Oxidative Stress Status and Placental Implications in Diabetic Rats Undergoing Swimming Exercise After Embryonic Implantation

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Abstract

The potential benefits and risks of physical exercise on fetal development during pregnancy remain unclear. The aim was to analyze maternal oxidative stress status and the placental morphometry to relate to intrauterine growth restriction (IUGR) from diabetic female rats submitted to swimming program after embryonic implantation. Pregnant Wistar rats were distributed into 4 groups (11 animals/group): control—nondiabetic sedentary rats, control exercised—nondiabetic exercised rats, diabetic—diabetic sedentary rats, and diabetic exercised—diabetic exercised rats. A swimming program was used as an exercise model. At the end of pregnancy, the maternal oxidative stress status, placental morphology, and fetal weight were analyzed. The swimming program was not efficient to reduce the hyperglycemia-induced oxidative stress. This fact impaired placental development, resulting in altered blood flow and energy reserves, which contributed to a deficient exchange of nutrients and oxygen for the fetal development, leading to IUGR.

Keywords

physical exercise, diabetes, pregnancy, oxidative stress, placenta

Introduction

Diabetes mellitus is a group of metabolic diseases due to hyperglycemia, resulting from defects in insulin secretion and/or action.¹ Diabetic pregnancy is complicated due to the metabolic demand of fetal, placental, and enclosure development. In this case, compensatory mechanisms are needed, and in cases where no compensatory mechanisms occur, it can result in maternal and fetal impairment.^{2,3}

The balance between the fetal nutrient demand and the maternal-placental supply regulates the fetal growth.⁴ The altered fetal growth (macrosomia in human and intrauterine growth restriction [IUGR] in experimental animals) in pregnancies complicated by diabetes is the result of abnormal substrate availability in placental transfer capacity.^{4,5} Besides, diabetes causes an exacerbated oxidative stress in pregnancies, which is associated with an increase in embryonic oxygen-free radicals, because of its relatively weak antioxidant defense, especially at the early stages of organogenesis.^{2,6}

Physical activity has long been known for its role in controlling glycemic levels by direct or indirect effects on insulin action.^{7,8} However, a major question remains regarding the correlation between the potential benefits and risks of physical

exercise on fetal development during human pregnancy. A previous study demonstrated that swimming applied to diabetic rats from day 7 (after embryo implantation) to day 20 of pregnancy led to an improvement in maternal lipid metabolism, showing beneficial results.⁹ Besides, these rats presented reduced embryonic death rates (resorption) compared to diabetic nonexercised dams.¹⁰ Damasceno et al⁵ demonstrated that nondiabetic and diabetic rats exercised prior to and during whole pregnancy showed fetuses with IUGR.

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Therefore, the objective of this study was to analyze maternal oxidative stress status and the placental morphometry to relate to IUGR in diabetic female rats submitted to swimming program after embryonic implantation, considering that exercise might lead to changes in the maternal oxidative stress status.

Materials and Methods

Experimental Animals

Wistar female rats, obtained from the São Paulo State University (UNESP) Vivarium (São Paulo, Brazil), were maintained in an experimental room under conditions with controlled temperature ($22^{\circ}\text{C} \pm 2^{\circ}\text{C}$) and humidity ($50\% \pm 10\%$) and a 12-hour light–dark cycle, with ad libitum access to commercial diet (Purina rat chow; Nestlé, St Louis, Missouri) and water. The procedures and animal handling were performed in accordance with the guidelines provided by the Brazilian College of Animal Experimentation in agreement with the International Guiding Principles for Biomedical Research Involving Animals promulgated by the Society for the Study of Reproduction and were authorized by the Ethical Committee for Animal Research of the UNESP, Brazil (Process number 353).

Experimental Diabetes Induction

Diabetes was induced by streptozotocin (SIGMA Chemical Company, St Louis, Missouri). Streptozotocin was dissolved in citrate buffer (0.1 mol/L, pH 6.5) and administered (intravenously [IV]) at a dose of 40 mg/kg body weight. Nondiabetic rats received only (IV) citrate buffer. Blood glucose concentrations were measured by One Touch Ultra Johnson & Johnson glucometer (Johnson & Johnson, HDI Home Diagnostics Inc, Fort Lauderdale, Florida) 7 days after the induction of diabetes. For inclusion criteria, the diabetic state was confirmed by blood glucose levels ≥ 300 mg/dL. For nondiabetic adult rats that received only citrate buffer, the inclusion criteria used was blood glucose levels < 120 mg/dL.¹¹

Mating Procedure

All female rats were mated overnight with nondiabetic male rats. The day when sperms were found in the vaginal smear was designated as gestational day 0. The mating period consisted of 15 consecutive days, a period comprising approximately 3 estral cycles, until a replicate number of groups was obtained. However, during this period, nonmated female rats were considered to be infertile and were discarded from the study.¹²

Calculation of Sample and Experimental Groups

To calculate the sample size for this experiment, the blood glucose concentration was estimated in rats with severe diabetes, which were obtained from previous studies in the same laboratory. Considering a reduction of 10% and a power of 80%, the minimum number obtained was 11 participants per group.

Then, after mating, the female rats were randomly distributed (by lot) into 4 experimental groups that constituted 11 animals/group: control (C)—sedentary nondiabetic, control exercised (CEX)—exercised nondiabetic, diabetic (D)—sedentary diabetic, and diabetic exercised (DEX)—exercised diabetic.

Exercise Program

For exercise, we used a swimming program according to the procedure by Volpato et al.¹⁰ To familiarize the rats to the swimming system (water), the rats were daily exposed to water for 15 minutes for 5 days in a cage ($100 \times 70 \times 60$ cm) containing water at a depth of 10 cm at 32°C . This period corresponded to the interval between diabetes induction and the mating period. Afterward, the female rats that were familiarized to the swimming system were placed in a cage containing water at a depth of 40 cm. Exercise on the first day under these conditions was about 20 minutes, with progressive increases of 10 minutes each day until they completed 60 minutes. Following, the rats were trained to swim for 1 hour daily until the end of pregnancy. Training for swimming was provided in water with a temperature of 32°C between 9 AM and 10 AM for 6 days a week. The pregnant rats that remained in water at a depth of 10 cm at 32°C were classified as sedentary.

Evaluation at Term of Pregnancy

Blood glucose levels and maternal weight were measured at approximately 9 AM every 7 days until the end of pregnancy. At day 21 of pregnancy, after determination of maternal weight, the rats were anesthetized, and the uterine horns were exposed for weighing the fetuses and their respective placentas. The placental efficiency was calculated as the ratio of fetal weight and placental weight.¹³ One placenta from each uterine horn was sectioned medial sagittally and fixed in 10% buffered formalin before being processed for paraffin embedding.

Placental Morphometry

Formalin-fixed placentas were dehydrated in a graded ethanol series, embedded in paraffin according to a standard protocol, sectioned at 5 μm , and mounted on glass slides for hematoxylin–eosin staining. For histological analysis, 11 placental blocks (11 blocks/group—1 placenta/dam) were cut in the longitudinal direction. The placental morphometric analyses were performed in a computerized image system coupled to a photomicroscope through a digital camera. The slides were preselected to assure the presence of all placental layers in the sample. From each slide, 6 areas were randomly selected. The decidua and junctional zones (mm^2) were evaluated at $100\times$ magnification, while the labyrinthine region was evaluated at a magnification of $25\times$.

Determination of the Oxidative Stress Parameters

The samples of blood collected in heparinized tubes were processed and washed erythrocytes were collected for the

Figure 1. Blood glucose levels from nondiabetic or diabetic rats, not exercised or exercised, after the embryonic implantation period. Data shown as the mean $\pm$ standard deviation (ANOVA—Student-Newman-Keuls posttest) * $P < .05$ —Statistically significant difference compared with control group. # $P < .05$ —Statistically significant difference compared with control exercised group. ANOVA indicates analysis of variance.

determination of lipoperoxidation marker (malondialdehyde [MDA]) and antioxidant substances (superoxide dismutase [SOD] and glutathiones) according to the methodology of Damasceno et al.¹⁴

Statistical Analysis

Analysis of variance and Student-Newman-Keuls test were used for quantitative variables with normal distribution. Differences were considered statistically significant if $P < .05$.

Results

Maternal Glycemia

The blood glucose levels of the nondiabetic groups (C and CEx) were lower than 120 mg/dL during pregnancy. The swimming program did not alter the blood glucose levels of the nondiabetic (CEx vs C) and diabetic female rats (DEx vs D) throughout the pregnancy. The diabetic animals presented levels maintained above 300 mg/dL during pregnancy, regardless of the swimming program (Figure 1).

Maternal Oxidative Stress Parameters

Superoxide dismutase activity increased in the exercised nondiabetic animals compared to the sedentary group (C). The sedentary diabetic female rats (D) had increased level of MDA compared to C group. The DEx rats had decreased levels of glutathione peroxidase (GSH-Px) and elevated SOD activity compared to the C group. This same group presented increased MDA levels and decreased SOD levels when compared to the D group (Table 1).

Maternal Reproductive Outcomes and Placental Morphometry

The maternal weight gain, with and without uterine content, was lower in all experimental groups compared to C group (Figure 2). The fetal weight was also lower in all experimental groups than the C group. The CEx rats presented placentas with the lowest weights and the placental efficiency indexes in the diabetic rats (D and DEx) were decreased in relation to C group. The mean area of the placental decidua from dams of the D and DEx groups was significantly lower compared to that of the C group. The mean area of the placental labyrinthine was lower in all groups compared to the C group (Table 2). Figure 3 shows placental morphology and structural changes in the placenta of diabetic groups. The disarrangement observed in diabetic placentas (D and DEx) was characterized by aberrant cell size in placental layers, ectopic and spread giant cells, and presence of cystic spaces.

Discussion

In the present study, the effect of exercise on blood glucose level was not observed in the diabetic rats during pregnancy. The lack of exercise effect on maternal hyperglycemia in diabetic pregnant female rats was previously observed in other studies.^{5,9,10} Similarly, clinical investigations with diabetic pregnant women confirmed this fact.¹⁵

Among the complications of diabetes, oxidative stress has been widely studied. Oxidative stress is a condition in which the production of reactive oxygen species (ROS) is alarmingly high and the available antioxidant defenses is limited, resulting in damage to DNA, proteins, sugars, and lipids caused by the excessive free radicals.¹⁶ Reactive oxygen species include free radicals such as superoxide ($\text{O}_2^{\bullet-}$), hydroxyl ($\text{OH}^{\bullet}$), peroxy ($\text{RO}_2^{\bullet}$), and hydroperoxyl ($\text{HRO}_2^{\bullet}$) as well as nonradical species such as hydrogen peroxide (H_2O_2).^{17,18} Production of 1 ROS may lead to the production of others through radical chain reactions. Exposure to free radicals from a variety of sources has led organisms to develop a series of defense mechanisms,¹⁹ such as preventative and repair mechanisms, physical, and antioxidant defenses. Enzymatic antioxidant defenses include SOD, GSH-Px, and catalase (CAT). Under normal conditions, $\text{O}_2^{\bullet-}$ is quickly eliminated by antioxidant defense mechanisms. $\text{O}_2^{\bullet-}$ is dismutated to H_2O_2 by manganese SOD in the mitochondria and by copper SOD in the cytosol.²⁰ H_2O_2 is converted into H_2O and O_2 by GSH-Px or CAT in the mitochondria and lysosomes, respectively. H_2O_2 can also be converted into the highly reactive $\text{OH}^{\bullet}$ radical in the presence of transition elements like iron and copper. Glutathione is highly abundant in the cytosol (1-11 mmol/L), nuclei (3-15 mmol/L), and mitochondria (5-11 mmol/L) and is the major soluble antioxidant in these cell compartments. The following are the main protective roles of glutathione against oxidative stress²¹: (1) glutathione is a cofactor of several detoxifying enzymes against oxidative stress, for example, GSH-Px, detoxifying H_2O_2 , and lipid peroxides by the catalytic action of

Table 1. Oxidative Stress Status of Nondiabetic or Diabetic Rats, Not Exercised or Exercised, After the Embryonic Implantation Period.^a

	Groups			
	Control	Control Exercised	Diabetic	Diabetic Exercised
MDA, nmol/L/gHb	54.60 ± 40.15	65.80 ± 21.84	319.7 ± 191.78 ^b	544.2 ± 148.21 ^{b,c}
SOD, UI/mgHb	7.17 ± 3.74	19.63 ± 5.41 ^b	8.55 ± 4.99	16.34 ± 6.80 ^{b,c}
Thiol group, μmol/L/gHb	0.76 ± 0.66	1.23 ± 0.37	0.64 ± 0.56	1.09 ± 0.29
GSH-Px, UI/gHb	0.48 ± 0.36	0.23 ± 0.08	0.22 ± 0.15	0.10 ± 0.04 ^b

Abbreviations: MDA, malondialdehyde; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; ANOVA, analysis of variance.

^aData shown as the mean ± standard deviation (ANOVA—Student-Newman-Keuls posttest).

^bP < .05—statistically significant difference compared with control group.

^cP < .05—statistically significant difference compared with diabetic group.

Figure 2. Maternal and uterine weights from nondiabetic or diabetic rats, not exercised or exercised, after the embryonic implantation period. Data shown as the mean ± standard deviation (ANOVA—Student-Newman-Keuls posttest) *P < .05—Statistically significant difference compared with control group. ANOVA indicates analysis of variance.

GSH-Px. The capacity of glutathione to regenerate the most important antioxidants is linked with the redox state of the glutathione disulfide–glutathione couple (GSSG/2GSH).²² Considering the antioxidant defense of the endocrine pancreas, it was verified that β cells are particularly sensitive to ROS because they are low in free-radical quenching (antioxidant) enzymes such as CAT, GSH-Px, and SOD.²³ Therefore, the ability of oxidative stress to damage mitochondria and markedly blunt insulin secretion is not surprising.²⁴ Evidence in both, experimental and clinical studies, suggests that free radical-mediated oxidative stress plays a major role in the pathogenesis of both type 1 and type 2 diabetes.^{25,26}

Maritim et al²⁷ reviewed in detail that diabetes has multiple effects on the protein levels and activity of the antioxidant enzymes, which further augment oxidative stress by causing a suppressed defense response.

The oxidative stress may be analyzed in the red blood cells (RBCs). This might be explained because RBCs are vulnerable to oxidative damage because of their continuous exposure to oxygen and their high concentrations of polyunsaturated fatty

acids and heme iron.²⁸ In the present study, the diabetic groups presented increased MDA levels, a marker of lipid peroxidation, in analysis of washed RBCs, confirming oxidative stress.

Several research about oxidative damage indicate that exercise exacerbates the generation of ROS, some of which are free radicals.^{29,30} Numerous studies have shown that muscle cells also release superoxide into the extracellular space,³¹ so free radicals readily reach the blood and act on other cells.³² Several potential alternative sources of free radicals, such as oxidase systems associated with membranes, nitric oxide production, and phagocytic processes,³³ as well as an increase in lactate formation, as happens in exhaustive exercise,³⁴ have been proposed to contribute significantly to the overproduction of free radicals.³⁵

In relation to the influence of swimming on pregnant rats, this study showed that the exercise contributed to an increased SOD enzymatic activity in nondiabetic and diabetic rats. However, the swimming decreased GSH-Px activity in diabetic status. These results show that increased SOD activity was not sufficient to reduce the elevated lipid peroxidation in the diabetic dams as a function of the uncontrolled metabolism due to severe hyperglycemia. Witt et al³⁶ verified that the exercise resulted in an increased free radical concentration in muscle and other tissues and membrane damage, as evidenced by lipid peroxidation, which depends on the state of training, duration, intensity of exercise, and the tissue examined.

The exercised dams of the different experimental groups showed a lower gain of maternal weight associated with the lower corporal weight of the fetuses. This decrease is characteristic of IUGR, which is related to fetal hypoxia caused by the practice of exercise, and this was exacerbated by the uncontrolled hyperglycemia, corroborating the previous results of this research group.¹⁰ The effects of physical exercise during fetal development are controversial, mainly regarding the intensity level of the exercise that is undertaken (light, moderate, or intense), and there are several conflicting reports regarding the effects of intense exercise on the risk of IUGR.^{37–39} One possible explanation for confounder factors in the interpretation of results is the physiological stress caused by exercise in experimental animals.⁴⁰ Swimming during pregnancy increases the plasmatic corticosterone levels in rats,^{41,42} and

Table 2. Fetal and Placental Analysis From Nondiabetic or Diabetic Rats, not Exercised or Exercised, After the Embryonic Implantation Period.^a

	Groups			
	Control	Control Exercised	Diabetic	Diabetic Exercised
Fetuses				
Fetal weight, g	5.32 ± 0.48	4.53 ± 0.40 ^b	4.22 ± 0.59 ^b	3.84 ± 0.45 ^{b,c}
Placenta				
Placental weight, g	0.47 ± 0.08	0.39 ± 0.08 ^b	0.66 ± 0.16 ^b	0.55 ± 0.12 ^{b,c}
Placental efficiency	11.60 ± 1.63	11.85 ± 1.86	6.70 ± 1.65 ^b	7.24 ± 1.57 ^{b,c}
Decidual area, mm ²	0.06 ± 0.02	0.06 ± 0.01	0.05 ± 0.01 ^b	0.05 ± 0.02 ^b
Junctional area, mm ²	0.18 ± 0.04	0.16 ± 0.03	0.17 ± 0.03	0.16 ± 0.03
Labyrinthine area, mm ²	4.14 ± 0.41	3.93 ± 0.44 ^b	3.85 ± 0.47 ^b	3.86 ± 0.32 ^b

^aData shown as the mean ± standard deviation (ANOVA—Student-Newman-Keuls posttest) and proportions (%; Fisher exact test).

^bP < .05—statistically significant difference compared with control group.

^cP < .05—statistically significant difference compared with diabetic group.

Figure 3. Microscopic images of the placentas (hematoxylin and eosin) at day 21 of pregnancy from nondiabetic or diabetic rats, not exercised or exercised, after the embryonic implantation period. A indicates control group; B indicates control exercised group; C indicates diabetic group; D indicates diabetic exercised group. D, decidua area; JZ, junctional zone; L, labyrinthine area; CS, cystic spaces presented transudate; arrow, Giant cells; asterisk, glycogen cells (magnification ×40). ANOVA indicates analysis of variance.

the effects of swimming-induced stress during pregnancy persist on birth weight through 2 subsequent generations.⁴³ Vaughan et al⁴⁴ showed that fetuses of corticosterone-treated dams always weighed less than normal. The corticosterone induced fetal growth restriction, which was associated with morphological and functional changes in placental phenotype

that depends on gestational age, particularly related to placental amino acid transport.

The exercised nondiabetic rats presented reduced placental weight and labyrinthine area, leading to reduced fetal weight. In the diabetic female rats, it showed higher placental weights, showing reduced placental efficiency, which confirms the

inability of the larger placenta to transfer nutrients to the developing fetus, causing IUGR. The reduction in decidual area from diabetic dams confirms that the decidua is highly influenced by the hyperglycemic status.¹² Besides, these results show that swimming did not protect this area, whereas lipid peroxidation process is exacerbated, which might alter decidual zone, interfering with placental development. The exercised nondiabetic and diabetic rats presented a significant decrease in labyrinthine area, the site of maternal–fetal exchange. Hewitt et al⁴⁵ showed that administration of glucocorticoids causes a permanent deficit in labyrinthine blood vessels in the rat placenta. As discussed previously, the stress caused by exercise contributed to a deficit in nutrient transport confirmed by decreased exchange area in the placenta. In addition, the hyperglycemia associated with exercise caused structural disarrangement in placenta, which demonstrated ectopic and spread giant cells and presence of cystic spaces.

The placental alterations verified in this study could justify that IUGR was caused by exercise program, which is related to a reduction in placental blood flow in the rats submitted to acute strenuous exercise. Although the literature showed that swimming program used in this experiment is moderate,⁴⁶ the impaired maternal and fetal outcomes suggest an exercise of strong/severe intensity.

Thus, the swimming program was not efficient to reduce the hyperglycemia-induced oxidative stress. This fact impaired placental development, resulting in altered blood flow and energy reserves, which contributed to a deficient exchange of nutrients and oxygen for the fetal development, leading to IUGR. These findings reinforce the necessity to reach a good glycemic control combined with interdisciplinary and professional discussion about exercise intensity and time of exposure for women during pregnancy.

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Declaration of Conflicting Interests

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