

## L-Alanyl-Glutamine dipeptide pretreatment attenuates ischemia–reperfusion injury in rat testis<sup>1</sup>

Pré-tratamento com o dipeptídeo L-Alanil-glutamina atenua a lesão por isquemia e reperfusão no testículo do rato

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### ABSTRACT

**PURPOSE:** To investigate the effect of alanyl-glutamine dipeptide (L-Ala-Gln) pre-treatment on ischemia-reperfusion (I/R) injury after unilateral testicular torsion-detorsion in a comparative controlled experiment.

**METHODS:** Forty-eight rats (150-200 g) randomly distributed into 4 groups (n=12), and distributed in 2 subgroups (n=6) each, were treated with saline 2.0 ml (G-1, G-3) or L-Ala-Gln 20%, 0.75g/kg dissolved in saline (total volume 2.0 ml) administered in the left saphenous vein 30 minutes before ischemia. Anesthetized rats were subjected to I/R induced by torsion (720°) of the right spermatic cord lasting 1h (G-1, G-2) or 3 hours (G-3, G4). Anesthesia was again applied at the end of ischemia time (T-0) for testis detorsion and 6 hours later (T-6) for orchietomy. All operations were performed on the right testes through transverse scrotal incisions. Right orchietomy was carried out at the end of ischemia (T-0), and 6 hours later (T-6) to evaluate the concentrations of malondialdehyde (MDA) and reduced glutathione (GSH) in the testis.

**RESULTS:** Pretreatment with L-Ala-Gln reduced MDA contents in rat testis at the end of ischemia lasting 3 hours. There was significant increase of GSH levels in T-6 time-point after 1 hour of ischemia. GSH levels also increased in T-0 and T-6 time-points in rats subjected to ischemia for 3 hours.

**CONCLUSION:** L-Ala-Gln administered before torsion/detorsion of the spermatic cord decreases lipid peroxidation during ischemia and protects the testis from oxidative stress by upregulating GSH levels during reperfusion.

**Keywords:** Oxidative Stress. Glutamine. Testis. Ischemia. Reperfusion. Rats.

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### RESUMO

**OBJETIVO:** Investigar o efeito do pré-tratamento com o dipeptídeo L-alanil-glutamina (L-Ala-Gln) sobre a lesão de isquemia e reperfusão (I/R), induzida por torção/destorção do testículo em um experimento controlado e comparativo.

**MÉTODOS:** Quarenta e oito ratos (150-200 g) divididos em quatro grupos (n=12) e distribuídos em dois subgrupos (n = 6) cada, foram tratados com 2,0 ml de solução salina (G-1, G-3) ou L-Ala-Gln 20%, 0,75g/kg dissolvida em solução salina (volume total de 2,0 ml), administrada na veia safena 30 minutos antes da isquemia. Ratos anestesiados foram submetidos à torção (720°) do cordão espermático direito durante 1h (G-1, G-2) ou 3 horas (G-3, G4) para indução da I/R. A anestesia foi reaplicada no final do tempo de isquemia (T-0) para destorção do testículo e 6 horas depois (T-6) para orquiectomia. Todas as operações foram realizadas nos testículos direitos através de incisões escrotais. Orquiectomia direita foi realizada no final de isquemia (T-0), e seis horas depois (T-6) para avaliar as concentrações de malondialdeído (MDA) e glutathiona reduzida (GSH) no testículo.

**RESULTADOS:** O pré-tratamento com L-Ala-Gln reduziu os níveis de MDA no testículo de ratos no final da isquemia (3 horas). Entretanto os níveis de GSH aumentaram significativamente no T-6 após 1 hora de isquemia e também no T-0 e T-6 em ratos submetidos à isquemia por 3 horas.

**CONCLUSÃO:** L-Ala-Gln administrada antes da torção/destorção do cordão espermático diminui a peroxidação lipídica na isquemia e protege o testículo contra o estresse oxidativo, promovendo aumento dos níveis de GSH durante a reperfusão.

**Descritores:** Estresse Oxidativo. Glutamina. Testículo. Isquemia. Reperfusão. Ratos.

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## Introduction

Testicular torsion is a urologic emergency that requires immediate surgical intervention to prevent testicular damage<sup>1</sup>. However, treatment by detorsion may further damage the testis leading to a burst of free radicals that enhance additional injuries to the testes. Therefore, testicular T/D is identified as a typical case of ischemia-reperfusion injury<sup>2,3</sup>.

Biological systems are equipped with several antioxidant mechanisms aimed at controlling the oxidative processes. Reactive oxygen species (ROS) can be scavenged in an aqueous environment by small molecules such as vitamin C, reduced glutathione (GSH) and uric acid. In a lipid environment, vitamin E scavenges ROS-derived radicals and protects membranes<sup>4</sup>. 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<sup>5</sup>.

Glutamine (GLN) is the most abundant amino acid in blood and in the intracellular free amino acid pool of most tissues and can be synthesized by most tissues<sup>6</sup>. The intense utilization of GLN by various organ systems and the marked reduction in its plasma concentration during stress conditions has led to the proposal that it should be considered a conditionally essential amino acid<sup>7</sup>. Alanyl-glutamine (Ala-Gln) is a highly stable dipeptide, can be heat-sterilized and when infused intravenously is promptly hydrolyzed to glutamine and alanine<sup>8</sup>.

To our knowledge no study has investigated the role of pre-treatment with Ala-Gln in testicular T/D injury. Thus, the aim of our experimental study was to evaluate the assumed protective effect of Ala-Gln pretreatment on the ipsilateral testis submitted to T/D.

## Methods

Approval for experimental use of laboratory animals was obtained from the local Ethics Committee on Animal Use (CEUA, former CEPA) (protocol #07/2006). 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](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 24°C on a 12 h 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.

Forty-eight rats prepubertal (55-65 days old) male Wistar (150-200 g) randomly divided into 4 groups (n=12), and distributed into 2 subgroups (n=6) each, were treated with saline 2.0 ml (G-1, G-3) or L-Ala-Gln 20%, 0.75g/kg dissolved in saline (total volume 2.0 ml) administered in the left saphenous vein 30 minutes before torsion of the testis. Surgery was conducted under intraperitoneal ketamine 90mg+xyllasine 10mg/Kg anesthesia. Anesthesia was

again applied at the end of ischemia time (T-0) for testis detorsion and 6 hours later (T-6) for orchietomy. Anesthetized rats were subjected to I/R induced by torsion (720°) of the right spermatic cord lasting 1h (G-1, G-2) or 3 hours (G-3, G4). All operations were performed on the right testes through transverse scrotal incisions. Right testes were collected at the end of ischemia (T-0), and 6 hours later (T-6) to evaluate the concentrations of malondialdehyde (MDA) and reduced glutathione (GSH) in the testis.

Tissues were snap-frozen in polypropylene tubes within 2 min after removal and stored at -70°C for later determination of lipid peroxidation and reduced glutathione (GSH) levels. At the end of experiments the animals were sacrificed by an overdose of anesthetics.

### Chemicals and drugs

Ala-Gln (Dipeptiven™) was purchased from Frenesius Kabi Áustria GmbH Graz / Áustria. All other chemicals were purchased from standard commercial sources and were of the highest quality available.

### Biochemical determinations

Lipid peroxidation, a measure of free radical damage, was assayed by measuring malondialdehyde (MDA) as thiobarbituric acid-reactive substances (TBARS) levels using the thiobarbituric acid method<sup>9</sup>. In brief, H<sub>3</sub>PO<sub>4</sub> (1%, 3ml) 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 1200 g for 15 min its optical density was determined in a spectrophotometer (Beckman DU 640 B) with 535 and 520nm 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 µmol MDA per gram of wet tissue. GSH levels were estimated by the method of Sedlak and Lindsay<sup>10</sup> which is based on the reaction between thiol groups and 5-5'-dithiobis-(2-nitrobenzoic acid) to give 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.

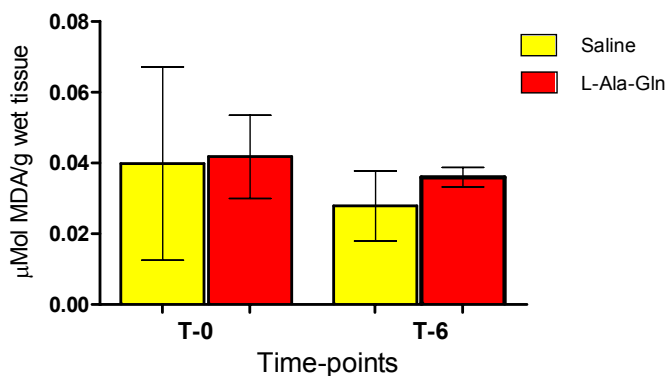
### Statistical analysis

Graphpad Prism 5.0 (GraphPad Software, San Diego California USA, [www.graphpad.com](http://www.graphpad.com)) was used for computation and statistical analysis. All results were expressed as means±SD. All data were tested for distribution (Kolmogorov-Smirnov test with Dallal-Wilkinson-Lilliefors *P* value). Unpaired t-test or Mann Whitney test where indicated were used for comparisons between control and experiment groups. *P* values of less than 0.05 were considered significant.

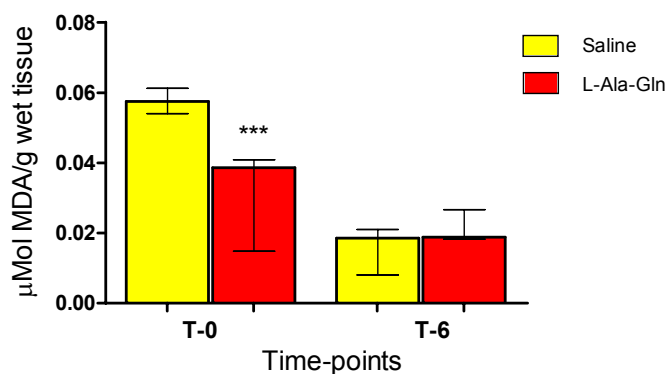
## Results

### MDA assay

There were no significant changes in MDA concentrations in the testis of rats subjected to ischemia (1 hour) followed by reperfusion (6 hours) and treated with saline or L-Ala-Gln in any of the time-points studied (Figure 1). MDA levels were significantly different ( $P < 0.01$ ) during reperfusion in T-6 compared with T-1 in control, in rats submitted to ischemia for 3 hours (Figure 2).



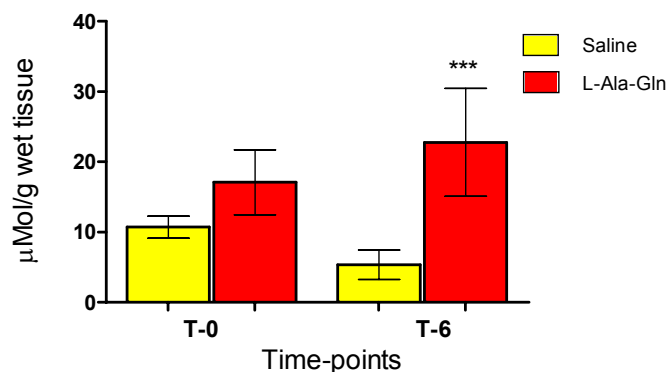
**FIGURE 1** - Thiobarbituric acid-reactive substances levels (micromoles of Malondialdehyde per gram of fresh tissue) in the testis of saline and Ala-Gln pretreated rats (Ischemia 1 h/Reperfusion 6 hours). Bars represent mean±SD of control (yellow bars) and Ala-Gln (red bars) groups at the end of the ischemia (1 hour, T-0) and at the end of reperfusion (6h, T-6). L-Ala-Gln group is not different from control group by Mann-Whitney test.



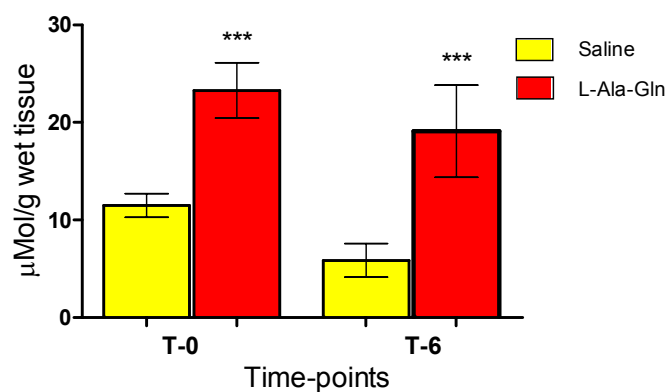
**FIGURE 2** - Thiobarbituric acid-reactive substances levels (micromoles of Malondialdehyde per gram of fresh tissue) in the testis of saline and Ala-Gln pretreated rats (Ischemia 3 h/Reperfusion 6 hours). Bars represent median with interquartile range of control (yellow bars) and Ala-Gln (red bars) groups at the end of the ischemia (3 hour, T-0) and at the end of reperfusion (6h, T-6). MDA levels decreased significantly ( $**P < 0.011$ ) in T-0 in L-Ala-Gln treated rats. Values were not different at the end of reperfusion (T-6) by unpaired t-test.

### Reduced glutathione assay

The pretreatment with L-Ala-Gln promoted a significant increase in testis GSH contents in T-6 ( $P < 0.001$ ) time-point compared with saline pretreated rats submitted to testicular 1-hour ischemia followed by 3 hours reperfusion (Figure 3). After 3 hours of ischemia, increased levels of GSH occurred in both time-points (T-0 and T-6) (Figure 4).



**FIGURE 3** - Reduced glutathione levels (micromoles per gram of fresh tissue) in the testis of saline and Ala-Gln pretreated rats (Ischemia 1 h/Reperfusion 6 hours). Bars represent mean±SD of control (yellow bars) and Ala-Gln (red bars) groups at the end of the ischemia (1 hour, T-0) and at the end of reperfusion (6h, T-6). Ala-Gln group is significantly different from control group by unpaired t-test.  $***P < 0.001$ , compared with control.



**FIGURE 4** - Reduced glutathione levels (micromoles per gram of fresh tissue) in the testis of saline and Ala-Gln pretreated rats (Ischemia 3 h/Reperfusion 6 hours). Bars represent mean±SD of control (yellow bars) and Ala-Gln (red bars) groups at the end of the ischemia (1 hour, T-0) and at the end of reperfusion (6h, T-6). GSH levels decreased significantly ( $**P < 0.01$ ) in T-6 compared with T-0 in control rats. Ala-Gln group is significantly different from control group by unpaired t-test.  $***P < 0.001$ , compared with control.

## Discussion

The susceptibility of mammalian testis to ischemic damage is well documented<sup>11</sup>. It is believed that testis sensibility to hypoxia may be related to its terminal blood supply and the presence of an inelastic shell (tunica albuginea) that would limit its compensatory expansion in the course of the traumatic edema<sup>12</sup>.

Spermatic cord torsion represents a form of I/R injury. During torsion spermatic blood flow is gradually obstructed leading to ischemia of the gonad. Surgical correction by detorsion releases blood flow to the testis. During I-R injury the fresh supply of oxygen to ischemic cells induces activated xanthine oxidase releases of superoxide anion and toxic oxygen metabolites in endothelial cells resulting in additional cell damage<sup>13</sup>.

The identification of therapeutic applications to rescue the testis from I/R injury and preserving it from other forms of oxidative stress are potentially useful. To date, a number of chemicals and drugs, such as oxygen radical scavengers, have been successfully used to reduce I/R injuries in animal models of testicular torsion, but few of these substances are currently in clinical use<sup>14</sup>. Karakaya *et al.*<sup>15</sup> evaluated the effects of Rosuvastatin, a synthetic statin, in rats torsted testes (2 hours ischemia / reperfusion 1 hour) and concluded that Rosuvastatin can protect tissue perfusion in the experimental testicular torsion model. Erol *et al.*<sup>16</sup> investigated the effect of intraperitoneal vardenafil (1 mg/kg) administration during an ischemic period in a rat model of testicular torsion/detorsion (1 hour ischemia/4 hours reperfusion) and concluded that vardenafil reduced MDA levels and decreased ischemia/reperfusion cellular damage.

The production of the oxygen-free radicals and their effect on polyunsaturated fatty acids located in cell membrane are the well-known damaging effects of I/R. Increase in TBARS expressed as MDA levels has been identified as a reliable marker of lipid peroxidation<sup>17</sup>. Guimaraes *et al.*<sup>3</sup> studied the protective effects of lipoic acid (LA) in a testis I/R rat model (3 hours ischemia, 6 hours reperfusion) and concluded that LA pretreatment using multiple doses significantly decreases MDA levels up to 6 hours following detorsion.

In this study the Ala-Gln pretreatment did not reduce MDA levels in the testis of rats subjected to ischemia (1 hour) followed by reperfusion (2 hours). We believe that a lengthened reperfusion time could alter MDA levels. In fact, 1- to 2-hours torsion in rats causes minimal damage to the testicular tissue. Three- to 6-hour periods of torsion are long and cause severe I/R injury in TT<sup>18</sup>. Wilhelm Filho *et al.*<sup>19</sup> found a significant increase (300-400%) of TBARS in the torsion testicle after 1 hour ischemia and 24 hours reperfusion, compared with the control group. When reperfusion time was reduced to 1 hour no significant alterations were observed. Wei *et al.*<sup>20</sup> demonstrated that curcumin, a potent antioxidant, reduces MDA levels in a rat model of ischemia (2 hours)/reperfusion (4 hours). In our study Ala-Gln pretreatment promoted no change in testis MDA concentrations after 6 hours of reperfusion in rats subjected to 3 hours of ischemia. However, there was a significant reduction of testicular MDA concentrations ( $P < 0.001$ ) in rats treated with GLN at the end of ischemia (T-0).

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<sup>21</sup>. 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<sup>22</sup>. The importance of glutathione in protecting various cells against free radical injury or chemically induced damage is now well established<sup>22,23</sup>.

During reperfusion after ischemia, the testicular tissue can counteract oxidative stress by upregulating antioxidant defenses with antioxidant enzymes<sup>24</sup>. Increasing concentrations of GSH in rats subjected to 1 hour or 3 hours of ischemia (Figures 3 and 4), as verified in our study, clearly demonstrate the protective effect of glutamine in testicular torsion. Hence, the present study demonstrates that unilateral testicular T/D causes testicular damage in ipsilateral testis. Ala-Gln pretreatment reduced testis damage during reperfusion in this animal model.

## Conclusion

The L-Ala-Gln administered before torsion/detorsion of the spermatic cord decreases lipid peroxidation during ischemia and protects the testis from oxidative stress by upregulating GSH levels in during reperfusion.

## References

- Burgher SW. Acute scrotal pain. *Emerg Med Clin North Am.* 1998;16(4):781-809.
- Blank ML, O'Neill PJ, Steigman CK, Cobb LM, Wilde RA, Havenstein PJ, Chaudry IH. Reperfusion injury following testicular torsion and detorsion in prepubertal rats. *Urol Res.* 1993;21(6):389-93.
- Guimaraes SB, Santos JMV, Aragão AA, Kimura OS, Barbosa PHU, Vasconcelos, PRL. Protective effect of alpha-lipoic acid in experimental spermatic cord torsion. *Nutrition.* 2007;23(1):76-80.
- Biewenga GP, Haenen GRMM, Bast A. The pharmacology of the antioxidant lipoic acid. *Gen Pharmacol.* 1997;29(3):315-31.
- Romeo C, Antonuccio P, Esposito M, Marini H, Impellizzeri P, Turicao N, Altavilla D, Bitto A, Zuccarello B, Squadrito F. Raxofelast, a hydrophilic vitamin E-like antioxidant, reduces testicular ischemia-reperfusion injury. *Urol Res.* 2004;32(5):367-71.
- Perriello G, Jorde R, Nurjhan N, Stumvoll M, Dailey G, Jenssen T, Bier DM, Gerich JE. Estimation of glucose-alanine-lactate-glutamine cycles in postabsorptive humans: role of skeletal muscle. *Am J Physiol.* 1995;269(3 Pt 1):E443-50.
- Darmaun D, D'Amore D, Haymond MW. Determination of glutamine and alpha-ketoglutarate concentration and specific activity in plasma using high-performance liquid chromatography. *J Chromatogr.* 1993;620(1):33-8.
- Albers S, Wernerman J, Stehle P, Vinnars E, Fürst P. Availability of amino acid supplied by constant intravenous infusion of synthetic dipeptide in healthy man. *Clin Sci.* 1989;76(6):643-8.
- Uchiyama M, Mihara M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Anal Biochem.* 1978;86(1):271-8.
- Sedlak J, Lindsay RH. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal Biochem.* 1968;25(1):192-205.

11. Ahmed FA, Whelan J, Jequier AM, Cummins JM. Torsion-induced injury in rat testes does not affect mitochondrial respiration or the accumulation of mitochondrial mutations. *Int J Androl.* 2000;23(6):347-56.
12. Regadera J, Nistal M, Paniagua R. Testis, epididymis, and spermatic cord in elderly men. Correlation of angiographic and histologic studies with systemic arteriosclerosis. *Arch Pathol Lab Med.* 1985;109(7):663-7.
13. McCord JM. Oxygen-derived free radicals in postischemic tissue injury. *N Engl J Med.* 1985;312(3):159-63.
14. Bozlu M, Acar D, Cayan S, Aktas S, Tunckiran A. Protective effect of trapidil on long-term histologic damage in a rat model of testicular ischemia-reperfusion injury. *World J Urol.* 2009;27(1):117-22.
15. Karakaya E, Ates O, Akgür FM, Olguner M. Rosuvastatin protects tissue perfusion in the experimental testicular torsion model. *Int Urol Nephrol.* 2010;42(2):357-60.
16. Erol B, Tokgoz H, Hanci V, Bektas S, Akduman B, Yencilek F, Mungan G, Mungan A. Vardenafil reduces testicular damage following ischemia/reperfusion injury in rats. *Med Sci.* 2009;25(7):374-80.
17. Akgür FM, Kilinç K, Aktug T, Olguner M. The effect of allopurinol pretreatment before detorting testicular torsion. *J Urol.* 1994;151(6):1715-7.
18. Blank ML, O'Neill PJ, Steigman CK, Cobb LM, Wilde RA, Havenstein PJ, Chaudry IH. Reperfusion injury following testicular torsion and detorsion in prepubertal rats. *Urol Res.* 1993;21(6):389-93.
19. Wilhelm Filho D, Torres MA, Bordin ALB, Crezcynski-Pasa TC, Boveris A. Spermatic cord torsion, reactive oxygen and nitrogen species and ischemia-reperfusion injury. *Mol Aspects Med.* 2004;25(1-2):199-210.
20. Wei SM, Yan ZZ, Zhou J. Curcumin attenuates ischemia-reperfusion injury in rat testis. *Fertil Steril.* 2009;91(1):271-7.
21. Grosshans K, Calvin HI. Estimation of glutathione in purified populations of mouse testis germ cells. *Biol Reprod.* 1985;33(5):1197-205.
22. Meister A, Anderson ME. Glutathione. *Annu Rev Biochem.* 1983;52:711-60.
23. Reed DJ. Glutathione: toxicological implications. *Annu Rev Pharmacol Toxicol.* 1990;30:603-31.
24. Sarica K, Bakir K, Yagci F, Erbagci A, Topcu O, Uysal O. Unilateral testicular torsion: protective effect of verapamil on contralateral testicular histology. *Urol Int.* 1999;62(3):159-63.

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Conflict of interest: none

Financial source: CNPq

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<sup>1</sup>Research performed at Experimental Surgical Research Laboratory (LABCEX), Department of Surgery, Postgraduate Program, Federal University of Ceara (UFC), Brazil.