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Exercise protects rat testis from cyclophosphamide-induced damage

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ABSTRACT. To investigate the effect of chronic moderate exercise on male reproductive tract of Wistar rats submitted to a single dose of cyclophosphamide (CP). Animals were submitted to swimming exercise during 21 days or maintained at sedentary state. Trained (TCP) and sedentary (SCP) groups received a single dose of CP (200 mg kg^{-1}, i.p.). Trained (TCo) and sedentary (SCo) control animals received sterile PBS. Animals were killed after one week and testis, epidydimis and seminal vesicle contend were weighted. Testis were embebbed in parafim and stained with hemotaxilin and eosin. Fifty seminiferous tubules of each animal were analyzed by Johnsen score. Mean Sertoli cells counts per tubule and Leydig cells counts per area were evaluated. CP treatment impairs body weight gain in trained and sedentary animals. Liver and seminal vesicle contend were reduced only in SCo group. SCP animals presented decreased Johnsen scores, indicating a slight toxicity over germinative cells, whereas trained (TCo and TCP) animals presented increased Johnsen scores. Training increased Sertoli cell counts and prevented their loss in TCP group. Leydig cells counts were increased in trained animals, but decreased in CP treated ones (TCP). We conclude that exercise have some protective effect on male reproductive tract submitted to a single dose of CP.

Keywords: cyclophosphamide, exercise, reproductive tract.

O exercício físico protege o testículo de ratos de lesões teciduais induzidas por ciclofosfamida

RESUMO. Objetivou-se investigar o efeito de exercício moderado crônico no trato reprodutivo masculino de ratos Wistar submetidos a uma dose única de ciclofosfamida (CP). Os animais foram submetidos ao exercício de natação por 21 dias ou mantidos em estado sedentário. Os grupos treinados (TCP) e sedentários (SCP) receberam uma única dose de CP (200 mg kg^{-1}, ip). Os animais treinados (TCO) e sedentários (SCO) receberam PBS estéril. Os animais foram sacrificados após uma semana e os testículos, epidídimo e conteúdo da vesícula seminal foram pesados. As amostras foram embebidas em parafina e coradas com hematoxilina e eosina. Cinquenta túbulos seminíferos de cada animal foram analisados pelo escore Johnsen. A contagem média das células de Sertoli por túbulo e contagem das células de Leydig por área foram avaliadas. O tratamento CP prejudicou o ganho de peso corporal em animais treinados e sedentários. O fígado e o conteúdo da vesícula seminal foram reduzidos apenas no grupo SCO. Os animais SCP apresentaram menores escores de Johnsen, indicando uma toxicidade moderada sobre as células germinativas, enquanto os animais treinados (TCO e TCP) apresentaram escores de Johnsen mais altos. O treinamento aumentou a contagem de células de Sertoli e impediu a sua perda no grupo TCP. A contagem das células de Leydig foram aumentadas em animais treinados, mas reduzidas nos animais tratados com CP (TCP). Concluiu-se que o exercício tem algum efeito protetor sobre trato reprodutivo masculino de animais submetidos a uma dose única de CP.

Palavras-chave: ciclofosfamida, exercício, trato reprodutiva.

Introduction

Cyclophosphamide (CP) is an alkylating agent widely used as an anticancer drug as well as an immunosupressive drug. The cytotoxic effect of CP targets rapidly dividing cells. The spermatogenic lineage cells are particularly susceptible to CP damaging effects due to its constantly turnover from the germ line cell pool and the impairment of new Leydig cells maturation (ANDERSON et al., 1995; COLVIN, 1999; JAHNUKAINEN et al., 2011) Human studies showed a long term male gonadal damage after CP chemotherapy, including reduced hormone production and infertility due to spermatogonial depletion (NURMIO et al., 2009; RIDOLA et al., 2009).

Rodents models has been employed to study cytotoxic effects of CP on testis and to evaluate new therapeutic strategies to avoid its deleterious effects (CARMELY et al., 2009; ILBEY et al., 2009; MOTAWI et al., 2010; REZVANFAR et al., 2008;

SELVAKUMAR et al., 2006; TURK et al., 2010). CP toxic effects on testis were mainly attributed to oxidative stress on seminiferous tubules and Sertoli cells, impairing spermatogenis and androgenesis, and inducing germinal cells apoptosis (ILBEY et al., 2009; MOTAWI et al., 2010; REZVANFAR et al., 2008; TURK et al., 2010). It was also observed decreased testis, seminal vesicles and epididymal weights, damage and decreased number of spermatogonial cells in the seminiferous tubules, low levels of plasma testosterone and infertility (ELANGOVAN et al., 2006; REZVANFAR et al., 2008). Increased levels of lipid peroxidation and malondialdehyde and reduced levels of antioxidant enzymatic systems (gluthatione reductase, gluthatione peroxidase, catalase, superoxide dismutase) were detected in CP damaged testis (ILBEY et al., 2009; MANDA; BHATIA, 2003; SELVAKUMAR et al., 2004; 2005; REZVANFAR et al., 2008; MOTAWI et al., 2010). The main findings on testicular morphology in CP treated rats were the impairment of germ cell line maturation, highlighted by decreased Johnsen's testicular score, atrophy and degeneration of seminiferous tubules, vacuolization in Sertoli cells, interstitial edema and degeneration of Leydig cells (CERIBASI et al., 2010; MOTAWI et al., 2010; REZVANFAR et al., 2008). In rats, a single dose of CP (200 mg kg^{-1}) can induce atrophy and degeneration of germinative epithelium, haemorrhage and edema replacing Leydig cells (MOTAWI et al., 2010).

The use of antioxidant agents seemed partially to protect male reproductive organs from CPb toxic effects in animals models (CARMELY et al., 2009; SELVAKUMAR et al., 2004, 2005; CERIBASI et al., 2010; ILBEY et al., 2009; MOTAWI et al., 2010; TURK et al., 2010). These studies suggest that increasing antioxidant defenses may have some protective effects over drug cytotoxicity in male reproductive tract.

Regular exercise has been associated to improvement of quality of live, reduced tissue damage induced by oxidative stress, improvement of tissue growth, remodeling, regeneration, revascularization and differentiation of stem cells (BAKER et al., 2011; BOVERIS; NAVARRO, 2008; BRANDT et al., 2010; ELLISON et al., 2011; ITOH et al., 2011; NAKAMOTO et al., 2007; TEIXEIRA et al., 2008; TOTH et al., 2011). The cells respond to low levels of exercise-induced oxidative stress by increasing the expression, production and activation of antioxidant systems (AKSOY et al., 2006; CHICCO et al., 2005, 2006; CHIGURUPATI et al., 2008; GOMEZ-CABRERA et al., 2008; GUNDUZ et al., 2004; KAKARLA et al., 2005; SOMANI; HUSAIN, 1996). Trained animals presented increased levels and activation of antioxidant enzymes, reduced levels of malondialdehyde (a final product of oxidative stress) on testis and were protect from free radical attack induced by drugs (AKSOY et al., 2006; CHIGURUPATI et al., 2008; SOMANI; HUSAIN, 1996). The beneficial effects of exercise on heart, kidney, brain and liver protection against oxidative stress has been proven in several experimental and clinical data (FISHER-WELLMAN et al., 2009; GUNDUZ et al., 2004; KAKARLA et al., 2005; SRIMAHACHOTA et al., 2010; TEIXEIRA et al., 2008). These studies suggest that exercise may protect male reproductive tract from cytotoxic effect of drugs that induce oxidative damage.

We hypothesized that chronic moderate intensity exercise may protect male reproductive tissues from citotoxicity induced by CP. The aim of this work was to analysis the morphology of the testis of sedentary or trained rats exposed to a single dose of CP.

Material and methods

Experimental protocol

Twenty-four male Wistar rats, weighing 100 ± 10 g, were kept at standard conditions at temperature 20-22ºC, photoperiod 7 am to 7 pm, free access to standard rodent chow (Nuvilab® CR1, Nuvital, Colombo, Brasil) and tap water. The animals were divided into four groups (n = 6): sedentary control (SCo), sedentary treated with CF (SCP), trained control (TCo) and trained and treated with CF (TCP) groups. First, TCo and TCP groups were submitted to training protocol, during 21 days, while SCo and SCP animals were not submitted to training. All experiments were performed in accordance with the Committee for Ethical Animal Research of the State University of Londrina.

Exercised groups (TCo and TCP) were subjected to swimming in a plastic container (depth 37 cm, diameter 35 cm) and continuously supervised, with the water temperature set at 30 to 32°C, 5 times per week during 6 weeks. Prior to beginning the formal exercise protocol, rats were progressively habituated to water environment (water depth 15 cm) during five consecutive days. The swimming exercise time increased about 5 min.

every three days, until it reached 40 min. per day, which was maintained until the sixth week. On day five, a charge of 5% body weight was attached to chest of each trained animal and weekly adjusted until the end of training. This swimming protocol is equivalent to a moderate exercise program, where first lactate threshold was reached (GOBATTO et al., 2001) and it has been proven to increase antioxidant defenses and decreases oxidative stress in trained rats (RAVI KIRAN et al., 2004).

One day after the last training, all the animals of SCP and TCP groups received a single dose of CP (200 mg kg^{-1}, Fosfaseron; Laboratório Filaxis; Buenos Aires, Argentina), intraperitoneally. The SCo and TCo received the same amount of sterile phosphate buffered saline, intraperitoneally. All animals were sacrificed after one week.

Testis, epididymis, seminal vesicle content and liver were removed and immediately weighted.

Histological analysis

The removed testis were fixed in Bouin's fixative solution After 24h, testis were washed three times and maintained in 70% ethanol. Samples were then embedded in histological paraffin and sectioned. Ten random tissue sections (7 μm) of the testis were then stained using hematoxylin and eosin and examined under a light microscope (Olympus, Tokyo, Japan) coupled to a digital camera (Moticam, Motic Company, Xiamen, China) at 100 and 400X magnification.

A total of 50 cross-sections of seminiferous tubules from each sample were examined.at 100X magnification. To evaluate spermatogenesis, seminiferous tubules were scored by means of the Johnsen score (JOHNSEN, 1970), whereby seminiferous tubules are scored on a scale of 1 to 10, with tubules having complete inactivity scored as 1 and those with maximum activity (at least five or more spermatozoa in the lumen) scored as 10. The number of Sertoli cells were scored at 400X magnification in 50 seminal tubules per sample.

The mean number of Sertoli cells were examined in 50 tubules of each sample at 400X magnification. The mean number of Leydig cells were scored in interstitial tissue and evaluated by the ratio of cells per area (mm^2), in 10 images at 400X magnification.

Statistical analysis

The normality distribution were assessed by Shapiro-Wilks' test, considering $p < 0.05$ for non normalized data. Differences among groups were observed using ANOVA and *post hoc* Tukey's test (parametric data) and expressed as means and standard deviation. The Kruskal-Wallis' and *post hoc* Dunn's test were applied on non parametric data and expressed as median and quartis. Differences were considered significant if $p < 0.05$.

Results

All rats survived the experimental period. CP treated rats (SCP and TCP) showed a significant reduction in body weight gain during the last week. Liver and seminal vesicle content where also reduced in CP treated animals when compared to the SCo and TCo groups (Table 1). It was not observed significantly differences of testicular and epididymis weights among different groups.

Histological analysis demonstrated that SCP animals presented decreased Johnsen scores in relation to SCo animals, indicating that CP induced toxic effects in testis. Trained animals submitted or not to CP treatment presented best scores than sedentary groups (Figure 1). Training appears to improve germ cell line maturation and to inhibit the toxic effects on germ cell line observed in SCP group.

Table 1. Effect of exercise training and cyclophosphamide on animal characteristics and reproductive system.

	SCO	SCP	TCO	TCP
Starting BW (g)	116.6 ± 08.6	118.5 ± 10.7	119.2 ± 14.9	123.0 ± 08.0
Injection BW (g)	254.6 ± 29.3	253.8 ± 29.8	227.7 ± 16.6	232.3 ± 19.7
Final BW (g)	283.6 ± 33.3	258.2 ± 31.9	249.2 ± 16.8	238.3 ± 20.9
BW gain (Final/injection)	1.10 ± 0.02	1.00 ± 0.05 †★★	1.09 ± 0.02	1.02 ± 0.03‡★
Liver weight (g)	12.82 ± 1.17	10.93 ± 0.83†★	10.56 ± 0.70	10.04 ± 1.84
% Liver weight (g 100 final^{-1} BW)	4.54 ± 0.19	4.25 ± 0.31	4.24 ± 0.28	4.20 ± 0.48
Testis weight (g)	1.40 ± 0.13	1.40 ± 0.15	1.39 ± 0.23	1.38 ± 0.14
% Testis weight (g 100 final^{-1} BW)	0.49 ± 0.13	0.54 ± 0.08	0.55 ± 0.08	0.57 ± 0.06
Epididymis (g)	0.47 ± 0.11	0.55 ± 0.14	0.42 ± 0.09	0.49 ± 0.11
% Epydidymis weigh (g 100 final^{-1} BW)	0.15 ± 0.03	0.22 ± 0.06	0.16 ± 0.03	0.19 ± 0.04
Seminal vesicle content (g)	0.13 ± 0.03	0.05 ± 0.01†★★	0.19 ± 0.02★	0.13 ± 0.04

Values are expressed as means ± SD. SCO: sedentary control; SCF: sedentary + CP; TCO: trained control; TCP: trained + CP. BW: Body weight. †★SCO x SCP, $p < 0.05$; †★★SCO x SCO, $p < 0.01$. ANOVA two–way and *post hoc* Tukey test. ‡★TCO x TCP, $p < 0.05$; ‡★★TCO x TCP, $p < 0.01$. ANOVA two-way and *post hoc* Tukey test. ★SCO X TCO, $p < 0.05$. ANOVA two-way and *post hoc* Tukey test.

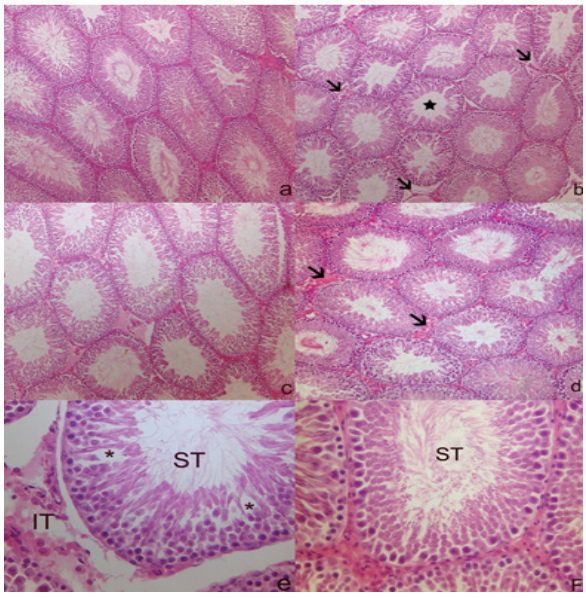

Figure 1. Seminal tubules and interstitial tissue of Wistar rats testis. a) Sedentary animals (SCo). Well organized seminiferous tubules and intersticial tissue were observed. b) Sedentary + CP (200 mg kg^{-1} cyclophosphamide) animals (SCP). Delayed spermatogenesis was observed in some tubules (star) and edema areas were present in interstitial tissue (arrows). c) Trained control animals (TCo). Seminal tubules structures were preserved. d) Trained + CP animals (TCP). Presence of some areas of interstitial edema (arrows) and well organized seminal tubules. Hematoxylin-eosin staining, 100X. e) Seminal tubules (ST) of SCP animal demonstrated areas of disorganization in germinative epithelium (⋆) and edema in interstitial tusse (IT). F) Seminal tubules (ST) of TCP animal showing a better organization of germinative epithelium. Hematoxylin-eosin, 400X.

Histological analysis of seminiferous tubules is shown in Figure 2. It was observed a better organized germinative epithelium and several tubules containing luminal sperms in SCO, TCo and TCF. SCF group presented edema areas among germinal cells, delayed cell line maturation and areas of degenerated interstitial tissue.

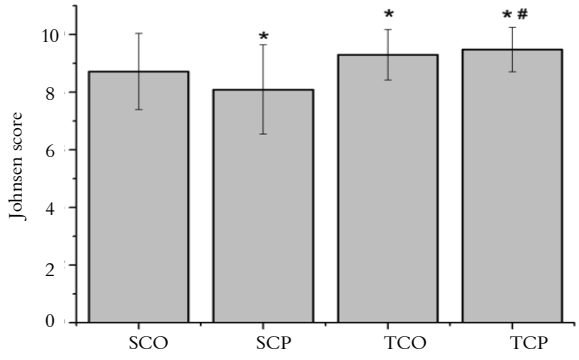

Figure 2. Johnsen score of Wistar rats testis. It was observed significant decrease in Johnsen scores in sedentary + CP animals (SCP) in relation to control (SCO, ⋆p < 0.05) and trained + CP animals (TCP, #p < 0.05). Trained control (TCO) and TCP animals presented increased Johnsen scores in relation to SCo group (⋆p < 0.05). ANOVA and *post hoc* Tukey's test.

There were no significantly differences between SCO and SCP groups, and TCO and TCP groups in relation to Sertoli cells per seminiferous tubules. There were increased cell counts in TCO group in relation to SCO animals, and in TCP in relation to SCP animals (Figure 3).

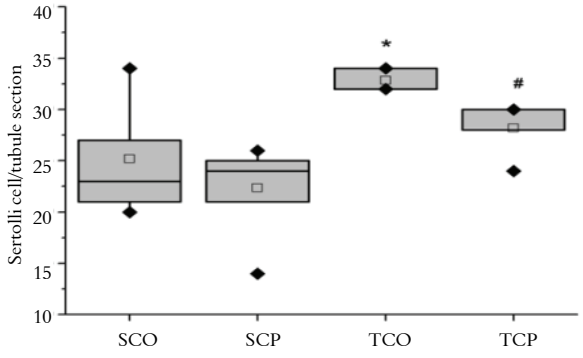

Figure 3. Number of Leydig cells per mm^2 of interstitial tissue of Wistar rats testis. Increased number of Leydig cells were observed in trained control (TCO) group in relation to sedentary control (SCO; ⋆p < 0.05, Dunn's test) and trained + CP (TCP; $^{\&}$p < 0.05, Dunn's test) animals. The box represents 25 to 75 per cent of the values, the horizontal bar represents the median, (□) represents the mean, vertical bars are 1 to 99 per cent of the values and (♦) the extreme values.

The Leydig's cells were counted in intersticial areas and expressed by the mean number of cells per mm^2. It was observed increased number of Leydig cells in TCO group in relation to SCO and TCP (Figure 4).

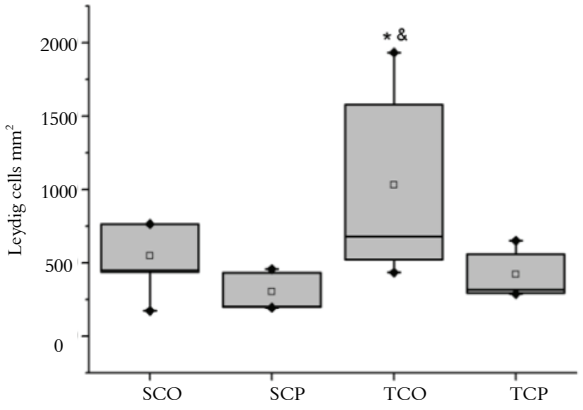

Figure 4. Number of Sertoli cells per seminiferous tubule of Wistar rats. Increased number of Sertoli cells were observed in seminifeous tubules of trained control (TCO; ⋆p < 0.05, Dunn's test) group in relation to sedentary control (SCO) group, and trained + CP (TCP; #p < 0.05, Dunn's test) in ration to sedentary + CP (SCP) animals. The box represents 25 to 75 per cent of the values, the horizontal bar represents the median, (□) represents the mean, the vertical bars are 1 to 99 per cent of the values and (♦) the extreme values.

Discussion

The main findings of this work are that moderate chronic exercise could improve spermatogenis in trained animals and partially protect testis from CP

induced damage. The effect of exercise seems to be more prone in protecting seminiferous tubules (spermatogenic cell lineage and Sertoli cells) than Leydig cells.

Despite its wide spectrum of clinical uses, CP has several adverse effects including reproductive toxicity in humans and experimental animals. CP itself is devoid of alkylating activity and must first undergo bioactivation by hepatic microsomal cytochrome P450 mixed function oxidase system (COLVIN, 1999; PASS et al., 2005; XIE et al., 2005). The main alkylating metabolite, phosphoramide mustard, is responsible for the therapeutic activity. However, another metabolite, acrolein, causes the inactivation of microsomal enzymes and results in increased ROS generation and lipid peroxidation in several tissues (COLVIN, 1999). The bioactivation of CP in hepatocytes targets liver to its primary cytotoxic attack, resulting in CP-induced hepatoxicity and decreased liver weight (TRIPATHI; JENA, 2010). However, we did not find significantly difference in liver weight of trained animals, suggesting that training may have some effect on CP induced injury. This same finding was reported by others authors who employed exercise protocols to demonstrate its protective effect against oxidant agents and aging, and improvement in DNA repair, in liver of exercised animals (DA SILVA et al., 2009; NAKAMOTO et al., 2007).

Leydig cells, Sertoli cells and germinative cells were susceptible to oxidative stress and inflammatory damage (ALY et al., 2010; CERIBASI et al., 2010; CHIGURUPATI et al., 2008; ELANGOVAN et al., 2006). Experimental studies demonstrated impairment of maturation of germinal epithelium, highlighted by atrophy of seminiferous tubules and amorphous Sertoli cells, interstitial edema and decreased number of Leydig cells (ELANGOVAN et al., 2006; REZVANFAR et al., 2008; MOTAWI et al., 2010). Reduced weight of mice and rat testis were reported as the consequences of testicular damage and dysfunction (CARMELY et al., 2009; ELANGOVAN et al., 2006; ILBEY et al., 2009; SELVAKUMAR et al., 2004). However, we did not observe weight differences among studied groups. This finding may be related to differences in animal age, dose of CP, chronic or acute administration, and day post-treatment when specimens were collected in the present study. Our study is in agreement with Motawi et al. (2010) who did not observed difference in testis weights in animals treated with a single dose of 200 mg kg^{-1} of CP, although histological analyses revealed a significant damage of seminiferous tubules and interstitial tissues.

CP-induced toxicity is observed in rat testis as decresead Johnsen score, evidencing low numbers of type B spermatogonias, spermatids and spermatocytes and reduced sperm (CARMELY et al., 2009; SATOH et al., 2002). We observed decreased Johnsen score suggesting that it was a slight toxicity in CP treated testis of sedentary animals. This finding is accordingly to Ilbey et al. (2009) who reported decreased Johnsen score in rats treated with a single dose of CP. However, trained animals presented best scores, even when they were treated with CP. Chigurupati et al. (2008) reported that mice engaged in voluntary wheel-running presented increased number of spermatogonia and spermatids in different stages of maturation in seminiferous tubules, in relation to sedentary group. This finding suggests that exercise stimulates germinal cells maturation and may have protective effects against CP induced damage.

The CP-induced citotoxicity on Sertoli cells are characterized by decreased number, morphological changes due to disruption of cytoskeleton, vacuolization and apoptosis (LIU et al., 2011; REZVANFAR et al., 2008; SAKR et al., 2011). We did not observed significant either reduction in number or aberrant morphology of Sertoli cells, probably due to acute protocol employed in the study that did not exposed these cells to CP chronic effects. However, the results suggest that the Sertoli cells counts were increased in exercised animals. This find were observed in exercised mice (CHIGURUPATI et al., 2008). The Sertoli cells were important in several key steps of spermatogenic lineage maturation and their stability during the free radical attack may help maintain seminiferous tubule integrity in TCP animals.

We observed reduced number of Leydig cells in CP treated animals. The Leydig cells seemed to be more sensitive to CP and were severely decreased in CP treated animals leading to hormonal dysfunction (CERIBASI et al., 2010; REZVANFAR et al., 2008). In the present study, exercise increased Leydig cell numbers, but it was not able to avoid cell loss after a single dose of CP. The reduction in Leydig cells counts may explain why SCP treated animals presented decreased seminal vesicle content since its function is hormone dependent. However, it was not found decreased seminal contend in TCF animals, indicating a potential effect of exercise on seminal vesicle activity. Exercise could release lactate into serum when animals where submitted to moderate training (RAVI KIRAN et al., 2004) and lactate can independently enhance testosterone

release from Leydig cells *in vitro* and during exercise (LIN et al., 2001; LU et al., 1997). Lactate production may decrease the impact of Leydig cell depletion, stimulating activity of reminiscent cells, and maintaining hormonal stimulus to seminal vesicles. Moreover, other authors have demonstrated that swimming trained rats presented better adaptation of mitochondrial oxydoreductive enzymes in Leydig cells, improving testis function and testosterone production (HU et al., 2004). Exercise training increased seminal vesicle contend in TCo animals in relation to sedentary controls. It is in agreement with previous reports (CHATURAPANICH et al., 2011), and it may also compensate for decreased vesicle weight found in SCP group.

We could not observe significant weight changes on epidydimis neither in CP treated animals nor in exercise animals, although these findings were reported by others authors (ELANGOVAN et al., 2006; CARMELY et al., 2009; CHATURAPANICH et al., 2011; REZVANFAR et al., 2008). Protocols treatment reveals epidydimis changes only when CP in chronically administered (ELANGOVAN et al., 2006; REZVANFAR et al., 2008; TRIPATHI; JENA, 2010) and acute administration could not induce fast weight changes, in a short time period, as observed in the present study.

Chronic moderate exercise has been suggested to have several health promoting properties in animals and human studies (GOMEZ-CABRERA et al., 2008). Chronic moderate exercise can induce low levels of free radicals production by mitochondria and xantine-oxidase system during muscle contraction and it has a physiological role in the adaptation to exercise. The presence of a small stimulus such as low concentrations of ROS is able to induce the expression of antioxidant enzymes and other defense mechanisms in several tissues including male reproductive tract (AKSOY et al., 2006; CHICCO et al., 2005; CHIGURUPATI et al., 2008; GOMEZ-CABRERA et al., 2008; GUNDUZ et al., 2004; KAKARLA et al., 2005). Exercise inhibited oxidative stress damage on brain, kidney, liver and heart in animal studies (CHICCO et al., 2005, 2006; GUNDUZ et al., 2004; KAKARLA et al., 2005; NAKAMOTO et al., 2007; RAVI KIRAN et al., 2004; SOMANI; HUSAIN, 1996; TEIXEIRA et al., 2008). The main effects of exercise are upregulation of manganese superoxide dismutase systems (MnSOD), catalase, glutathione peroxidase, Glutathione-S-transferase expression in testis (AKSOY et al., 2006; CHIGURUPATI et al., 2008). Exercise can improve antioxidant systems and decrease oxidative damage induced by ethanol in rat testis (HUSAIN; SOMANI, 1998). These results suggest that protective effect of exercise against free-radical attack evoked by CP metabolites may be related to adaptations in testicular antioxidant system.

Another exercise-induced tissue protection mechanism may be related to its anti-inflammatory properties. Systemic and local inflammation could impairs spermatogenesis, and affect Leydig and Sertoli cells functions (LIEW et al., 2007; HEDGER, 2011; REDDY et al., 2006). Exercise could decrease expression of inflammatory cytokines attenuating the inflammatory process and oxidative stress, preventing tissue injury and improving tissue repair (FUNK et al., 2011; HOFFMAN-GOETZ et al., 2010; SERRA et al., 2010).

Conclusion

We conclude that chronic exercise may protect male reproductive tract from toxic effects of CP, decreasing citotoxicity scores on spermatogenic cells and increasing Sertoli cell counts. Leydig cells are susceptible to drug injury even in exercised animals. The study suggests that exercise has beneficial effects on male reproductive tract exposed to CP.

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