Adult exercise effects on oxidative stress and reproductive programming in male offspring of obese rats.

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Abstract

Introduction/Purpose. Exercise improves health but few data are available regarding benefits of exercise in offspring exposed to developmental programming. There is currently a worldwide epidemic of obesity. Obesity in pregnant women predisposes offspring to obesity. Maternal obesity has well documented effects on offspring reproduction. Few studies address ability of offspring exercise to reduce adverse outcomes. We observed increased oxidative stress and impaired sperm function in rat offspring of obese mothers. We hypothesized that regular offspring exercise reverses adverse effects of maternal obesity on offspring sperm quality and fertility. Methods. Female Wistar rats ate chow (C) or high energy, obesogenic diet (MO) from weaning through lactation, bred at postnatal day (PND) 120 and ate their pregnancy diet until weaning. All offspring ate C diet from weaning. Five male offspring (different litters) wheel-ran 15 min, 5 times/week from PND 330 to 450, and were euthanized at PND 450. Results. Average distance run per session was lower in MO offspring who had higher body weight, adiposity index and gonadal fat and showed increases in testicular oxidative stress biomarkers. Sperm from MO offspring had reduced antioxidant enzyme activity, lower sperm quality and fertility. Exercise in MO offspring decreased testicular oxidative stress, increased sperm antioxidant activity and sperm quality and improved fertility. Conclusions. Exercise intervention has beneficial effects on adiposity index, gonadal fat, oxidative stress markers, sperm quality and fertility. Thus, regular physical exercise in male MO offspring recuperates key male reproductive functions even at advanced age: it’s never too late.

Keywords: developmental programming, oxidative stress, interventions, fertility.
Introduction

Exercise is well known to improve health (7, 43). Programming by adverse conditions during development is now well accepted as a major determinant of offspring life-course health. Developmental programming can be defined as the response to a specific challenge to the mammalian organism during a critical developmental time window that changes the trajectory of growth altering phenotype with resulting effects on health that can persist throughout life (14, 57). Developmental effects of suboptimal maternal nutrition have been extensively investigated (15, 28, 44) including programming resulting from maternal obesity (50). The incidence of obesity has increased substantially worldwide over recent years and obesity is now recognized as a major, universal health problem by organizations such as WHO (53) with over 1.1 billion individuals throughout the world classified as obese (37). Increased prevalence of obesity is also reflected among women of reproductive age and recognized as a major complication in pregnant women (30, 55).

Maternal overnutrition and obesity during pregnancy and/or lactation affects offspring metabolic phenotype (56), cardiovascular function (46), anxiety behavior, associative learning and motivation (36). In contrast, programming of offspring reproductive capacity by maternal obesity is poorly documented. We have reported that male offspring of pregnant rats fed a low protein diet develop obesity and have lower sperm counts and a 50% decrease in fertility rate (58). At the other extreme of maternal nutrition, maternal over-nutrition and obesity during pregnancy and lactation decreases sperm viability, motility and concentration in adult male offspring accompanied by manifestation of increased testicular oxidative stress (35, 55). Female rat offspring from mothers fed with high fat diet during pregnancy and lactation have earlier puberty onset (42), higher leptin and insulin serum levels (21) and altered reproductive function...
reflected in an increased incidence of prolonged or persistent oestrus (10). Maternal obesity in humans leads to earlier onset of puberty (20) and affects semen quality in male offspring (34). In mice, Founder generation F₀ paternal obesity compromises F₁ female pancreatic function (29). F₁ females oocytes show increased oxidative stress and altered mitochondrial function and F₁ males have altered sperm function with reduced motility, increased ROS levels and decreased in vitro fertilization rates (13).

Human (23, 49) and animal studies (31, 38, 56) show that offspring of obese mothers are themselves predisposed to obesity. Obesity and male infertility have increased in parallel (25), which potentially explains the positive correlation between male obesity and low sperm quality and fertility (32). Human (8, 19, 45) and animal studies (33) show that spermatozoids from obese male have decreased motility, more morphologic defects, increased DNA damage and higher oxidative stress. While the etiology of male infertility is clearly multifactorial, oxidative stress is now considered to play an important role. In humans, paternal obesity is associated with increased oxidative stress in sperm (41), reduction in semen quality and decreased fertility (6). Similarly, obese male rats have lower sperm quality and higher epididymal lipoperoxidation (48).

Given the existence of multiple negative effects of adverse programming on life-course health, there is a pressing need for development of interventions that effectively prevent or reverse unwanted outcomes. Adult exercise provides numerous health benefits (43) and we have shown that exercise in obese female rats before and during pregnancy has beneficial effects on maternal and male and female offspring metabolism (47). Lifestyle changes that induce weight loss such as dietary and exercise interventions can improve semen parameters and therefore fertility in obese men (6). In light of the lack of information available about the beneficial exercise effects
on negative developmental programming outcomes in offspring, the present study aimed to determine the effects of maternal obesity on male offspring metabolism and fertility and determine whether developmental programming outcomes are permanent or are reversible by offspring lifestyle modification. We hypothesized that regular offspring exercise even in early adult life, would reverse the adverse effects of maternal obesity on offspring metabolism, sperm quality and fertility.

Methods

Standardization of phenotype of females (F₀) recruited to produce study F₁ offspring.

All procedures were approved by the Animal Experimentation Ethics Committee of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INNSZ), Mexico City, Mexico. Female albino Wistar rats were born and maintained in the animal facility of the INNSZ, which is accredited by and adheres to the standards of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Rats were maintained at 22–23°C under controlled lighting (lights on 07:00 to 19:00 h) and fed normal laboratory chow (Zeigler Rodent RQ22-5, USA) containing 22.0% protein, 5.0% fat, 31.0% polysaccharide, 31.0% simple sugars, 4.0% fiber, 6.0% minerals and 1.0% vitamins (w/w), energy 4.0 kcal g⁻¹.

At age 14 – 16 weeks, when they weighed 200 – 240 g, females were bred to randomly assigned, non-litter mate, proven male breeders. At delivery (day 0) litters that provided F₀ mothers were culled to 10 pups, containing at least four females. Litters with less than 10 or more than 14 pups were excluded. At weaning (day 21) one female F₀ pup from each litter was randomly assigned to either a control (C; n=5) group that received the laboratory chow or to a maternal obesity group (MO; n=5) fed a high energy, obesogenic diet containing 23.5% protein, 20.0% animal
lard, 5.0% fat, 20.2% polysaccharide, 20.2% simple sugars, 5.0% fiber, 5.0% mineral mix, 1.0% vitamin mix (w/w), energy 4.9 kcal g\(^{-1}\). Thus each F\(_0\) group contained only one female from any litter and F\(_0\) females in different groups, but not within groups, were sisters, providing maximal possible homogeneity in F\(_0\) mothers’ own developmental programming and genetics.

**F\(_1\) offspring**

F\(_0\) female rats were placed with proven male breeders on day 120 and conceived during the next cycle. To minimize consumption of the obesogenic diet by the males during the mating period males were placed with females at night and removed during the morning. Lactating mothers were maintained on their pregnancy diet. Litter size and pup weight were recorded at birth. Anogenital distance was measured to identify males and females (54). Litters with less than 10 or more than 14 pups were excluded. To ensure F\(_1\) offspring homogeneity, on postnatal day (PND) 2 all litters studied were adjusted to 10 pups with equal numbers of males and females as closely as possible. Offspring were weaned at PND 21, housed 5 per cage and fed Chow diet throughout the study. Only male offspring were studied. At PND 330 five males per group from different litters were randomly selected to begin wheel-running exercise (C exercised – Cex; MO exercised - MOex) (Fig. 1).

**Voluntary Moderate Exercise**

F\(_1\) Cex and MOex rats were trained to wheel run (15 min sessions) on two days in the week before PND 330. A pilot study established an optimum running schedule of 15 min which was always completed. Throughout to ensure some recovery time as in human athletic training schedules the study rats were allowed two non-consecutive rest days weekly. Rats ran for only
one 15-minute session 5 days per week during four months (PND 330 – PND 450). Distances run were quantified using a bicycle odometer (47). The last bout of exercise took place 24 hours prior tissue collection.

**F<sub>1</sub> offspring at PND 450**

At PND 450, following a 6 hour fast, male rats were decapitated by trained personnel, experienced in using a rodent guillotine (Thomas Scientific, USA) between 12.00 to 14.00 hours. Testes were dissected, cleaned of fat, weighed, frozen and stored at -70°C until analyzed. The sternal, pancreatic, perirenal, retroperitoneal and gonadal fat pads were dissected and weighed individually. Adiposity index was calculated as total adipose tissue (g) ÷ body weight (g). At PND 450 the cauda epididymis and vas deferens were rapidly removed and placed in saline at 37°C to release sperm by squeezing the vas deferens with tweezers and triturating the cauda epididymis with scissors.

**Tissue and sample preparation**

Frozen testes were homogenized in saline and aliquots were frozen at -70°C for no more than 24 hours for later protein quantification using the Bradford method and for determination of antioxidant enzymes activity lipid peroxidation was determined at the time of homogenization of the testis. All determinations were performed in duplicate and averaged for statistical analysis.

Sperm aliquots containing 5x10<sup>6</sup> and 1x10<sup>7</sup> spermatozoa underwent 6 thermal shock cycles from -70°C to 45°C, sonication for two minutes with 6 intervals of 20 seconds each, and were then placed in ice and frozen at -70°C for no more than 24 hours for later quantification of antioxidant enzymes.
Lipid peroxidation assay

Aliquots containing 5 million sperm were adjusted with saline to 100 µl. Lipid peroxidation was determined in 100 µl aliquots of either homogenized testes or 5x10^6 sperm by measurement of malondialdehyde (MDA) by the thiobarbituric acid-reactive substances assay (TBARS). All samples were read at 532 nm in a Perkin-Elmer LS50-B luminescence spectrometer. Results are expressed as either nmol MDA per mg of protein or nmol MDA per 5 million sperm (47). Intra and inter assay coefficients of variation were <6 and <8%, respectively.

Superoxide dismutase (SOD) activity

SOD activity was determined in 10 µl aliquots of homogenized testes or sperm with a RANSOD kit (RANDOX laboratories limited, UK). A standard curve was obtained according to the manufacturer’s instructions. All samples were read at 505 nm in a Perkin-Elmer LS50-B luminescence spectrometer at 0, 30 sec and 3 min at 37°C to obtain the deltas of absorbance. Results are expressed as either units of activity per mg of protein or % of activity per 5 million sperm (47).

Glutathione peroxidase (GPx) activity

GPx activity was determined in 10 µl aliquot of homogenized testes or sperm with the RANSEL kit (RANDOX laboratories limited, UK). All samples were read at 304 nm in a Perkin-Elmer LS50-B luminescence spectrometer at basal, 1, 2 and 3 min at 37 °C to obtain the deltas of absorbance. Results are expressed as either milliunits per mg of protein in the testes or activity units per 10 million sperm (47).
Sperm measurements

Sperm viability was assessed by mixing 10 μl of saline (0.90% w/v of NaCl) containing spermatozoids with 10 μl of eosin. Live spermatozoa were identified by absence of staining as a sign of membrane integrity. Results are expressed as the percentage of live cells. Sperm concentration and motility were evaluated with a computerized sperm analyzer (Sperm Quality Analyzer). All sperm measurements performed were according to the World Health Organization guidelines (52).

Evaluation of fertility rate

At PND 440 individual experimental males were placed for one week with two non-experimental group females aged 4 months. Males were then separated from the females who were kept individually until day 15 of any potential gestation. The male was considered fertile when at least one of the two females became pregnant (58). Results are expressed as percentages of fertile males and as percentages of pregnant females.

Statistical analysis

All data are presented as mean ± SEM. For differences within maternal diet and exercise intervention, data were analyzed using one way multiple analysis of variance (ANOVA) followed by the Tukey test. Fertility rate was analyzed using the $\chi^2$ test. A $p<0.05$ was considered significant. One male per litter was chosen randomly to form a group of 5.

Results
**Voluntary exercise**

The average distance run during the first month of exercise was (Cex: 12.2 ± 2, MOex: 7.5 ± 1.06 m/15min, p<0.05); at the fourth month the average distance run increased 2.2 and 2 times respectively. The average distance run per 15 minutes session in the 4 months was lower in male offspring of MO mothers compared to C offspring (Fig. 2A).

**Body weight, adiposity index and gonadal fat**

Unexercised MO male offspring had increased body weight, adiposity index and gonadal fat compared to C and Cex offspring (Fig. 2B-D) while testicular weight was similar in all groups (C: 1.6 ± 0.05, Cex: 1.5 ± 0.05, MO: 1.7 ± 0.05, MOex: 1.7 ± 0.04 g). At the end of the voluntary exercise program, none of these parameters were different between Cex and C group (Fig. 2B-D). Exercise in the MOex group did not decrease body weight (Fig. 2B) but it partially ameliorate adiposity index and gonadal fat compared to C and Cex (Fig. 2C and D).

**Testicular oxidative stress biomarkers**

Unexercised MO offspring increased testicular MDA levels as well as SOD and GPx antioxidant enzyme activity in comparison with C offspring (Fig. 3A-C). Exercise in Cex and MOex groups decreased MDA concentration and GPx activity in comparison to C and MO respectively (Fig. 3A and C). Exercise had no effect on SOD activity (Fig. 3B).

**Sperm oxidative stress biomarkers**
Sperm MDA was increased and SOD and GPx activity decreased in unexercised MO compared with C offspring (Fig. 4A-C). MDA levels were lower in Cex and MOex in comparison to C and MO respectively (Fig. 4A). There were no differences in the antioxidant enzyme activity in the sperm between C and Cex (Fig. 4B and C). Exercise in MOex group increased SOD and GPx activity compared to MO but values remained lower than C and Cex values (Fig. 4B and C).

**Sperm parameters**

Sperm quality evaluated by concentration, viability and motility was lower in the unexercised MO compared to C offspring (Fig. 5A-C). Sperm concentration increased in Cex and MOex compared with C and MO offspring (Fig. 5A). MOex improved viability and motility compared with MO but values did not reach those in C and Cex (Fig. 5A-C).

**Fertility rate**

Fertility rate reported as the percentages of fertile males was similar between groups (Fig. 6A). However when expressed as percentage of pregnant females, the fertility rate was lower in MO offspring compared with C and Cex. MOex partially improved male fertility since it was not different than C (Fig. 6B). No differences were observed in F2 litter size among the 4 groups (C = 10.4 ± 0.2, Cex = 10.6 ± 0.2, MO = 10.2 ± 0.6, MOex = 11 ± 0.3 pups/litter).

**Discussion**

There is growing evidence that the offspring of obese women have an increased risk of metabolic diseases in adult life. However, it has not been established if obesity during pregnancy programs male F1 reproductive capacity. There is also a need to determine whether adverse outcomes can
be reversed by later life behavioral modification. In a previous study conducted in young adult 
250 male offspring of obese mothers at PND 110 despite differences from controls in some hormones 
251 (e.g. testosterone) and sperm quality by this age, fertility rate was not different (35). Therefore 
252 we decided to exercise the rats after reproductive function is affected, around PND 330. The 
253 present study shows that maternal obesity impairs sperm quality and fertility in F₁ male rat 
254 offspring and that these adverse outcomes can be ameliorated by increasing physical activity 
255 even in later life.

257 It is well known that body weight can be affected by individual’s diet and physical activity. 
258 Epidemiological and animal studies have shown that the environment experienced in utero and 
259 in early neonatal life can increase the risk not only of developing metabolic diseases but also of 
260 adult obesity (24). We have reported that male offspring from obese mothers are fatter with 
261 higher leptin and insulin serum levels than offspring of controls (47, 56). Others have reported 
262 similar observations (11, 22). In this study we report that body weight, gonadal fat and adiposity 
263 index were increased in the MO F₁.

264 Obesity is an important factor for infertility (18). In humans, male obesity is associated with 
265 decreased serum sex hormone-binding globulin, testosterone concentrations and increased 
266 estrogen levels (32). These changes are accompanied by higher incidence of azoospermia or 
267 oligozoospermia (39), and structural changes in germ cells and sperms (32). Changes in scrotal 
268 temperature are associated with lower fertility (40). In the present study we found that male 
269 offspring from the MO group had an increase in gonadal fat and a decrease in sperm 
270 concentration and viability. These results may be due by the higher fat accumulation around the 
271 testes in the MO group with a resulting elevation in scrotal temperature which impairs sperm 
272 function. Scrotal lipectomy in infertile patients with scrotal lipomatosis improves sperm count
and motility (40). The improvement of sperm parameters in the MOex group might therefore be a consequence of the loss of gonadal fat.

Sperm are more susceptible to oxidative stress because they lack cytoplasmic antioxidant enzymes and have high membrane polyunsaturated fatty acid levels (3). Therefore, the lipid environment to which spermatozoa are exposed likely has an important role in sperm development and function (3). Lipoperoxidation and its products (e.g. MDA) are very toxic for sperm (2), damaging the plasma membrane, proteins and DNA and increasing apoptosis (1). Male mice fed with high fat diet show decreased sperm motility and capacitation as well as increased oxidative stress and DNA damage (5). In this context, we found that both the testes and sperm from the F1 MO had an increase in oxidative stress. Testes contain a sophisticate antioxidant defense system to ensure that oxidative stress does not affect spermatogenic and steroidogenic functions (4). However, sperm mitochondria have little capacity to respond to ROS attack because they are unable to respond by producing more enzymes to counteract oxidative stress and repair DNA damage. We attribute the different antioxidant responses in sperm from those in the testes to the fact that sperm are much less transcriptionally active due to the relative lack of cytoplasm (12). Thus mature sperm do not produce antioxidant enzymes de novo due to the lack of the required synthetic machinery even in the presence of increased ROS. In contrast, testicular somatic cells play an important role in antioxidant defenses to protect spermatogenic cells, and therefore respond to oxidative stress by producing new enzymes to counteract the increased ROS. As a result in our study SOD and GPx were higher in the testes and lower in sperm from MO offspring compared to C. Paternal obesity impairs embryo development and implantation (26) and decreases the reproductive function of both male and female in the next
two generations, probably through germ cell epigenetic alterations (13). However, effects of maternal obesity on offspring reproductive capacity have been generally overlooked. Female F1 offspring of rats fed a high fat diet during pregnancy and lactation exhibit early sexual maturation and a longer proestrous phase (57). The present study provides evidence that maternal obesity negatively programs fertility in the male offspring.

Human and animal studies indicate that obesity during pregnancy predisposes the offspring to chronic diseases in later life (57). Therefore there is a need for effective interventions that can be used to prevent or reverse the adverse outcomes of maternal obesity. There is growing evidence in human (27) and animal studies (47, 56) that indicates that changes in lifestyle of obese mothers such as reducing calorie intake or increasing exercise prevents excessive weight gain during pregnancy and the subsequent adverse outcomes in offspring health. However, if these interventions cannot be applied in obese pregnant women it is necessary to determine whether interventions in offspring are beneficial.

To date few studies in obese men have examined the effects of weight loss and exercise alone or separately on reproductive capacity. Exercise has been reported to increase serum testosterone levels and decreasing adiposity improves sperm quality (17). In young men, regular activity is associated with higher sperm concentration than men who are sedentary (16). Diet and exercise intervention in obese male rats improved sperm motility and morphology and diminished sperm DNA damage and ROS (33). In mice seminiferous tubules of lifelong regular runners exhibited abundant Sertoli cells and well-organized stratification of spermatogenesis and a larger number of luminal sperm, which may be associated with the decrease in testicular oxidative stress (9). However, not all physical activity is beneficial for reproductive health. In humans strenuous exercise has been proposed as a risk factor for male factor infertility since long-distance runners
and cyclists have lower testosterone levels and semen quality due to a negative energy balance. (16). In another study in men attending a fertility clinic bicycling 5 hours or more per week was associated with lower sperm concentration (51). Palmer et al. have reported that either diet and/or swimming exercise intervention in obese mice improve sperm motility and morphology and reduce sperm damage and ROS levels which were correlated with the metabolic status (32, 33). In the present study we found that the negative effects of maternal obesity in male reproductive capacity were ameliorated by offspring lifestyle exercise modification since regular voluntary exercise reduced body weight and fat accumulation, oxidative stress in the testes and sperm and increased sperm quality and improved fertility rate.

Finally, an important finding in the present study in relation to its potential as an intervention is the observation that MOex F₁ males exercised less than Cex. The male MO offspring weighed more and had higher adiposity index than the C offspring. In addition to the fact that exercise is more difficult in the presence of obesity and increased weight it is also possible that MO offspring are less motivated to exercise than C F₁ males. Further studies will be needed to explore this interesting question in a controlled animal model as well as to determine whether longer individual bout duration and overall period of exercise modify the outcomes we have observed.

**Perspectives and Significance**

In conclusion, the present study shows that maternal obesity impaired offspring reproductive capacity and that regular physical voluntary exercise even in old male offspring of obese mothers
ameliorated the fertility rate that is the functional end point of reproduction. The encouraging feature of these data is the indication that it is never too late for exercise to be beneficial.

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Conflict of interest

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Author contributions

M.S. researched data; G.L.R.G. researched data; C.I. researched data; C.C.V. researched data; P.W.N. study design, manuscript writing; E.Z. study design, wrote the manuscript.

References


**Figure legends**

**Figure 1.** Study design. d = days and PND = postnatal days. Control (C), control with exercise (Cex), maternal obesity (MO), and maternal obesity with exercise (MOex).

**Figure 2.** Voluntary exercise and weights at postnatal day 450 in male offspring of different groups of mothers: control (C), control with exercise (Cex), maternal obesity (MO), and maternal obesity with exercise (MOex). A) average distance run, B) body weight, C) adiposity index and D) gonadal fat. Mean ± SEM, n=5 from different litters. p<0.05 for data not sharing at least one letter.

**Figure 3.** Testicular oxidative stress biomarkers at postnatal day 450 in male offspring of different groups of mothers: control (C), control with exercise (Cex), maternal obesity (MO), and maternal obesity with exercise (MOex). A) malondialdehyde (MDA), B) superoxide dismutase (SOD), and C) glutathione peroxidase (GPx). Mean ± SEM, n=5 rats from different litters. p<0.05 for data not sharing at least one letter.

**Figure 4.** Oxidative stress biomarkers in sperm at postnatal day 450 in male offspring of different groups of mothers: control (C), control with exercise (Cex), maternal obesity (MO), and maternal obesity with exercise (MOex). A) malondialdehyde, (MDA) B) superoxide dismutase (SOD), and C) glutathione peroxidase (GPx). Mean ± SEM, n=5 rats from different litters. p<0.05 for data not sharing at least one letter.
SOD) and C) glutathione peroxidase (GPx) Mean ± SEM, n=5 rats from different litters. p<0.05 for data not sharing at least one letter.

Figure 5. Sperm at postnatal day 450 in male offspring of different groups of mothers: control (C), control with exercise (Cex), maternal obesity (MO), and maternal obesity with exercise (MOex). A) sperm concentration, B) viability, C) motility. Mean ± SEM, n=5 rats from different litters. p<0.05 for data not sharing at least one letter.

Figure 6. Fertility rate at postnatal day 450 in male offspring of different groups of mothers: control (C), control with exercise (Cex), maternal obesity (MO), and maternal obesity with exercise (MOex). A) % fertile males (# of fertile males/5) and B) % pregnant females (# of pregnant females/10). n = 5 male rats from different litters. p<0.05 for data not sharing at least one letter.
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