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Small-for-pregnancy-age rats submitted to exercise: DNA damage in mothers and newborns, measured by the comet assay

A.O. Netto[a], R.B. Gelaleti[a], S.B. Corvino[a], R.G. Serrano[a], S.C. Hernández[b], G.T. Volpato[a,c], M.V.C. Rudge[a], M.G. Braz[d], D.C. Damasceno[a,*]

[a] Postgraduate Course on Gynecology, Obstetrics and Mastology, Laboratory of Experimental Research on Gynecology and Obstetrics, Botucatu Medical School, São Paulo State University - UNESP, Botucatu, São Paulo State, Brazil
[b] Biochemical Department, Havana Medical Science University, ICBP Victoria de Girón, Havana, Cuba
[c] Laboratory of System Physiology and Reproductive Toxicology, Institute of Biological and Health Sciences, Federal University of Mato Grosso (UFMT), Barra do Garças, Mato Grosso State, Brazil
[d] Department of Anesthesiology, Botucatu Medical School, São Paulo State University - UNESP, Botucatu, São Paulo State, Brazil

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ABSTRACT

Background: Fetal impairment caused by a deleterious intrauterine environment may have long-term consequences, such as oxidative stress and genetic damage. Rats born as small-for-gestational-age (SPA) were submitted to exercise (swimming) before and during pregnancy. The animals exhibited glucose intolerance, reduced general adiposity, and increased maternal and offspring organ weight, showing the benefit of exercise for these rats. We hypothesised that regular exercise in SPA during gestation could prevent DNA damage in these animals and in their offspring, contributing to altered fetal programming of metabolism in the offspring. Severe diabetes was induced by streptozotocin treatment, to obtain SPA newborns. At adulthood, pregnant SPA rats were randomly distributed into two groups: exercised (SPAex – submitted to swimming program) or not-exercised (SPA – sedentary rats). Post-partum, blood was collected for analysis of DNA damage (comet assay) and oxidative stress. SPAex rats presented lower DNA damage levels, decreased lipid peroxidation, and a lower rate of newborns classified as large-for-pregnancy-age. DNA damage was also lower in SPAex newborns. We conclude that swimming applied to SPA pregnant rats contributes to decreased DNA damage and lipid peroxidation in the dams, and decreased DNA damage and macrosomia in their offspring.

1. Introduction

The interaction of environmental and genetic factors defines health conditions through an individual's life. Environmental factors can modify phenotype as early as the intrauterine milieu. Maternal diabetes affects embryonic and fetal development, reducing the metabolic energy supply needed to protect essential organs, as described in the "Thrifty Phenotype" hypothesis [1,2] that the later (adult) development of type 2 diabetes or "metabolic syndrome" may be a result of inadequate intrauterine nutrition that causes permanent changes in glucose/insulin metabolism.

In uncontrolled maternal diabetes, fetal growth is impaired [1] (intrauterine growth restriction; IUGR). IUGR describes a fetal phenotype resulting from failure of the foetus to reach its growth potential, leading to low body weight (LBW). Small-for-gestational-age (SPA) refers to birth weight below a certain cut-off for a specific gestational age [3,4].

Fetal impairment caused by a sub-optimal intrauterine environment may lead to long-term consequences for the glucose tolerance of the adult offspring and may even extend to subsequent generations. These outcomes in later life have been demonstrated in humans and in several animal models [5]. Individuals with lowest birth weights were found to be 6-fold more likely to develop type 2 diabetes or impaired glucose tolerance when compared to those who were heavier at birth [6].

Embryonic development occurs in a relatively low-oxygen/ low-antioxidant-capacity environment. A delicate balance between optimal oxygen levels and the generation of reactive oxygen species (ROS) exists during the gestational period. ROS can interact directly with DNA, resulting in oxidative damage and DNA breaks [2]. IUGR foetuses present oxidative stress, as indicated by increased lipid peroxidation

* Corresponding author at: Lab. Pesquisa Experimental de Ginecologia e Obstetrícia, Faculdade de Medicina de Botucatu _UNESP, Distrito de Rubião Junior s/n, CEP. 18618970 Botucatu, Estado de São Paulo, Brazil.
E-mail address: debora.damasceno@unesp.br (D.C. Damasceno).

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and DNA damage levels, as well as increased antioxidant indices: superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) [7]. These foetuses may also show impaired tissue development: pancreatic beta cells are vulnerable to oxidative stress because of low antioxidant capacity and increased ROS production [8], which might lead to postnatal complications [2].

Experimental models can reproduce adverse foetal environments: deficient maternal nutrition, placental insufficiency, stress, and diabetes [9]. In our laboratory, we have used the streptozotocin (STZ)-induced severe diabetes model to obtain IUGR and/or SPA offspring [10,11]. In adulthood, IUGR rats presented glucose intolerance; however, when these rats were submitted to a swimming program before and during pregnancy, glucose tolerance and reduced general adiposity were observed, showing the benefit of physical activity [12]. We hypothesised that a swimming program in pregnant SPA rats could affect maternal metabolism, contributing to adequate fetal programming and preventing DNA damage. Therefore, the objective of the present study was to evaluate the effects of swimming on pregnant SPA rats, with respect to DNA damage and oxidative stress in the mothers and DNA damage in the offspring.

2. Materials and methods

2.1. Ethical experimentation

The animals were maintained in accordance with the principles of the Guide for Care and Use of Experimental Animals. All experimental procedures were approved by the Ethics Committee in Animal Experimentation of UNESP_Botucatu, São Paulo State, Brazil (Protocol number 952/2012). All efforts were employed to minimise animal suffering.

2.2. Animals

Twelve-week-old female and male Wistar rats were obtained from CEMIB. The animals were housed two or three per cage and kept under standard conditions (22 ± 3 °C, 12-h light/dark cycle), received standard rat chow diet (Purina rat chow, Purina®, São Paulo, SP, Brazil), and were given tap water to drink *ad libitum*.

2.3. Experimental diabetes induction for obtaining small-for-gestational-age rats

Diabetes was induced in female rats by intravenous (i.v.) administration of streptozotocin (STZ; Sigma Chemical Co., St. Louis, MO, USA), which was dissolved in citrate buffer (100 mM, pH 4.5), 40 mg/kg body weight (Fig. 1). Blood glucose concentrations were measured with a glucometer. Rats presenting blood glucose concentrations ≥300 mg/dL were included in the severely diabetic group [13].

After STZ-induction of diabetes, female rats were mated overnight with healthy males. The day when sperm was found in the vaginal smear was designated as gestational day 0. The mating period consisted of 15 consecutive days, comprising approximately three oestral cycles, until a replicate number of groups was obtained [14]. During the pregnancy and lactation periods, the rats were maintained in individual cages until weaning of their offspring. Subsequently, the dams were anaesthetised with sodium thiopental (Thiopentax®) and sacrificed by decapitation.

2.4. Selection of SPA offspring

The offspring were born by spontaneous delivery from severely diabetic rats. After birth, sex determination and body weight assessment of newborns were performed. Female offspring were separated and classified according to the mean values

of the fetal weights of the non-diabetic (ND) group [mean ± 1.0 × standard deviation (SD)]. The newborns were classified as SPA, appropriate-for-gestational-age (APA), or large-for-gestational-age (LPA) when their weights were less than (mean − 1.0 × SD), similar to (mean ± 1.0 × SD), or greater than (mean + 1.0 × SD), the mean value of the ND group, respectively [15]. Female offspring born from severely diabetic dams and classified as SPA were included in this study.

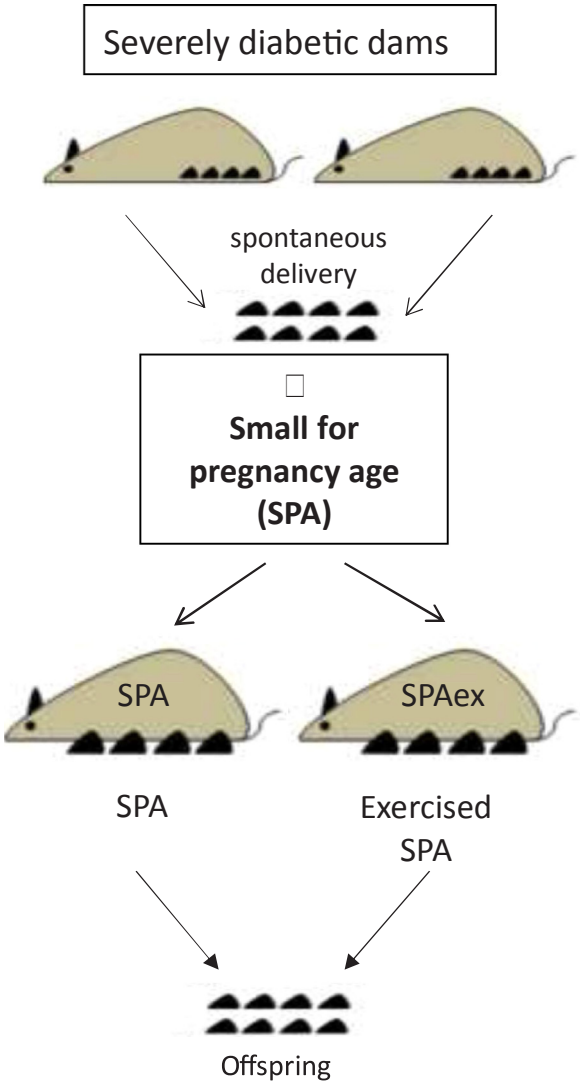

Fig. 1. Experimental design of small-for-gestational-age (SPA) rat mothers submitted to exercise during pregnancy (SPAex) or not exercised (SPA) and their offspring.

2.5. Experimental groups

In adulthood, the female SPA rats were mated with healthy male rats, using similar methods to those described above. During pregnancy, the SPA rats were maintained in individual cages and distributed into two experimental groups (n = 5 animals/group): 1) not-exercised SPA rats (SPA) – sedentary rats; and 2) exercised SPA rats (SPAex) - submitted to swimming during pregnancy.

2.6. Physical exercise: swimming program

The SPAex rats were submitted to swimming during pregnancy. The exercise program was administered six times per week, 9:00-10:00 a.m., in water with depth = 40 cm, 32 ± 2 °C. The initial duration was

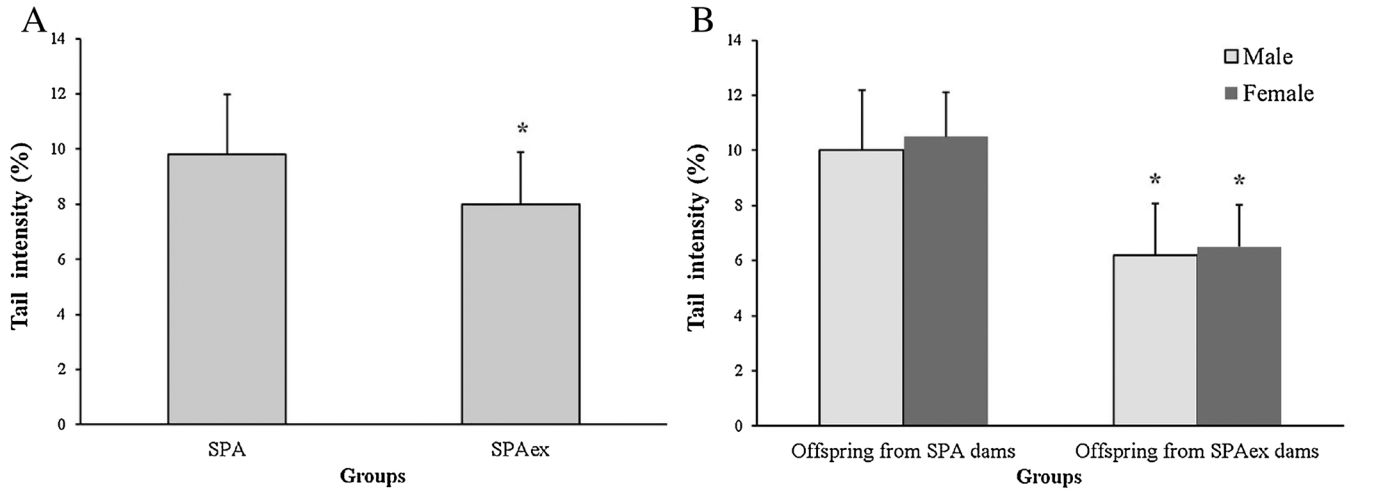

Fig. 2. A DNA damage in rats submitted to exercise (SPAex) or not exercised (SPA) during pregnancy. B DNA damage in offspring of mother rats submitted (SPAex) or not exercised (SPA) to exercise during pregnancy. Data are expressed as percentage mean (X) ± standard error of the mean (SEM).
*p < 0.05 compared to SPA group (gamma distribution).

20 min with a gradual increase of 10 min per day to a maximum of 60 min, and this time was maintained until pregnancy day 20 [11].

2.7. Post-partum/lactation period: maternal DNA damage and oxidative stress analyses

At approximately pregnancy day 21, the SPA and SPAex rats presented spontaneous delivery to produce the next generation. The newborns were classified for body weight according to the methods described above. After 10 d lactation, the dams were anaesthetised with sodium thiopental (Thiopentax®) and decapitated for the collection of maternal blood for DNA damage and oxidative stress measurements. Whole blood (0.5 mL) was collected into EDTA-vacutainer tubes and stored in Eppendorf tubes with RPMI 1640 medium, 0.4 mL, and dimethyl sulphoxide (DMSO, 0.1 mL). Samples were frozen gradually and stored in a freezer (−80 °C) until DNA damage analysis.

Additional blood samples were collected in heparinised tubes and centrifuged (185 × *g* for 10 min at 4 °C for oxidative stress evaluation. The supernatant was discarded and the erythrocytes were washed with phosphate-buffered saline (PBS) (0.01 M, pH 7.4) followed by centrifugation at 263 × *g* for 1 min at 4 °C. This procedure was repeated three times and the final erythrocyte sedimentation was used for the evaluation of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities, thiol groups (SH), and malonaldehyde (MDA) concentrations, according to de Souza's modified protocol [16] related to sample volume. Absorbances were measured with a spectrophotometer.

2.8. Blood samples for glycaemia and DNA damage analysis

At postnatal d 10, a small cut on the tail tip of one male newborn and one female newborn of each dam was performed to collect blood samples (0.5 mL) for glycaemic determination (glucometer). Blood samples were processed similarly to the methods used for the maternal samples [17] for DNA damage analysis by the comet assay [18]. Then, for measuring newborn DNA damage, one male sample and one female sample were collected from each dam, totaling 10 animals/group.

2.9. Comet assay

All the comet assay steps were carried out under minimal illumination, according to a modified protocol [17], and the slides were coded. Frozen blood was stored and proceeded according to the protocol of Netto et al. [17]. Slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10; 1% Triton 100-X and 10% DMSO added just before use) and then washed. Under alkaline conditions (pH > 13), and after a 20 min DNA unwinding period, electrophoresis was conducted at 4 °C for 30 min at 0.7 V/cm and 300 mA. Following 15 min neutralization (0.4 M Tris, pH 7.5), the slides were fixed in absolute ethanol, allowed to air dry, and stored at 4 °C. The slides were stained with ethidium bromide and immediately analysed in a fluorescence microscope connected to a computer-based analysis system (Comet Assay IV, Perceptive Instruments, UK). One hundred randomly selected nucleoids were scored per animal (50 from each of two replicate slides). Results were expressed as tail intensity (% tail DNA). According to Collins et al. [19], positive control cells were incubated with H_2O_2 (200 μM, 30 min, ice-cold).

2.10. Statistical analysis

DNA damage levels are presented as means ± standard error of mean (SEM); oxidative stress biomarkers as mean ± standard deviation; classification of fetal body weight as percentage. For the comparison of DNA damage levels, the gamma distribution was used. Biomarkers of maternal oxidative stress were analysed by the t-test and fetal body weight classification by Fisher's exact test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Maternal data

Fig. 2A and Table 1 summarise the maternal DNA damage and

Table 1
Maternal oxidative stress biomarkers in day 10 post-partum of small-for-gestational-age rat mothers submitted to exercise (SPAex) or not exercised (SPA) during pregnancy.

	SPA	SPAex
MDA (nM/g Hb)	1120 ± 89	923 ± 104*
SH (mM/g H)	74.0 ± 5.6	63.4 ± 6.8
SOD (UI/mg Hb)	14.6 ± 8.0	12.5 ± 9.5
GSH-Px (UI/mg Hb)	0.41 ± 0.02	0.33 ± 0.03

MDA = malondialdehyde; SH = reduced thiol groups; SOD = superoxide dismutase; GSH-Px = glutathione peroxidase.
Data are expressed as mean ± standard deviation (SD).
* $p < 0.05$ compared to SPA rat mothers (*t*-test).

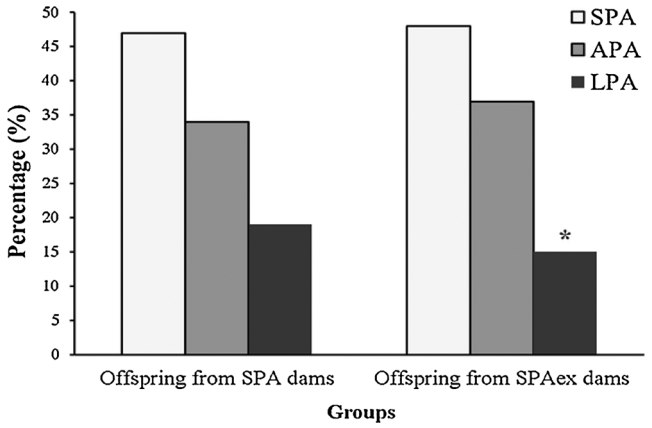

Fig. 3. Classification of body weight of offspring from mother rats submitted to exercise (SPAex) or not exercised (SPA) during pregnancy. Data are presented as the ratio between the number of fetuses classified as: small-for-gestational-age (SPA); appropriate-for-gestational-age (APA), or large-for-gestational age (LPA), and the total numbers of newborns. Data are expressed as percentage of newborns in the respective classifications. * $p < 0.05$ compared to LPA offspring from SPA rat mothers (Fisher's exact test).

oxidative status at postpartum d 10. The SPAex animals showed lower DNA damage (Fig. 2A) and MDA levels (Table 1) than the SPA rats. The comparisons of SH, SOD, and GSH-Px showed no differences between the groups (Table 1).

3.2. Newborns

Fig. 2B shows the DNA damage levels of male and female offspring from SPA mother rats. No statistically significant difference in glycaemia at post-natal d 10 was observed between the groups (data not shown). Male and female offspring from SPAex mother rats showed lower DNA damage levels in relation to their respective subgroups from SPA dams (Fig. 2B). The percentage of newborns classified as LPA was reduced in SPAex mother rats compared with SPA dams (Fig. 3).

3.3. Positive control for DNA damage levels

Samples of three rats of each group were used as positive control. Thus, the positive control (H_2O_2) showed higher levels of DNA damage (tail intensity mean > 20% - data not shown), as expected.

4. Discussion

We observed decreased DNA damage in SPA mother rats and their offspring, and reduced maternal lipid peroxidation (MDA concentration), 10 d after the swimming program was completed. Both general and specific changes result from adaptation induced by exercise [20], as a result of poorly understood cellular and molecular changes. Exercise increases the formation of reactive oxygen and nitrogen species (RONS) [21]. Moderate regular exercise is likely to cause adaptations of the antioxidant and oxidative-damage repair systems [22]. Even 10 d after the end of the swimming program, the SPAex rats and their offspring showed reduced DNA damage. Oxidative stress-induced adaptations might play an important role in the beneficial effects of regular exercise.

SPA rats submitted to swimming at pregnancy had unchanged antioxidant systems (SOD and GSH-Px activities and protein oxidation indicated by thiol levels), 10 d post-partum. It has been suggested that temporal changes in enzyme activity, both transitory and biphasic in nature, may occur, and such changes might contribute to decreased oxidative status, as indicated by reduced MDA levels. Siu et al. [23] observed decreased in MDA levels in the experimental group after 16 weeks of exercise training and an increased resistance of lymphocytes to oxidant-induced DNA damage in rats after 20 weeks of training. These authors confirmed exercise-induced elevated expressions of antioxidant enzymes (mitochondrial superoxide dismutase and catalase) and DNA repair enzymes (APEX1 nuclease; protein kinase, DNA activated, catalytic polypeptide, Prkdc; and O^6-methylguanine-DNA methyltransferase), after the training period.

In summary, SPAex rats showed lower DNA damage levels following a swimming program, which may be explained by an increase in antioxidant and metabolic capacity and/or stimulation of DNA repair enzyme activity. In addition, we demonstrated that a swimming program leads to reduced glycaemia and increased insulin sensitivity during pregnancy in IUGR rats, confirming the beneficial effects of exercise [24]. The intrauterine environment has a strong influence on pregnancy outcome. These findings suggest that SPA rats submitted to exercise benefit from an adequate intrauterine environment contributing to better fetal development. Our results also showed a lower rate of macrosomic offspring (LPA). Human studies show that exercise during pregnancy may be associated with changes in newborn weight at birth, either reduced weight [25,26] or increased weight [27]. Regular exercise during pregnancy may elicit maternal and fetal adaptations with the potential for either positive or negative long-term effects on offspring. Regular exercise (moderate or vigorous activity) during pregnancy was associated with decreased risk of large infant size for gestational age (LGA) [28]. Reducing the risk of LGA by physical activity might indirectly mitigate LGA-induced complications. Conflicting results have been observed, depending on the design and extent of the population study and the type, intensity, and frequency of regular exercise performed during pregnancy [29–31].

5. Conclusion

The present study shows that exercise (swimming) applied in SPA pregnant rats contributes to reduced DNA damage and lipid peroxidation. In addition, maternal physical exercise resulted in less genotoxicity in their offspring, reducing/ preventing DNA damage and macrosomia. Additional studies to study other interventions that may protect against diseases in adulthood caused by IUGR.

Disclosure statement

The authors report no conflicts of interest.

Author contributions

Netto, Corvino, and Damasceno designed the study. Netto, Corvino, and Serrano managed and conducted the tests. Netto, Gelaleti, Hernandez, Braz, Volpato, and Damasceno drafted the manuscript. All authors revised and approved the final manuscript.

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