Dimethylsulfoxide attenuates ischemia-reperfusion injury in rat testis[1]

Dimetilsulfóxido atenua a lesão de isquemia-reperfusão em testículo de ratos

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ABSTRACT
Purpose: To evaluate the protective role of dimethylsulfoxide (DMSO) in a rat model of testis ischemia/reperfusion (I/R). **Methods**: Twenty-four male Wistar rats were randomized in two equal groups. Control rats (G-1) received saline 2.0 ml intraperitoneally (ip) 21, 9 and 1 h before torsion. Experimental rats (G-2) received ip injections of 3% aqueous solution of DMSO, 0.1ml/10g body weight. Saline was added to complete 2.0ml when necessary. I/R injury was induced in anesthetized rats by torsion of the right testis lasting 3 hours. Testis and blood samples were collected at the end of ischemia (T-0) and 3 hours later (T-3) for assessment of testis malonaldehyde (MDA), reduced glutathione (GSH), and plasma total antioxidant power (TAP). **Results**: MDA levels decreased significantly in G-2 rats compared with G-1 animals in all time-points. GSH levels increased significantly in T-0 and T-3 time-points in DMSO pretreated rats compared with G-1 rats. GSH levels increased significantly during reperfusion in G-2 rats. TAP was similar in both groups denoting absence of systemic effects in this study. **Conclusion**: Pretreatment with DMSO reduces testis lipid peroxidation and oxidative stress caused by torsion/detorsion of the testis.
Key words: Antioxidants. Testis. Oxidative Stress. Malondialdehyde. Glutathione. Rats.

RESUMO
Objetivo: Avaliar o papel protetor do dimetilsulfóxido (DMSO) utilizando um modelo de isquemia/reperfusão (I/R) do testículo do rato. **Métodos**: Vinte e quatro ratos machos Wistar, randomizados em dois grupos iguais e tratados com solução salina 2,0 ml (grupo Controle-G-1) ou solução de DMSO (3%, 0,1ml/10g peso corporal), acrescida de solução salina até completar 2,0 ml (grupo Experimento-G-2) por via intraperitoneal, as 21, 9 e 1 h antes da torção. .A lesão de I/R foi induzida por torção do testículo direito e mantida por 3 horas, em ratos previamente anestesiados. Amostras (sangue e testículo direito) foram coletadas ao término da isquemia (T-0) e após 3 horas de reperfusão (T-3) para determinação das concentrações de malonaldeído (MDA), glutationa reduzida (GSH) e capacidade antioxidante total do plasma (CAT). **Resultados**: As concentrações de MDA diminuíram significativamente nos ratos do G-2. comparados ao G-1, nos tempos estudados. As concentrações de GSH aumentaram significativamente nos testículos dos ratos do G-2 comparado aos controles no T-0 e no T-3 e durante a reperfusão no G-2. Os valores obtidos na análise do CAT foram semelhantes. **Conclusão**: O pré-tratamento com DMSO reduz a peroxidação lipídica e o estresse oxidativo decorrentes da torção/destorção do testículo, no rato.
Descritores: Antioxidantes. Testículo. Estresse Oxidativo. Malondialdeído. Glutationa. Ratos.

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Introduction

Spermatic cord torsion is a surgical emergence that affects newborns, children and adolescents. Apparently, there are two types of testicular torsion, one occurring between prenatal and the first year of life, and the other, which shows the highest incidence, during puberty[1,2]. It has been is known that treatment by detorsion may further damage the testis. With the resumption of blood flow, large amounts of free radicals are produced. These free radicals react with lipids in the cell and mitochondrial membranes, changing their permeability and disrupting cell integrity[3]. Under normal conditions, free radicals are produced and their effects are counterbalanced by the endogenous antioxidant system. When reactive oxygen species (ROS) generation exceeds the defense mechanisms capacity to control, oxidative stress is generated and contributes to reversible or irreversible cell injury. One method of effective pharmacological therapy against reperfusion injury is by reestablishing cell membrane integrity via antioxidant therapy[4]. Antioxidants would theoretically have a dual effect in testis ischemia-reperfusion injury as they would limit the development

of damage by decreasing free radicals generated by lipid peroxidation and counteracting ROS mediated activation of inflammatory reaction[5].

Dimethylsulfoxide (DMSO) [$(CH_3)^2SO$], a by-product of the wood industry, is an amphipathic molecule with a highly polar domain and two apolar groups, making it soluble in both aqueous and organic media. Due to these physic-chemical properties, it is widely used as a solvent for hydrophobic drugs. Also, DMSO is an efficient hydrogen-bound disrupter[6]. Several antioxidants like superoxide dismutase, catalase and DMSO have proven to be efficient in attenuating the changes in microvascular permeability, which is the final outcome of ischemia/reperfusion (I/R) injury[3,4]. DMSO has been used in many studies for its scavenger and cell membrane protectant properties. De la Torre *et al.*[7] utilized a combination of DMSO and fructose 1,6-diphosphate (FDP) in rodents with persistent visuo–spatial memory (VSM) dysfunction secondary to chronic cerebrovascular ischemia and concluded that daily DMSO–FDP therapy for one week appeared to be effective in improving severe ischemic-induced memory dysfunction in aging rats. Koksal *et al.*[8] demonstrated protective effects of DMSO in a rat model of hind limb ischemia/reperfusion. More recently, Koksal *et al.*[9] found a significant decrease in plasma thiobarbituric acid reactive substances values in rats subjected to lower limb ischemia/reperfusion and treated with DMSO.

Different therapeutic strategies have been investigated with the aim of reducing testis ischemia/reperfusion damage[10-13]. The aim of the present study was to evaluate, in an experimental model of testis I/R the effects of DMSO on tissue and plasma oxidative stress in twisted testis after unilateral torsion and detorsion. To our knowledge, the effects of DMSO treatment on testicular oxidative stress in an experimental torsion model have not been studied before.

Methods

Approval for experimental use of laboratory animals was obtained from the Commission of Ethics in Animal Research, Federal University of Ceará. Throughout the study, the guidelines of the Brazilian Animal Experiments College (COBEA) were followed.

The animals were housed in groups of five in plastic cages (39 x 33 x 16 cm) at controlled temperature (22 ± 1ºC) on a 12 h light/dark cycle with constant air exchange and free access to food and water until 12h before ischemia. During the course of experiment only water was provided *ad libitum*. The weight of the animals were subjected to Kolmogorov-Smirnov statistical evaluations and considered as a normal distribution. Twenty-four young (2 month old) male Wistar rats (weight :179 ±13g) were randomly selected in two groups (n=12). G-1-Saline (Control) and G-2 (DMSO). Anesthetized rats were subjected to 3 h torsion of the testis and received intraperitoneal injections of 0.9% NaCl (G-1) or 3% aqueous solution of DMSO, 10ml/Kg body weight (G-2) 21, 9 and 1 h before torsion of the testis. DMSO solutions were adjusted for treatment to give the rats the same volume (2.0ml) by adding saline when necessary. Testis was fixed to the scrotal wall by placing a suture through the mesorchium, avoiding trans-parenchymal stitches. Right testes were harvested at the end of ischemia (T-0) and 3 h following detorsion (T-3). Tissues were snap-frozen in polypropylene tubes within 2 min after removal and stored at -70°C for later determination of malondialdehyde (MDA) and reduced glutathione (GSH) levels. Arterial blood samples were collected from the abdominal aorta, put on ice and centrifuged promptly at 4000 rpm for 10 min at 10°C. The plasma was removed and frozen until analyzed.

Chemicals

DMSO (purity grade: 99.9%) was purchased from Cromato Produtos Químicos Ltda (Diadema, SP, Brasil). Total Antioxidant Power kit (TA-01) was purchased from Oxford Biomedical Research (Oxford, MI, USA). All other chemicals were purchased from standard commercial sources and were of the highest quality available.

Biochemical analysis

Lipid peroxidation, a measure of free radical damage, was assayed by MDA as thiobarbituric acid-reactive substances (TBARS) levels using the thiobarbituric acid method[13]. 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 levels were estimated by the method of Sedlak and Lindsay[14] 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. Plasma TAP was measured with the TA-01 kit from Oxford Biomedical Research. In this procedure, the Cu_ derived from Cu^{2+} by the combined action of all antioxidants present in the sample is detected after complex formation between Cu+ and bathocuproine. The complex is stable and has an absorption maximum between 480 and 490 nm. The absorbance values obtained from the samples are compared with a standard curve obtained using uric acid as a typical reductant.

Data analysis

All results were expressed as mean±SEM. All data were tested for distribution (Kolmorogov-Smirnov test with Dallal-Wilkinson-Lilliefor P value). One-way ANOVA was performed to determine differences among groups in MDA and GSH (testis) and plasma TAP concentrations. In the post hoc analysis (Tukey), a probability value of $p < 0.05$ was considered to indicate statistical significance.

Results

No animal died throughout the course of the experiment. TBARS and GSH were assayed in testis homogenates from control and experiment groups. Total antioxidant power (TAP) was assayed in the blood plasma.

MDA Assay

The DMSO pretreatment promoted a significant reduction ($P < 0.001$) in testicular concentrations of malondialdehyde (micromoles per gram of fresh tissue) during ischemia/reperfusion compared with control group in T0 and T3 time points (Figure 1).

GSH Assay

The pretreatment with DMSO promoted a significant increase ($P < 0.001$) in GSH testes contents compared with saline pretreated rats subjected to testicular torsion/detorsion during ischemia/reperfusion injury in T0 and T3 time points. GSH levels increased significantly during reperfusion (T0 vs. T3) in DMSO treated rats (Figure 2).

TAP Assay

The reflow of blood to the ischemic testis pretreated with DMSO induced no significant difference in plasma TAP concentrations (Figure 3).

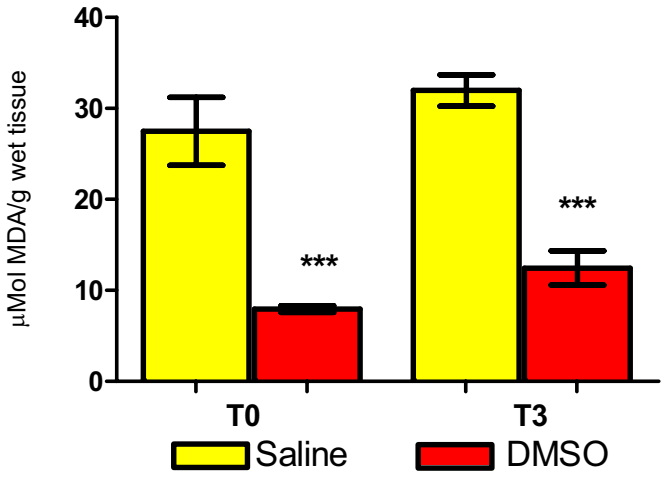

FIGURE 1 - Malondialdehyde (MDA) levels (micromoles of per gram of fresh tissue) in the testis of saline and DMSO pretreated rats. Bars represent mean ±SEM of control (yellow bars) and DMSO (red bars) groups at the end of the ischemia (T-0) and at the end of reperfusion (T-3). ***$P < 0.001$, DMSO group is significantly different from control group by ANOVA test. T-0, immediately before detorsion; T-3, 3 h after detorsion.

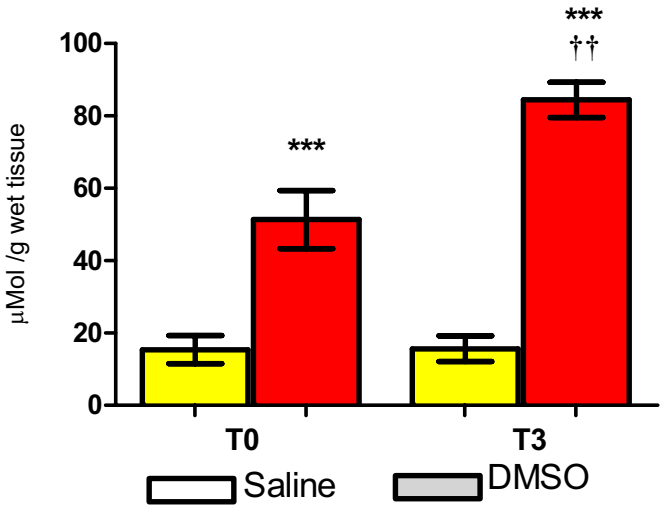

FIGURE 2 - Reduced glutathione (GSH) levels (micromoles per gram of fresh tissue) in the testis of saline and DMSO pretreated rats. Bars represent mean±SEM of control (yellow bars) and DMSO (red bars) groups at the end of the ischemia (T-0) and at the end of reperfusion (T-3) time points. ***$P < 0.001$, DMSO group is significantly different from control group by ANOVA test. ††$P < 0.001$, T-3 value is significantly different from T-0 time point by Tukey's test. T-0, immediately before detorsion; T-3, 3 h after detorsion.

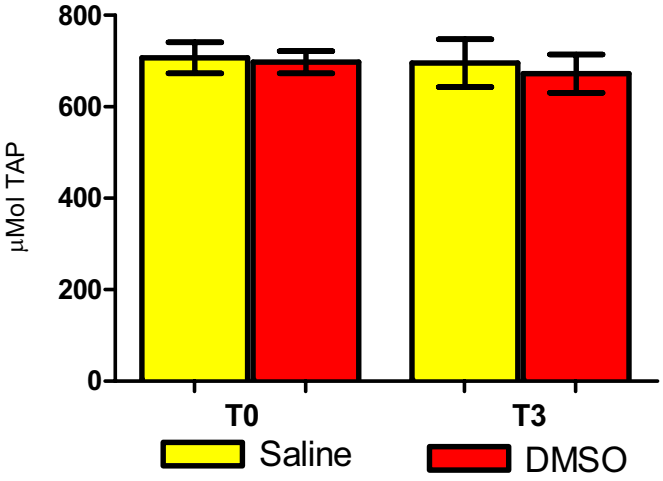

FIGURE 3 - Total antioxidant power (TAP) levels (micromoles). Bars represent mean± SEM of control (yellow bars) and DMSO (red bars) groups at the end of the ischemia (T-0) and at the end of reperfusion (T-3) time points. Values are not significantly different in DMSO-treated rats compared with controls. T-0, immediately before detorsion; T-3, 3 h after detorsion.

Discussion

The primary pathophysiologic event in testicular torsion is ischemia of the testis followed by reperfusion upon repair[15]. Thus, testicular torsion can be thought of as an ischemia/reperfusion injury to the testis. There is increasing evidence to suggest that toxic oxygen radicals play a role in the pathogenesis of ischemia/reperfusion injury in the testis. These free radicals react with lipid in cells and mitochondrial membranes forming lipid peroxides. Peroxidation of the lipid in membrane changes membrane permeability or disrupts membrane and cell integrity. Lipid peroxidation is probably the most extensively investigated process induced by free radicals[16].

The biochemical markers of oxidative stress are reported to increase just 4 hours after reperfusion in testicular torsion/detorsion model[17]. However, in our previous study[12] we have demonstrated an increase in MDA levels just 1 h after detorsion of the rat testis subjected to 3 h of torsion.

GSH (L-g-glutamyl-L-cysteinylglycine) is the predominant low molecular weight thiol in mammalian cells and is present in high concentration in adult mouse testis[18]. This tripeptide has different functions which contribute to cell defense and protection: free radical scavenger, coenzyme for several antioxidant enzymes, maintenance of the thiol-disulfide status, and detoxication of electrophilic xenobiotics via conjugation[19]. The importance of glutathione in protecting various cells against free radical injury or chemically induced damage is now well established[18].

Dimethylsulfoxide (DMSO) was used clinically as an adjuvant to treat some musculoskeletal diseases[20] but its use clinically has been banned since 1965 by the American authorities (FDA). Despite this fact many experimental studies have been carried out worldwide. Park *et al.*[21] studied the mechanism of DMSO in the prevention of acetaminophen-induced hepatotoxicity in mice treated with DMSO 1ml/Kg and conclude that it could be related to the ability to competitively inhibit acetaminophen-induced hepatotoxicity (APAP) bioactivation or to directly scavenge free radicals produced during APAP metabolism. Authier *et al.*[22] assessed DMSO neurotoxicity in rats, using increasing doses of DMSO (1.8%, 3.6% and 7.2% solutions) for each group of 7 rats. DMSO was given daily for 10 days. Clinical status of the animals was good throughout the experiment and no motor deficits were observed. Extremely high doses of DMSO (1mg/g) were used to study the effects of DMSO in inducing acute-phase response, a non-specific reaction of an organism to stress, in ten weeks old male Wistar rats[23]. In the present study the DMSO was dissolved in saline, resulting in a solution of 3%, quite below the maximum concentration used by Authier *et al.*[22] Therefore, the maximum dose of DMSO administered to a 200g rat was 6 mg.

DMSO was efficient in preventing rat striated muscle peroxidation due to ischemia, in an experimental model of rat hind limb ischemia for 4 hours followed by one-hour reperfusion. The protection offered by the DMSO was similar to N-Acetylcysteine, a known GSH precursor and a scavenger of radical oxygen species[8].

G-1 MDA levels were significantly increased in torted testis ($p<.0001$) at the end of the ischemia (T-0). No further increase of MDA levels occurred during reperfusion in control rats. Pretreatment with DMSO reduced MDA levels ($p<0.001$) in all time points compared with control rats (Figure 1). Similar results were presented by Guimarães *et al.*[12] in rats pretreated with α-lipoic acid. GSH levels decreased abruptly in torted testis of saline pretreated rats compared with DMSO-treated rats. DMSO pretreatment increased significantly GSH levels (Figure 2).

Akgur *at al.*[10] experiment elicited a significant increase in TBARS in torted testis ($p<0.01$) and further increase was caused by detorsion (reperfusion). Pretreatment with allopurinol before detorsion prevented reperfusion injury. In the present study, TBARS levels did not increase after 3 hours of reperfusion. Abes *et al.*[11] investigated the effect of ATP-Mg Cl_2) administered before and after detorsion on the prevention of reperfusion injury after unilateral testicular torsion. Testicular torsion and detorsion caused a significant increase in the TBARS levels. TBARS levels decreased to approximately normal levels following ATP-$MgCl_2$) administration before or after detorsion. Ozkan *et al.*[24] studied the effect of zinc aspartate (ZA) pretreatment in testicular torsion/detorsion. Again TBARS level decreased following ZA administration. A recent paper studied the effect of Raxofelast, a hydrophilic vitamin e-like antioxidant administered 15 min before and 15 min after torsion i.p. Significant reduction of ischemia/reperfusion cell damage was observed[5].

The torsion of the testis resulted in significant tissue oxidative stress. Increased testicular levels of TBARS along with reduction of GSH levels in T-0 are the results of the oxidative stress derived from the ischemia imposed by the torsion of the testis. The increase in GSH levels during reperfusion in DMSO-treated rats favors the hypothesis that the use of this substance in the pre-torsion period may attenuate the damaging effects of the I/R injury. The absence of significant differences in the total antioxidant capacity of the plasma suggests that the ill repercussions of the testis torsion are more important in the ischemic tissue, at least under the conditions of the present experiment (Figure 3).

Conclusion

The results of the present study support the view that DMSO can exert a protective effect against testis lipid peroxidation injury caused by ischemia/reperfusion in rats subjected to ischemia lasting three hours.

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