Protective effects of abdominal electroacupuncture on oxidative stress and inflammation due to testis torsion/detorsion in rats[1]

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ABSTRACT

PURPOSE: To evaluate the effects of acupuncture (Ac) and electroacupuncture (EAc) on oxidative stress and inflammation in testis torsion/detorsion (T/D) model in rats.

METHODS: Thirty male Wistar rats were randomized into five groups. G1 Group (Sham) served as control. The remaining groups were submitted to spermatic cord torsion (720°) for 3 hours, followed by detorsion and reperfusion for 4 hours. Before detorsion G3, G4 and G5 rats were treated with Ac, EAc 2Hz and EAc 10 Hz, respectively, applied to acupoint Gulai (S-29) bilaterally under anesthesia for 5 minutes. Next, the testes were detorsioned and reperfused for 4 hours. Afterwards, blood samples and the right testis were collected for biochemical assays: reduced Glutathione (GSH), Malonaldehyde (MDA), Myeloperoxidase (MPO).

RESULTS: EAc stimulation (2 and 10 Hz) promoted significant increase in concentrations of GSH in plasma and testis of G4-G5 rats, compared with G1. There was significant increase of tissue MDA in groups G4-G5 and plasma MDA in all groups, compared with G1. There was a significant reduction in MPO activity in groups G4-G5 compared with G1.

CONCLUSION: Electroacupuncture stimulation (2 and 10 Hz) attenuates oxidative stress and inflammatory response in rats subjected to testicular torsion/detorsion.

Key words: Spermatic Cord Torsion. Ischemia. Reperfusion Injury. Electroacupuncture. Rats.

Introduction

Testicular torsion is a frequent urologic emergency that affects infants, children and adolescents requiring the prompt action of the surgeon to prevent testicular damage[1]. The resulting ischemia leads to an increase of free radicals. Upon detorsion, with return of blood flow, these free radicals are released, enhancing additional injuries to the testis[2]. The testes of mammals and especially the germ cells, may suffer serious injuries, often irreversible, when exposed to the action of free radicals produced in the body, in the course of life processes or in situations of stress[3]. These free radicals are neutralized by radical scavengers such as glutathione peroxidase, glutathione reductase, superoxide dismutase and by catalase. Oxidative stress occurs when the balance between pro-oxidant agents and antioxidant defenses is not attained[4].

Acupuncture (Ac) is an ancient therapeutic method that has been used for centuries in the treatment and prevention of many diseases. It has been demonstrated that one cycle of acupuncture treatment (twice a week for five weeks) may improve parameters of males suffering from subfertility related to low sperm activity[5]. A more recent controlled study reinforced the findings reported previously by the same authors[6].

Electroacupuncture (EAc) is a modality of treatment that utilizes previously inserted needles in specific points connected to a generator of electric current, capable of producing electrical stimuli with intensities ranging between 0.5 and 50 mA and voltage up to 20 V. Stener-Victorin *et al*.[7] studied the effects of the EAc on the ovarian blood flow in rats. Burst (trains of pulses) 10 mA electrical stimulation was applied during 30 seconds to needles inserted in the abdominal muscles at the level of the 12th rib, 2 cm from the midline. The researchers reported positive effects of electrical stimulation on ovarian blood flow[7]. Cakmak *et al*.[8] studied the effect of abdominal acupuncture on blood flow of the testicular artery in humans and concluded that the simple needle insertion in acupoint Gulai (S-29), located approximately 8 cm below the umbilicus and 4.4 cm lateral to the midline of the abdomen or the application of EAc (2 Hz, burst-type trains of pulses) induced no significant changes in blood flow. However when the stimulation frequency was augmented to 10 Hz a significant increase in the blood flow of the spermatic artery was obtained[8].

Published studies have demonstrated the effects of Ac in attenuating oxidative stress in experimental spinal cord injury[9] and in rats in menopause[10]. Whereas previous studies have proved the attenuating effects of the EAc on the oxidative stress on liver[11] and kidneys[11], skin[12], and in the ovaries[3], it is expected that protection also may occur in rats submitted to testis torsion/detorsion. No similar studies were found in the literature, hence the importance of this investigation.

Methods

Approval for experimental use of laboratory animals was obtained from the local Ethics Committee on Animal Use (CEPA), protocol #18/2012, May 2012. All surgical procedures and animal handling were conducted in accordance with the Brazilian Federal Law No. 11794 of October 8, 2008 (http://www.planalto.gov.br/ccivil_03/_Ato2007-2010/2008/Lei/L11794.htm). The study was designed so as to minimize the number of animals required for the experiments. All animals were housed in polypropylene cages at ambient temperature of 24ºC on a 12h light-dark cycle. Rats were allowed free access to food (Purina chow) and fasted 12h before the experimental procedure. Tap water was offered *ad libitum* until the beginning of the experiment.

Materials

Needles (sterilized stainless steel, 0.25 mm in diameter, 1.5 cm long) were purchased from Xu Li Comércio, Importação e Exportação Ltda, São Caetano do Sul – SP, Brazil. EL-608 portable electronic stimulator was purchased from NKL Produtos Eletrônicos Ltda., Brusque, Santa Catarina, Brazil.

Design of the experiment

Thirty male Wistar rats were randomized into five groups. G1 Group (Sham) served as control. G2 group was submitted to torsion of the testis (720º) for 3h. After detorsion the testis was allowed to reperfuse for 4 hours before removal. G3Ac, G4EAc2 and G5EAc10 were anesthetized and submitted to torsion of the testicle (720º) during 3h, followed by the application of the Ac (G3Ac) or EAc 2Hz or 10 Hz (G4EAc2, G5EAc10) for five minutes. The needles were inserted in the S-29 (Gulai) acupoint, bilaterally. Next, the testes were detorsioned and reperfused for 4h. At the end of the experiment blood samples and the right testis were collected for biochemical assays of reduced Glutathione (GSH), Malonaldehyde (MDA) and Myeloperoxidase (MPO). Figure 1 depicts groups and treatments.

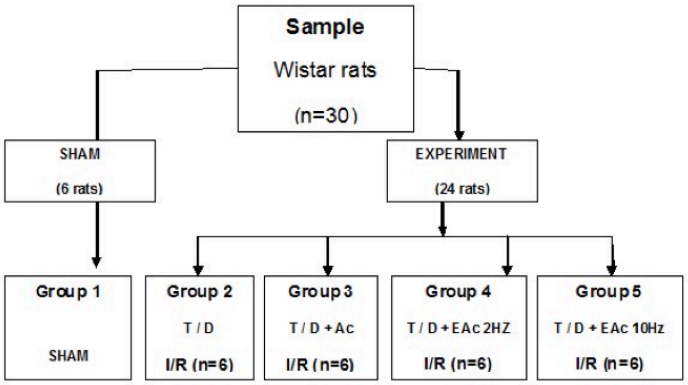

FIGURE 1 - Distribution of the study groups, specifying the treatments applied.

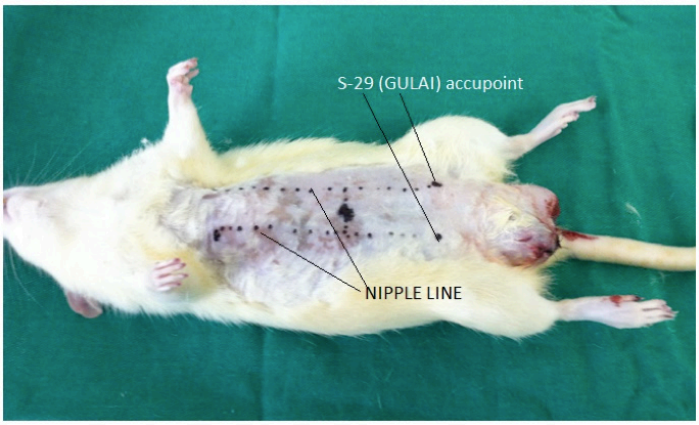

FIGURE 2 - Photo (5 rat #5, Group 2) identifying the reference points (nipple line) and the position of acupoints GULAI on the abdomen of the Wistar rat.

Surgical procedure

Rats (G2-G5) were anesthetized with a solution of Ketamine 90 mg/Kg+Xylazine 10 mg/Kg i.p. and were kept in a supine position. The right testis was exposed through a longitudinal incision. Ischemia was obtained by torsion of the spermatic cord, two complete turns (720º), around its axis, clockwise. To avoid spontaneous detorsion, the right testicle was fixed to the fold of peritoneum which attaches the testis to the dorsal wall of the scrotal sac (mesorchium), using the technique recommended by Ryan *et al.*[14], thus avoiding the transparenquimal suture. The incision was closed and was reopened after 3h. The testis was counter-rotated to its natural position and allowed to reperfuse for 4h. At the end of the experiment, following blood samples and right testis harvesting, the rats were killed by cervical dislocation.

Application of acupuncture/eletroacupuncture

After induction of anesthesia, acupuncture needles (0.25/15mm) were inserted bilaterally, in S-29 (Gulai) acupoints at a depth sufficient to reach the muscle tissue, maintained for 5 minutes and subsequently removed (Figure 2). Time of stimulation and the selection of acupoint were based on work by Cakmak *et al.*[8]. In the rat, the acupoint S-25 is located lateral to the umbilical scar at the midpoint between the navel and the nipple line[15]. In the human, the acupoint S-29 lies on the same height of the Zhongji (CV-3) acupoint, at the midpoint between the navel and the nipple line[16]. Thus, based on the system proposed by Yin *et al.*[15], whereas the acupoint E-29 lies on the same line of S-25 and next to CV-3, one can find the acupoint described above (Figure 2).

Biochemical determinations

MDA assay

Tissue samples were snap-frozen in liquid nitrogen and stored in glass tubes at -70º until subsequent preparation and analysis of testis homogenates. Lipid peroxidation was assayed by measuring malondialdehyde as TBA-reactive substances[17]. In brief, H_3PO_4 (1%, 3 mL) and aqueous TBA solution (0.6%, 3 mL) were added to the 10% homogenate (0.5 mL). The assay medium was shaken and heated on a boiling-water bath for 45 min. After cooling, 4 mL of *n*-butanol was added and the mixture shaken. After separation of the *n*-butanol layer by centrifugation at 1200g for 15 min, its optical density was determined in a spectrophotometer (Beckman DU 640 B; Beckman Instruments, now Beckman Coulter, Inc., Fullerton, CA, USA) with 535 and 520 nm as absorption wavelengths, respectively. The difference between the results of the two optical density determinations was taken as the TBA value and the amount of malondialdehyde (MDA) in the testis was calculated, comparing with MDA standards and expressed as micromoles of MDA per gram of wet tissue.

GSH assay

GSH levels were estimated by the method of Sedlak and Lindsay[18] which is based on the reaction between thiol groups and 5-5-dithiobis-(2-nitrobenzoic acid) to produce a compound that absorbs light at 412 nm. The amount of GSH was determined from a standard curve simultaneously obtained under the same conditions with various concentrations of GSH.

MPO activity

In brief, after collection, the sample was placed in an Eppendorf tube with cold buffer (0.1 M NaCl, 0.02 M NaPO, NaEDTA 0.015 M, pH 4.7). The tissue was weighed and then homogenized in a Polytron PT 3.100 to 13.000 rpm. Hypotonic lysis was carried out in the pellet using 2 % NaCl solution; after 30s, NaCl 1.6 % and glucose 5% were added. After centrifugation, the pellet was suspended and homogenized in 0.05 M $NaPO_4$ buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide. After centrifugation at 13.000 rpm, 5 mL of supernatant diluted in 45 mL of 0.08 was used to measure MPO activity in a 96-well plate. MPO activity in the supernatant was measured using tetramethylbenzidine (TMB) and 1.6 mM H2O (0.5 mM) and read on the 96-well-plate reader at 450 nm. The final concentration of MPO was obtained by comparing the absorbance value with a standard scale of MPO, prepared in advance. The concentrations of MPO were expressed as units/mg of tissue.

Statistical analysis

Statistical analysis was performed using Graphpad Prism 5.0 (GraphPad Software, San Diego California USA, www.graphpad.com). All data were tested for distribution (Kolmorogov-Smirnov test with Dallal-Wilkinson-Lillie for *P* value), Next, one-way analysis of variance followed by Dunnett test was used. $p<0.05$ was considered to be significant.

Results

GSH assay

As shown in Figure 3, EAc stimulation using 2 and 10 Hz promoted significant increase in tissular GSH levels in the testis of G4-G5 rats, compared to G1. The use of Ac did not alter significantly the concentrations of GSH in the G3 group compared to G1. G2 and G1 were not significantly different at the end of the study.

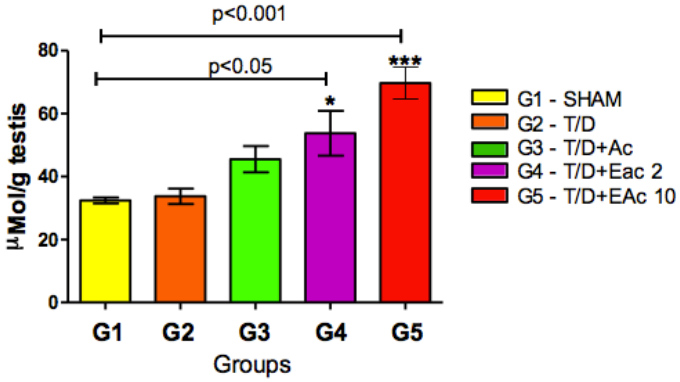

FIGURE 3 – Testis glutathione (GSH) levels at the end of the experiment. Bars represent mean±SD values for each group (G1-Sham; G2-Torsion/Detorsion; G3-Acupuncture; G4-Electroacupuncture 2Hz and G5-Electroacupuncture 10Hz). GSH expressed as microMol/ml of testis tissue. Test: ANOVA/Dunnett. ***p<0.001, *p<0.05 compared to G1.

As shown in Figure 4, EAc (2 and 10 Hz) promoted significant increase in the concentrations of plasma GSH in G4-G5 rats, compared to G1. G2 and G1plasma concentrations were not significantly different at the end of the study.

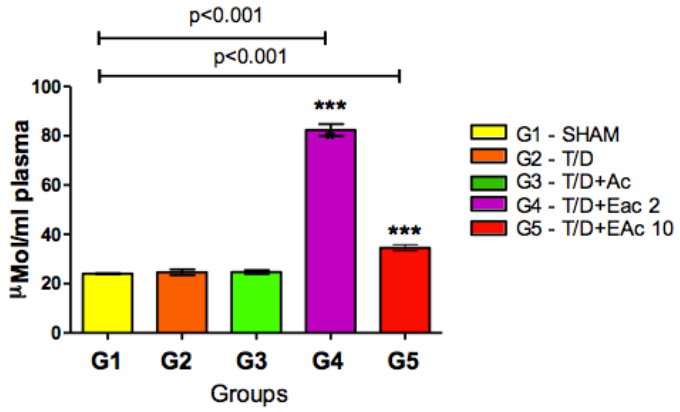

FIGURE 4 – Plasma glutathione (GSH) levels at the end of the experiment. Bars represent mean±SD values for each group (G1-Sham; G2-Torsion/Detorsion; G3-Acupuncture; G4-Electroacupuncture 2Hz and G5-Electroacupuncture 10Hz). GSH expressed as microMol/ml of plasma. Test: ANOVA/Dunnett. ***p<0.001 compared to G1.

MDA assay

There was significant increase of plasma MDA in all groups (G2-G5), compared to G1 (Figure 5).

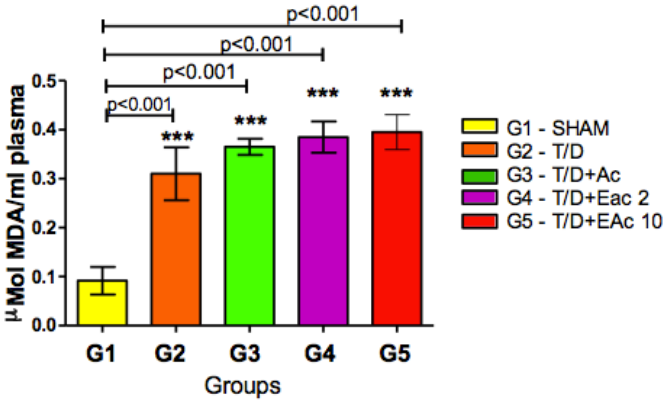

FIGURE 5 – Plasma malonaldehyde (MDA) levels at the end of the experiment. Bars represent mean±SD values for each group (G1-Sham; G2-Torsion/Detorsion; G3-Acupuncture; G4-Electroacupuncture 2Hz and G5-Electroacupuncture 10Hz). MDA expressed as microMol MDA/ml of plasma. Test: ANOVA/Dunnett. ***p<0.001 compared to G1.

As shown in Figure 6, EAc (2 and 10 Hz) promoted significant increase in the concentrations of tissue MDA in G4-G5 rats, compared to G1.

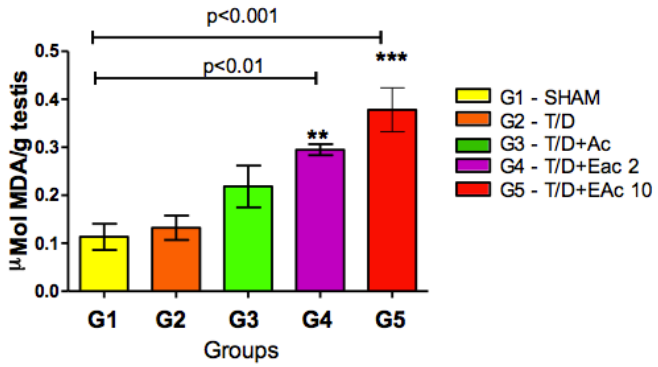

FIGURE 6 – Testis malonaldehyde (MDA) levels at the end of the experiment. Bars represent mean±SD values for each group (G1-Sham; G2-Torsion/Detorsion; G3-Acupuncture; G4-Electroacupuncture 2Hz and G5-Electroacupuncture 10Hz). MDA expressed as microMol MDA/ml of plasma. Test: ANOVA/Dunnett. ***p<0.001, **p<0.01 compared to G1.

MPO assay

EAc (2 Hz and 100 Hz) significantly decreased MPO activity in the testes of G4-G5 rats compared to G1. No significant differences were observed in the activity of MPO comparing groups G2-G3 to group G1 (Figure 7).

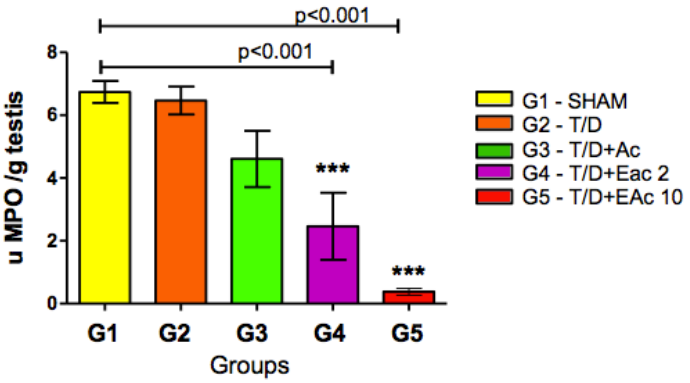

FIGURE 7 – Testis myeloperoxidase (MPO) activity levels at the end of the experiment. Bars represent mean±SD values for each group (G1-Sham; G2-Torsion/Detorsion; G3-Acupuncture; G4-Electroacupuncture 2Hz and G5-Electroacupuncture 10Hz). MPO expressed as uMPO /g testis. Test: ANOVA/Dunnett. ***p<0.001compared to G1.

Discussion

The experimental model used in this study to induce I/R by testicular torsion was described by Ryan *et al.*[14]. The time of ischemia was set at 3h. According to Akugür *et al.*[19] when ischemia duration exceeds 6 h, the reperfusion injury does not occur. This is due to the fact that periods of ischemia larger than 4-6h result in permanent tissue injury[20-21].

To evaluate the oxidative GSH and MDA concentrations were assayed in the testis and in plasma. Acupoint Gulai (E29) was selected for application of Ac/EAc, based on the study of Cakmak *et al.*[8]. We used the same electrical current intensities and frequencies for acupoint stimulation, hoping to attain the same effects observed in humans in a rat model. We hypothesized that the increased blood flow shown by those researchers after EAc application could reduce the oxidative stress and the inflammation present in the testis subjected to T/D.

In the present study, the use of EAc (2 and 10 Hz) promoted significant increase in tissue concentrations of GSH in G4 groups (53.8±1.76 vs. 32.4±3.33, p<0.05) and G5 (69.7±12.4 vs. 32.4±3.33, p<0.001). It was also observed a significant increase of plasma concentrations of GSH in rats of group 4 (G4xG1: 82.4 ± 5.95 vs. 24.1 ± 0.42, p<0.05) and group 5 (G5xG1: 34.6 ± 3.22 vs. 24.1 ± 0.42, p<0.01) compared with control G1. On other hand, Ac stimulation did not promote any significant alterations of tissue or plasma GSH levels in G3 group compared with G1.

Silva *et al.*[11] demonstrated that the EAc applied during 30 minutes in just one session, using low and high frequencies (10 and 100 Hz) induces a significant increase in plasma and tissue (liver and kidney) concentrations of GSH. The protective

effect of Ac/EAc also was demonstrated by Lima *et al*.[12] in randomized skin flaps in back of Wistar rats. Thus, both low frequencies (2 and10 Hz) in single or multiple applications may promote increased GSH concentrations in different tissues and in the plasma of rats submitted to the EAc, ensuring antioxidative protection to treated animals.

Concerning lipid peroxidation, there has been a significant increase in tissue concentrations of MDA in groups G4 (0.295±0.028 vs. 0.113±0.068, p<0.01) and G5 (0.378±0.112 0.068±0.113 vs., p<0.001) compared with G1. Increased lipid peroxidation was most marked in the plasma where there was an overall increase of MDA levels in all groups studied. These results are conflicting with a study by another researcher. Wang *et al*.[22] studied the effects of EAc in experimental model of Parkinson's disease in mice and found that the use of the EAc (100 Hz) significantly reduced brain concentrations of MDA on the 7th day of the experiment. How to explain so different findings? An explanation could be related to the size of the animals tested by Wang *et al*.[22], who used mice in the study, considering that the Wistar rat is much larger than the mouse.

With respect to inflammation the study showed that EAc (2 Hz and 100 Hz) significantly decreased the activity of MPO in the testis of rats subjected to torsion of the testicle (G4xG1:2.46±2.61 vs. 6.74±0.86, p<0.001 and G5xG1: 0.38±0.27 vs. 6.74±0.86, p<0.001), compared with G1. However there was no significant difference in the activity of MPO in rats submitted to needling (Ac).

Santos *et al*.[13] studies showed decrease of MPO activity when different types of stimulation were used (AC, EAc -2 and 100 Hz). Reducing the activity of MPO using Ac has already been demonstrated in work recently published by Da Silva *et al*.[23], using an experimental model of peritonitis in mice. Lima *et al*.[12] studied the effects of EAc in randomized skin flaps in rats and found significant reduction in MPO activity.

The analysis of the results obtained in the present study, where were clear the significant increase of GSH levels and the substantial decrease of MPO activity in rats treated with Ac or EAc, shows that this complementary therapy has protective action on the oxidative stress triggered by ischemia-reperfusion injury due to T/D of the rat testis.

Conclusion

Electroacupuncture stimulation (2 and 10 Hz) attenuates oxidative stress and inflammatory response in rats subjected to testicular torsion/detorsion.

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