



DNA damage in Wistar Kyoto rats exercised during pregnancy¹

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Abstract

Purpose: To evaluate DNA damage levels in pregnant rats undergoing a treadmill exercise program.

Methods: Wistar Kyoto rats were allocated into two groups (n= 5 animals/group): non-exercise and exercise. The pregnant rats were underwent an exercise protocol on a treadmill throughout pregnancy. Exercise intensity was set at 50% of maximal capacity during maximal exercise testing performed before mating. Body weight, blood pressure and glucose levels, and triglyceride concentration were measured during pregnancy. At day 10 post-natal, the animals were euthanized and maternal blood samples were collected for DNA damage.

Results: Blood pressure and glucose levels and biochemical measurements showed no significant differences. Increased DNA damage levels were found in exercise group compared to those of non-exercise group (p<0.05).

Conclusion: The exercise intensity protocol used in the study might have been exhaustive leading to maternal increased DNA damage levels, demonstrating the relevance of an adequate protocol of physical exercise.

Key words: Pregnancy. Exercise. DNA Damage. Rats.

■ Introduction

Animal studies have shown that normal fetal growth is critically dependent on adequate intrauterine environment because maternal metabolic abnormalities impair homeostasis, which might predispose development of disease in adulthood¹.

Pregnancy is per se an inflammatory condition with increased susceptibility to oxidative stress². Reactive oxygen and nitrogen species, derived from the inflammatory process, can cause DNA damage that might affect several physiological processes during pregnancy. As repair of DNA damage is suppressed during pregnancy, pregnant women become more susceptible to environmental and endogenous agents³. Furthermore, modern sedentary lifestyles can also cause excess inflammation and oxidative stress, which increases the risk of diseases and DNA damage⁴.

Physical activity brings health benefits, so pregnant women are advised to keep active during pregnancy, although a large number of them stop exercising when they are pregnant⁵. Physical fitness and regular prenatal exercise can enhance placental growth and vascularity, decrease oxidative stress and reduce maternal endothelial dysfunction⁶. Nonetheless, the American College of Obstetricians and Gynecologists⁷ recommends that in order to ensure safety of both mother and fetus, exercise intensity should be closely monitored during pregnancy. Better benefits are directly associated with higher levels of physical activity, but depending on its intensity, exercise can either prevent or cause DNA damage⁸. The underlying mechanisms are still unclear. Although some studies have evaluated the influence of exercise intensity on oxidative stress during pregnancy⁹, the effects of physical activity on DNA damage remain without further investigation. Thus, the aim of this study was to evaluate DNA damage levels in pregnant Wistar Kyoto rats under a treadmill exercise program.

■ Methods

The Ethics Committee for Animal Research of Botucatu Medical School-Universidade Estadual Paulista approved the protocols of this study (CEEA Number 889/2011).

Virgin female and male Wistar Kyoto (WKY) rats were acquired from the animal colony of Universidade de São Paulo and kept at the Laboratory of Experimental Research of the Department of Internal Medicine, Botucatu Medical School, UNESP, under controlled temperature ($22 \pm 2^\circ\text{C}$) and lighting (12h light/12h dark cycle) with free access to food and water.

Female rats were randomly assigned in two experimental groups (n=5 animals/group): non-exercise WKY – pregnant rats were submitted to no physical exercise, and exercise WKY- pregnant rats submitted to treadmill walking.

Adaptation to treadmill walking

At adult life (approximately at 80 days of life), the animals from the exercise group were familiarized with the exercise apparatus by being placed on the treadmill operating at zero speed as a mild electric stimulation and noise. Subsequently, familiarization to treadmill walking was performed at 5 meter (m)/minutes (min) for 10 min over a 1-week period. Electric stimulation was used to encourage and improve the ability to walk throughout the adaptation period. During pregnancy this stimulus was not carried out. Non-exercise rats were not submitted to a treadmill walking and were kept at the same inverted dark-light cycle to reproduce the *circadian* biological *clock of animals*.

Maximal exercise testing

Following familiarization to training, two days before mating, exercise animals were underwent to maximal exercise testing, which

is widely used to determine exercise intensity in experimental studies¹⁰. The test consisted of graded exercise on the treadmill, starting at 5 km/h with increments of 0.3 km/h every 3 min up to exhaustion¹¹. Low intensity exercise was set at 50%-60% of the speed reached during maximal exercise test prior to pregnancy¹⁰.

Mating

At approximately 90 days of life, every morning males and females rats were put in breeding cages for mating. By late afternoon, the male was removed and vaginal smears were taken from the females for examination under a light microscope. The presence of spermatozoa in vaginal smears was positive mating indicator, and this was designated as day zero of pregnancy. Following, the animals were weighed, and the blood pressure was measured. The pregnant rats were housed in individual cages during whole pregnancy.

Treadmill exercise during pregnancy

The pregnant rats were submitted to a motorized treadmill started on day 1 of pregnancy at 10 m/min for 20 min, with increments of 10 min every two days up to 60 min. Thereafter, 60-minute sessions were held up to the end of pregnancy. Speed was gradually increased by 1m/min every day until maximum (13 m/min - determined by maximum exercise testing).

At pregnancy_maternal analysis

At days 0, 7, 14 and 21 of pregnancy, maternal body weight was assessed for weight gain evaluation, and systolic blood pressure level was measured in conscious rats by indirect tail-cuff plethysmography (LE5001 Pressure Meter; Panlab®, Barcelona, Spain). At days 0, 14 and 20 of pregnancy, blood samples were obtained by tail cut for triglyceride level measurement (Accutrend® Plus, Roche Diagnostics GmbH, Mannheim, Germany) and blood glucose levels (One-Touch Ultra, Johnson

& Johnson®).

At day 10 post-partum_maternal analysis

At term pregnancy, the rats were closely monitored for newborn birth. Ten days after birth, mothers and pups were euthanized under sodium pentobarbital (Hypnol®, Cristália Chemicals, Brazil) and decapitated for collection of whole blood samples. 500µL were collected in EDTA vacutainer tubes and transferred to microtubes containing 400µL of RPMI 1640 medium and 100µL of dimethyl sulfoxide (DMSO, Sigma-Aldrich®). The samples were kept at -20°C for 24h, and then stored in a -80°C freezer. Thawing was achieved on a 37°C water bath, and pellets were washed with PBS. A 20-ml aliquot was mixed with 120ml low melting point (LMP) agarose, immersed into a lysis solution, electrophoresed, neutralized and stained for DNA damage assessment.

Comet assay

Chemicals

Normal Melting Point Agarose (NMPA) and Low Melting Point Agarose (LMPA)¹² (Collins 2004), sodium chloride (NaCl), ethidium bromide, dimethyl sulfoxide (DMSO), sodium ethylene diamine tetra-acetic acid (EDTA), hydrochloric Acid (HCl), sodium hydroxide (NaOH), N-lauroyl sarcosine, Calcium (Ca²⁺) and Magnesium (Mg²⁺)-Free phosphate-buffered saline (PBS), Tris and Triton X-100.

Treatment with hydrogen peroxide

(H₂O₂)

Treatment with H₂O₂ (400µM) was used to create positive controls, according to the protocol described by Blasiak *et al.*¹³ with modification regarding the molarity to the H₂O₂ solution (10µM).

Electrophoresis

The comet assay was conducted as described by Tice *et al.*¹⁴ with modification¹⁵.

Slides with test-samples were placed in an electrophoresis chamber and incubated with NaOH/EDTA buffer solution for 20 min at 4°C. Electrophoresis was run at 25 volts (300 mA) for 30 min. Subsequently, the slides were neutralized in Tris (pH=7.5) for 15 min, fixed in 100% ethanol, and dried at room temperature.

Staining

All slides were stained with ethidium bromide (20mg/mL), coated with a coverslip, and immediately analyzed.

Slide analysis

Slide samples were examined considering 5 different classes of DNA damage according to a visual score system, on a 0-4 scale (0 = no damage, 4= maximum damage). A total of 100 nucleoids per sample were analyzed. Each nucleoid analyzed received a score ranging from zero to 4 according to its characteristics. In addition, comet observations were made at x400 magnification using a fluorescence microscope. One hundred nucleoids per sample (50/slide) were assessed by automated image analysis (Comet Assay IV, Perceptive Instruments, UK). Tail intensity (defined as the percentage of DNA in the tail) was used as a measure of DNA damage.

Statistical analysis

Analysis of variance (ANOVA) followed by Student's t test was used for the analysis of the study population. DNA damage levels were assessed by the Wilcoxon two-sample test. The level of statistical significance adopted was $p < 0.05$

■ Results

During pregnancy, maternal body weight, systolic pressure, blood glucose and triglyceride levels, and heart weight showed no significant statistically difference between the

exercise and the non-exercise groups ($p > 0.05$). In addition, the mean number of live newborns of exercise group did not differ compared to that of non-exercise rats (Table 1).

Table 1 – Characteristics of the study groups during pregnancy (days 0, 7, 14 and 20) and day 10 post-partum of non-exercise and exercise Wistar Kyoto rats.

	NON-EXERCISE	EXERCISE
Body weight (g)		
Day 0	198.3 ± 10.35	213.4 ± 9.56
Day 7	213.6 ± 8.07	218.3 ± 10.71
Day 14	230.0 ± 7.96	242.2 ± 11.15
Day 20	267.5 ± 9.20	288.0 ± 22.49
Day 10 post-partum	234.7 ± 4.34	224.5 ± 41.57
Systolic pressure level (mmHg)		
Day 0	121.80 ± 4.09	130.50 ± 16.78
Day 7	120.80 ± 2.28	125.33 ± 6.66
Day 14	113.40 ± 7.16	118.33 ± 5.51
Day 20	112.75 ± 5.91	115.67 ± 6.66
Blood glucose level (mg/dL)		
Day 0	129.25 ± 57.27	107.25 ± 4.72
Day 7	115.20 ± 13.52	108.50 ± 14.48
Day 14	82.33 ± 8.62	102.50 ± 12.29
Day 20	84.60 ± 12.78	85.67 ± 7.23
Triglyceride level (mg/dL)		
Day 0	98.25 ± 18.96	98.75 ± 7.68
Day 14	144 ± 16.97	138 ± 19.82
Day 20	178.33 ± 109.56	136 ± 46.67
Number of live newborns		
Day 10 post-partum	9.0 ± 2.01	11.33 ± 1.53

$p > 0.05$ - No statistical difference between groups (Student's t test)

Data reported as mean ± standard deviation

Figure 1 shows maternal DNA damage levels. The exercise group presented higher DNA damage levels compared to those of non-exercise rats ($p < 0.05$). The DNA damage levels showed no significant correlation with body weight, systolic pressure, blood glucose and triglyceride levels ($p > 0.05$) (data not shown).

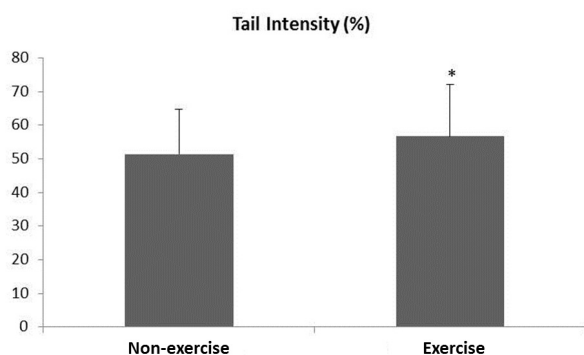


Figure 1 - DNA damage levels of non-exercise and exercise Wistar Kyoto rats at day 10 post-partum. Data reported as mean \pm standard deviation. * $p < 0.05$ – compared to non-exercise group (Wilcoxon two-sample test).

■ Discussion

Physical training during pregnancy in laboratory animals has been shown to influence fetal growth and to improve maternal metabolism in models of voluntary exercise¹⁶ and swimming¹⁷. However, such exercise models do not allow standardizing exercise intensity for animals, even if they are not pregnant. Therefore, the motorized treadmill has been used for establishing exercise duration/intensity, and the direct effect of the preferred intensity¹⁸.

Our results show that treadmill training did not affect pregnancy or fetal viability. Indeed, maternal body weight, systolic pressure, blood glucose and triglyceride levels showed no differences between experimental groups. Other studies have yielded conflicting

results with regard to maternal body weight gain and fetal growth. These results might be explained due to differences on exercise intensity, duration and type as well as to the presence/absence of preconditioning or familiarization with the type of exercise employed¹⁹. Some animal studies have suggested that, in contrast to exercise of low to-moderate intensity, strenuous exercise may affect fetal and maternal body weight gain²⁰. However, others have reported no effect on either maternal body weight gain²¹ or fetal body weight²².

Although the exercise type used in our study did not affect the maternal reproductive and offspring number, there was an increased DNA damage levels in the exercise group. In this group, maximal exercise testing was performed before mating, indicating the estimated capacity for low intensity training before pregnancy might be different of that applied during pregnancy. According to Veja *et al.*²³, the pregnant rats submitted to voluntary exercise showed reduced physical capacity as pregnancy advanced. Thus, it is possible that the exercise intensity used in our study, which was classified as low at early pregnancy, was strenuous during pregnancy, and might therefore be linked to the increased maternal DNA damage levels. This hypothesis is supported by the fact that physical training of moderate intensity has been reported to provide protection against free radical generation in the long term due to an adaptive mechanism to meet energy demands²⁴ that modulates oxidative responses, which become more effective with time. Nonetheless, in the event of overtraining, the homeostatic balance may be altered and to impair hormonal, metabolic and immune functions²⁵. DNA damage levels do not increase after low-to-moderate physical exercise, but can be induced by strenuous exercise in rats¹⁸. Exhaustive exercise can lead to blood flow misdistribution, contributing to increased free radical production. Excess production of

endogenous oxidants leads to tissue and DNA damage²⁶.

The increased levels of DNA damage observed in the exercise group have also been reported in other experiments. In a study of Sprague-Dawley rats exercising 30min/day on a treadmill for eight weeks, DNA damage was significantly higher as compared to sedentary controls. However, in rats receiving supplementation with vitamin E, exercise-induced DNA damage clearly was reduced, but it persisted higher than in sedentary animals²⁷. Pereira *et al.*¹⁸ examined the effects of aerobic training (60% maximal capacity) and overtraining (90% maximal capacity) in Swiss mice and found increased DNA damage in peripheral blood and skeletal muscle cells in the overtrained group as compared to the aerobic training group.

Strenuous exercise-induced DNA damage has been investigated with conflicting results. Our finding of increased DNA damage after exercising is consistent with those reported in human²⁸ and animal studies¹⁸. Nonetheless, it is noteworthy that, in such studies, DNA damage levels were measured either immediately following exhaustive exercising or later. In the latter case, damage reversal was found to occur after 72h of rest²⁸, whereas in our study DNA damage levels were still increased 10 days after exercising.

Tsai *et al.*²⁹ assessing oxidative DNA damage in peripheral blood mononuclear cells from 12 male runners completing a 42-km marathon race (3h median running time) observed a significantly increased level of oxidative DNA damage that persisted for more than one week after the race. The authors pointed out the exact mechanisms underlying this phenomenon required elucidation as their findings, as well as ours, contradict other studies reporting decreased damage levels following a 72h post exercise rest¹⁸. According to those authors, the duration and extent of DNA effects seemed to increase synchronously

with the amount and intensity of exercise. Furthermore, the fact that substantial increase in DNA damage still occurred in the subjects of their study, who undertook regular running training contradicts the notion that regular training confers protection against further exercise-induced DNA damage caused by adaptive responses²⁹.

Gandhi & Gunjan²⁶ in their study review suggested the delayed onset of DNA damage might be explained by the fact that the initial injury is mechanical (muscular) in origin and results in physiological changes similar to those seen in acute inflammation, as well as in activation of polymorphonuclear neutrophils³⁰. These polymorphonuclear neutrophils infiltrated in muscle damage sites release oxygen radicals and other reactive agents as part of the phagocytic process, leading to secondary tissue damage³¹. The infiltration of macrophages at the injury site rises steadily and is maintained through several days after exercise³².

■ Conclusions

The comet assay is a sensitive tool for assessing the effects of exercise during pregnancy on DNA damage and exercise has no adverse effects on both maternal and fetal health. In addition, no interference in body weight gain, pregnancy progress, delivery, and fetal viability were observed after treadmill training. However, an adequate intensity protocol is relevant to conduct an exercise program for laboratory animals and human.

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