So, maternal stress during the last week of a rat's pregnancy is hypothesized to lead to penile morphological alteration in the offspring. The objective of this study is to investigate the effects of maternal stress on the biometry, metabolism, and penile morphology of young Wistar rats.

Materials and methods

Animals and experimental model

This study followed the International Guiding Principles for Biomedical Research Involving Animals, published by the Council for International Organizations of Medical Sciences (CIOMS) (National Research Council, 2004), as well as with the Brazilian law on the scientific use of animals (Marques et al., 2009) and was approved by the ethics committee for animal experimentation of the Federal Fluminense University (UFF) under number 9518170621.

Ten virgin female Wistar rats and five males of the same lineage were obtained from the Central Animal Facility of UFF, (Niteroi, Brazil) and were housed under controlled conditions (21°C, 30% air humidity, 12/12h light-dark cycle), with water and food *ad libitum*. At 90 days of life, the animals were allowed to mate for 24 hours. After mating was confirmed (vaginal smear), the females were randomly placed into individual cages and divided into two groups: control (C) and stressed (S) (n=5 for each group).

The females in the stressed group were submitted to a variable stress protocol during the third week of pregnancy (14 to 21 days), which is considered a critical period in the development of the male reproductive system (Hashimoto et al., 2019; Hyuga et al., 2019). The protocol consisted of intercalating four different stressors: isolation in a metabolic cage for 60 minutes; wet bed for 24 hours; cage tilt (5 cm) for 24 hours; and food and water deprivations for six hours (Marin et al., 2007).

At birth, male offspring were weighed and litter size was adjusted to eight pups/mother (four males and four females) to standardized nutrition until weaning (Garcia-Vargas et al, 2019). After this (21 days), male pups (two per mother) were randomly divided into two groups:

from control dams (C; n=10) and stressed dams (S; n=9), followed up to 90 days.

Body mass and food intake were recorded weekly and daily throughout the experiment. Regarding body mass, the body mass delta was calculated by subtracting the animals' final (90 days) and weaning (21 days) body mass. Food intake was estimated by subtracting the amount of food left on the grid and the amount of spilled food from the initial weight of food supplied. At 30 and 60 days, the caudal vein (five animals from each group) was punctured to measure capillary blood glucose, using an appropriate glucometer (Accu-check performa®, Roche, São Paulo, Brazil). Before euthanasia (which was induced by anesthetic overdose), the animals underwent eight-hour fasting for blood glucose determination, performed again by caudal puncture. Fat deposits (retroperitoneal, epididymal, subcutaneous) and the penis were dissected and collected. The middle third of the penis was washed in saline solution (0.9% NaCl) and immersed in buffered formalin (4.0%) during 72h. Subsequently, the material was subjected to routine histological techniques and, after embedding in paraffin, histological sections were performed of five µm thickness and spacing of 50 µm between each section.

Penile morphometry

The areas of the penis (total area, area of the corpora cavernosa with and without the tunica albuginea, and the thickness of the tunica albuginea) were calculated using photomicrographs of cross-sections of the penis stained with Masson's trichrome. The images (five sections/animal) were captured by an Axiocam 506 color digital camera (Carl Zeiss, Gottingen, Germany) coupled to a Stereo DiscoveryV8 stereomicroscope (Carl Zeiss), at 8x magnification. All areas were measured using the "freehand" tool of Software ImageJ version 1.53k (NIH, Bethesda, United States). The thickness of the tunica albuginea was calculated from the difference between the areas of the corpora cavernosa (Felix-Patrício et al., 2015).

The surface densities (Sv) of the connective tissue, smooth muscle, and sinusoidal space (400x magnification) were evaluated in the same histological sections, stained with Masson's Trichrome, while the Sv of elastic system fibers were determined in sections stained with Weigert's resorcin fuchsin (600x magnification), both using manual staining techniques. All parameters (25 fields/animal) were quantified by the point counting method, using ImageJ software (version 1.53k, National Institute of Health, Bethesda, Maryland, USA) (Felix-Patrício et al., 2015).

Statistics

Data (mean ± standard deviation) were evaluated for normality using the Shapiro-Wilk normality test. Those not considered normally distributed were analyzed using the Kruskal-Wallis test with Dunn's post-test; normally

distributed data were analyzed by the student's t-test (unpaired). Differences were considered significant when $p<0.05$. All analyses were performed with GraphPad Prism (version 9.0, GraphPad Software, San Diego, USA).

Results

At birth, the litter body mass of the S group was lower compared with the C group (-33%). This difference was perpetuated in the offspring until weaning when the S group was -17% lighter compared to the C group. At 90 days, the final body mass and body mass delta (mass gain from weaning to euthanasia) were not different (Table 1).

Food intake ($p=0.7738$) and subcutaneous ($p=0.1021$), retroperitoneal ($p=0.6011$), and epididymal ($p=0.4658$) fat deposits in both groups were statistically similar. However, the glucose values measured at 30 [+20.59% (p=0.0042)] and 60 [+14.56% ($p=0.0079$)] days of life indicated higher glycemia in animals from the S group. This pattern was not observed between the respective groups at 90 days of age, as shown in Table 1.

The penile areas, the thickness of the tunica albuginea, and the Sv of the components of the corpora cavernosa and the elastic system fibers did not differ between groups (Fig. 1), although the results showed a tendency towards a reduction in the Sv of smooth muscle and of the elastic system fibers in the S group (Table 1).

Discussion

Pregnancy is a period that encompasses many changes in women's lives, which are physical, neuropsychic, hormonal, physiological, and metabolic (Morton, 2021). A large body of evidence indicates that gestational stress can directly impact the physical health of both those involved: mother and fetus (Hamada and Matthews, 2019; Gotlieb et al., 2022). Fetal programming theory identifies pregnancy as a critical period in the development of offspring, which is extremely dependent on and related to maternal health (Barker, 1998, 2007). The presence of stressful events during pregnancy increases the risk of low birth weight, intrauterine growth retardation, and prematurity (Wadhwa et al., 1993; Bussières et al., 2015; Medsker et al., 2015; Lima et al., 2018).

Here, we used different stressor stimuli in the last week of pregnancy, and as pointed out by studies, our group observed a reduction in the body mass of the animals in the S group, which extended from birth to weaning. Fetal growth is mediated by the placenta, which is the organ directly involved in the transport of nutrients and production of growth factors, including insulin-like growth factors (IGF1 and IGF2). During maternal stress, the increase in glucocorticoids (corticosterone) disrupts these actions (decreases placental flow) and makes the conceptus more susceptible to growth restriction (Cuffe et al., 2012, 2014; Briffa et al., 2017).

Intrauterine growth retardation has been linked to the development of future diseases, such as obesity and dysregulation of carbohydrate metabolism (Barker, 1998, 2007). Although we did not observe overweight in the stressed offspring at the end of the experiment, there was a weight compensation in these animals at 90 days; with no increase in food intake and fat deposits, suggesting the

Table 1. Metabolic and penile data of Wistar rats at birth, weaning, and adulthood (90 days).

	C	S	*p*-value
Litter birthweight (g)	45.11±9.48	29.90±13.15	0.0422*
Weaning mass (g)	49.22±3.06	40.82±7.42	0.0018*
Final body mass (g)	356.40±25.27	345.00±47.53	0.4835
Body mass delta (g)	307.20±25.40	304.10±44.92	0.7021
Food intake (g/animal/day)	020.77±6.03	019.98±06.03	0.7738
Blood glucose at 30 days (mg/dL)	118.00±3.94	142.30±13.16	0.0042*
Blood glucose at 60 days (mg/dL)	103.00±7.38	117.80±1.10	0.0079*
Blood glucose at 90 days (mg/dL)	93.67±13.23	103.30±9.87	0.1820
Retroperitoneal fat deposit (g)	3.76±1.28	3.41±1.74	0.6011
Epididymal fat deposit (g)	4.63±1.06	4.14±1.91	0.4658
Subcutaneous fat deposit (g)	6.07±2.96	4.01±2.32	0.1021
Cross-sectional penile area (μm^2)	7.56±2.29	7.32±2.50	0.9723
Area of the corpus cavernosum including tunica albuginea (μm^2)	5.66±0.67	5.93±0.70	0.3878
Area of the corpus cavernosum without tunica albuginea (μm^2)	2.56±0.39	2.656±0.34	0.5885
Area of the tunica albuginea (μm^2)	3.10±0.41	3.27±0.48	0.3754
Connective tissue Sv (%)	68.44±7.61	67.78±5.83	0.8312
Smooth muscle fiber Sv (%)	12.55±5.35	11.62±3.78	0.6617
Sinusoidal space Sv (%)	18.01±3.84	19.60±3.83	0.3589
Elastic system fiber Sv (%)	10.20±2.20	8.86±2.65	0.2850

Data presented as mean ± standard deviation (student's t-test). For each parameter, * indicates *p*-values with statistically significant differences. C, pups from control dams; S, pups from stressed dams; Sv, surface density.
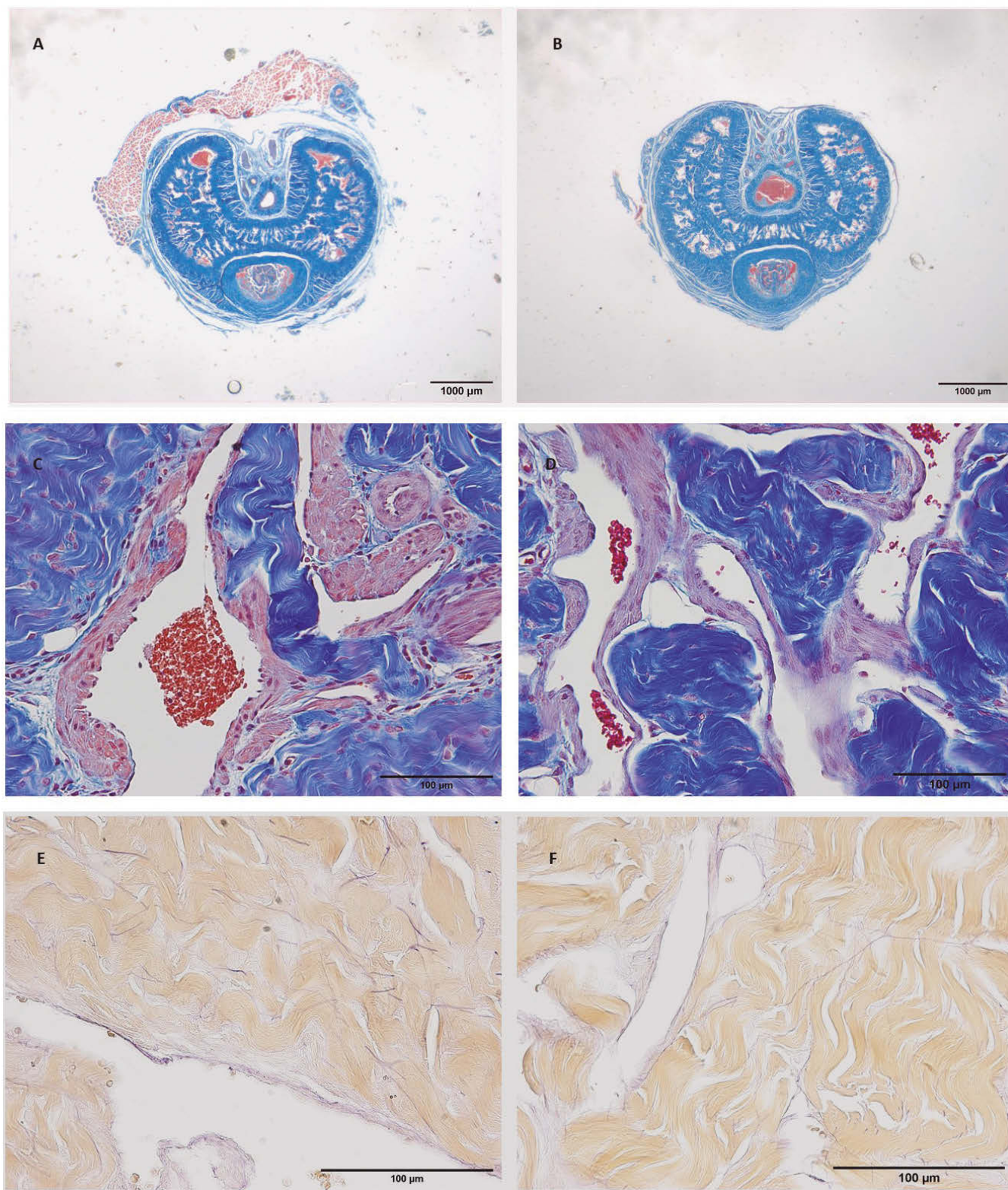
Fig. 1. Illustrations of the parameters analyzed in the penis of offspring from the C **(A, C, E)** and S groups **(B, D, F)**. **A, B.** Represent Masson's trichrome stained sections, where we did not observe differences in the total areas of the penis and corpus cavernosum (with and without tunica albuginea). **C, D.** Also show Masson's trichrome stained photomicrographs, with the same surface density of smooth muscle fibers, connective tissue, and sinusoidal space in both groups. **E, F.** Illustrate Weigert's resorcin-fuchsin sections stained, in which the elastic system fibers show a tendency to reduce in the S group. A, B, x 16; C, D, x 400; E, F, x 600.

occurrence of catch-up. Dulloo (2008) pointed out that catch-up growth early in life is the biggest risk factor for the development of obesity, type-2 diabetes, and cardiovascular diseases. Environmental exposures that impede growth early in life result in the programming of "thrifty mechanisms", which are adaptive during limited nutrient supply (or growth restriction), but which increase the risk of disease in the presence of better nutrition or recovery of growth in later stages of life (Dulloo, 2008). According to the literature, this event is not healthy, as the increase in cell division rates accelerates cell aging and contributes to the early development of metabolic disorders (Berends et al., 2018; Pericuesta et al., 2021; Lizarraga-Mollinedo et al., 2022).

Our results found hyperglycemia in the S group at 30 and 60 days of life, which was not seen at 90 days of life. Although we did not measure corticosterone and insulin levels in the offspring, it is suspected that the role of glucocorticoids in glucose metabolism is the main mechanism related to this metabolic alteration. Experimental studies of gestational stress found that the offspring of stressed mothers have higher levels of corticosterone in their circulation, this hormone being responsible for promoting greater glucose release by tissues and greater insulin resistance (Seckl and Holmes, 2007).

In addition, catch-up growth can also lead to dysfunction in the pancreatic beta cell mass, which directly interferes with carbohydrate metabolism (Chen et al., 2011). Even though at 90 days the glucose of animals in the S group had not changed, the metabolic alterations found in this work, and in others already published, lead us to believe that, in the long term, these animals may develop hyperglycemia and insulin resistance disorders (Berends et al., 2018; Pericuesta et al., 2021).

The penis is an organ responsive to changes in glycemic status and stress, which directly impact sexual function. Hyperglycemia can damage the vascular endothelium, which compromises penile erection and decreases penile sensitivity (Abidu-Figueiredo et al., 2011). Stress, on the other hand, can lead to changes in penile morphology in adult animals, reducing the number of smooth muscle fibers and increasing the amount of connective tissue, which compromises erection mechanisms (de Souza et al., 2012). Here, gestational stress did not impact the morphology of the male external genitalia at 90 days of age, since we did not obtain differences in the various morphometric measurements that were performed. One study showed that when stress is induced in adulthood, the animal's penis becomes less thick (with a smaller area), less muscular, and less elastic, making it more susceptible to erectile dysfunction (Ribeiro et al., 2019). Thus, it is possible that penile modifications in these animals (whose mothers were subjected to stress during pregnancy) only appear later than 90 days of age.

In rodent models, the development of the penis is dependent on androgen hormones, since it is from the production of testosterone that the genital tubercle differentiates into the penis. However, unlike humans, whose organs develop throughout uterine life, in rodents, penile formation begins approximately on the 16th day of gestation and completes development around the 10th postnatal day (Hyuga et al., 2019). Thus, it is possible to presume that the period of organogenesis is decisive in maternal stress responsiveness. Our experimental protocol ruled out any stressor stimulus during lactation, which may explain the morphological results found, which only illustrate the effects of gestational stress on the penis of adult animals. Even along these lines, the analyses showed a tendency towards a reduction in smooth muscle and fibers of the elastic system in the S group. Perhaps, if we had studied these animals at a more advanced age, we would have found adverse remodeling similar to that caused by the exclusive action of stress.

This work has some limitations that should be highlighted. In our study, we did not study maternal hormones and we used an animal model within controlled experimental conditions; therefore, the results of this study may not represent what occurs in the scenario of uncontrolled stress stimuli during pregnancy. Furthermore, we have to consider that the small sample size and the artifactual tissue shrinkage effects of formalin fixation and tissue processing can impact the measurements. Hence, the importance of further experimental work to support our results.

In conclusion, this study points to the importance of considering gestational stress in the etiology of fetal programming. Although variable stress in the last week of pregnancy did not cause changes in the penile structure of animals at three months of age, it is an important factor in future diseases. The low birth weight of the litter, the recovery of body mass (catch-up growth), and the presence of hyperglycemia during the growth phase indicate the negative and acute effects generated by stress. A longer lifetime (180 days) would be crucial to examine both the metabolism and the morphology of the penis.

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