Remote ischemic conditioning protects against testicular ischemia/reperfusion injury in rats

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Abstract

Purpose: To evaluate the effect of remote ischemic conditioning associated to N-acetylcysteine (NAC) on testicular ischemia/reperfusion (I/R) injury in rats.

Methods: Twenty-five adult male Wistar rats were randomly distributed into five experimental groups (n=5), as follows: Sham, I/R, Perconditioning (PER), NAC and PER+NAC. Two-hour ischemia was induced by rotating the left testis 720° to clockwise direction, followed by 4 hours of reperfusion. Perconditioning was performed by three I/R cycles of 10 min each on the left limb, 30 min before reperfusion. N-acetylcysteine (150 mg/kg) was administered 30 min before reperfusion.

Results: Statistical differences were observed in MDA levels between I/R group with all groups (p<0.01), in addition there was statistical difference between PER and Sham, and PER+ NAC groups (p<0.05) in plasma.

Conclusions: The protective effect of perconditioning isolated in the reduction of lipid peroxidation related to oxidative stress was demonstrated. However, when Perconditioning was associated with NAC, there was no protective effect against testicular injury of ischemia and reperfusion.

Key words: Acetylcysteine. Ischemia. Reperfusion. Spermatic Cord Torsion. Testis. Rats.
**Introduction**

Torsion of the spermatic cord is a common urologic emergency among infants and adolescents. It requires early diagnosis and surgical intervention to prevent subfertility and infertility.1 Torsional injury resulting from spermatic cord torsion/detorsion resembles the ischemia/reperfusion phenomenon (I/R).2 Moreover, it has been demonstrated that spermatic cord torsion in rats causes permanent aspermatogenesis.3

This loss of spermatogenesis has shown to be due to germ cell-specific apoptosis. I/R of the testis stimulates an intra-cellular signaling cascade in the testicular endothelial cells that results in neutrophil recruitment, increase of intra-testicular reactive oxygen species (ROS), and eventual germ cell-specific apoptosis.4 Although with not conclusive results, several anti-inflammatory drugs,5 antioxidants6 and ischemic conditioning have been used to prevent such I/R injury in testis.7

Among these conditionings, ischemic perconditioning has been highlighted as the most promising strategy to improve tolerance to I/R injury. It consists of multiple short periods of ischemia followed by the same periods of reperfusion, applied during prolonged ischemia.8 This method was found to be effective in the treatment of myocardial,9 kidney,10 and cerebral ischemia11, through temporary and short-term enhancement of cellular antioxidant defenses to avoid the deleterious consequences of future I/R injury.8

Moreover, several studies have shown that treatment of antioxidants and free radical scavengers prevents testicular injury and male infertility.6,7 Among them, N-acetylcysteine (NAC) has been proved as a powerful antioxidant that acts as a free-radical scavenging agent and precursor of glutathione synthesis. Its powerful antioxidant features led NAC to be tested on many studies of I/R injury.12

This study aimed to compare the effects of perconditioning combined to NAC on experimental testicular I/R injury through serum oxidative stress analysis.

**Methods**

All experimental procedures were conducted according to international ethics guidelines and were previously locally approved by the Ethics Committee of the State University of Pará (protocol 17/2015).

Twenty-five adult male rats aged 90 days (300-350g), were obtained from the Evandro Chagas Institute (IEC/PARÁ). Animals were maintained with free access to regular food and water, at 22± 1°C, under a 12h light/dark cycle. The rats were weighed and anesthetized using an intraperitoneal injection of ketamine hydrochloride 10% and xylazine hydrochloride 2% (70 mg/kg and 10 mg/kg, respectively). During the surgical procedures, additional doses were administered if necessary.13

**Experimental protocol**

Five groups comprised this study:

- **Sham group (SG):** animals were subjected to all operative procedures, except testicular torsion;
- **I/R group:** rats were submitted to 2-hours of ischemia followed by 4 hours of reperfusion.14
- **Perconditioning group (PER):** perconditioning was performed by three cycles of 10 min of I/R with tourniquet: 5 min under ischemia and 5 min of reperfusion on left hind limb, 30 min before reperfusion as in I/R group;
- **N-acetylcysteine group (NAC):** animals received an intraperitoneal injection of NAC (150 mg/kg) 30 min before reperfusion as in I/R group;
- **Perconditioning+NAC:** perconditioning was performed as in PER group and NAC administered 30 minutes before reperfusion.

**Surgical procedure**

The surgical procedures were performed under sterile conditions through standard ilioinguinal incisions. Left testes were exposed and torsion was performed by twisting the testicular cord 720° clockwise. After 2 hours, the left testicular cord was restored to its anatomical position with the testes replaced back to their normal position. Four hours after reperfusion, rats underwent euthanasia by anesthetic overdose. PER, NAC and PER+NAC groups are better explained in Figure 1.
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Biochemical parameters

Blood was collected by cardiac puncture just before euthanasia and serum was obtained after suitable centrifugation of blood samples and stored at -20°C until analysis.

Thiobarbituric acid reactive substances (TBARS)

TBARS is a method that evaluates lipid peroxidation and was used as an indicator of oxidative stress. This technique is based on the reaction of malondialdehyde (MDA), among other substances, with Thiobarbituric acid (TBA; Sigma-Aldrich® T5500), in low pH and high temperature, yielding MDA-TBA complex of pink colour, and absorbance peak at 535 nm. The technical procedure was performed according to the protocol proposed by Khon and Liversedge16, adapted by Percario et al.17. In brief, initial TBA solution (10 nM) was prepared in phosphate monobasic potassium (KH₂PO₄ 75 mM; Synth; cat. 35.210) adjusted to pH 2.5 with acetic acid. Two-hundred and fifty μL of sample was added to 500 μL of TBA solution, mixed and placed in a water bath (95°C x 60 min); after cooling at room temperature, 2.0 ml of 1-butanol was added, vortex mixed and subsequently centrifuged (175xg x 15 min); 1.0 ml of the supernatant was collected and read at 535 nm (Femto®, São Paulo, Brazil; 800 XI). 1,1,3,3, tetraethoxypropane (Sigma-Aldrich®; T9889) was used for the implementation of the standard curve.

Trolox Equivalent Antioxidant Capacity (TEAC)

TEAC evaluates the total anti-oxidative capacity of the serum. This technique is based on the inhibition of the absorbance of the free-radical ABTS⁺ (2,2’-Azino-bis 3-Ethylbenzthiazoline-6-Sulfonic Acid) by anti-oxidants, and on the relation of this radical cation with the anti-oxidant scavenger Trolox, which is the synthetic analogue of Vitamin E. The method modified by Re et al.18, in brief: Trolox (Aldrich Chemical Co® 23,881-3, USA), (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), was used as anti-oxidant. ABTS (Sigma-Aldrich®, A1888, USA) - and potassium persulfate K₂S₂O₈ (Sigma-Aldrich®, P5592, USA) were used to prepare a solution containing the radical cation ABTS⁺. A saline solution (phosphate buffer PBS) prepared by diluting 1.48g Na₂HPO₄ (Sodium dibase phosphate), 0.43g NaH₂PO₄ (Sodium monobase phosphate) and 7g NaCl (potassium chloride) in 1 L of distilled water, adjusting for a pH of 7.4 was used. A calibration curve for the Trolox equivalent antioxidant capacity was built by plotting different concentrations of Trolox (mM) versus its total equivalent antioxidant capacity. The final results

![Figure 1 - Schematic diagram of the interventions in PER (Perconditioning), NAC (administration of N-acetylcisteine) and PER+NAC (association of techniques) groups.](image-url)
will be expressed in micromoles per liter (mM/l) corresponding to trolox concentration with antioxidant capacity equivalent to that of the studied sample, a standard of measurement called TEAC (trolox equivalent antioxidant capacity).

**Statistical analysis**

All data were statistically analyzed using BioEstat® 5.4 software¹⁹. Data were expressed as mean + standard deviation. First, descriptive statistics were performed, showing arithmetic mean and standard deviation values. So, the normality test of Shapiro-Wilk was applied, confirming parametrical distribution of data. Finally, the Analysis of variance (ANOVA) was applied. ANOVA test was used for statistical analysis of data among all groups and post hoc Tukey test was applied to both variables. A value of p < 0.05 was considered statistically significant.

■ **Results**

Compared to Sham group, I/R showed a statistically significant difference in MDA between I/R and Sham (p=0.05); I/R and Per (p=0.01); Per and Per+NAC (p=0.05). However, There was not statistical difference between groups at serum antioxidant capacity (p=0.2239) (Figs. 2 and 3).

**Discussion**

On the present study, I/R group showed TBARS serum levels significantly higher than those of Sham group, demonstrating the experimental model was adequate in induction of testicular ischemic injury, promoting the oxidative pattern expected.

A parasympathetic neural response was identified as one of the effector mechanisms of ischemic conditioning techniques, reducing the lesion of non-perfusion by mechanism of vasospasm and contributing to the reestablishment of blood flow in the microcirculation of the organ submitted to I/R injury, which at the testicular level may be associated with an improvement of its physiology against oxidative damage²⁰,²¹.

It is suggested that this mechanism is the activation of a neurohumoral pathway against the reperfusion injury of organs submitted to ischemia²². The addition of remote ischemic conditioning may induce the release of humoral factors, such as adenosine²³, bradykinin²⁴ and opioids²⁵ which, under local innervation, would trigger activation of neural pathways to ensure testicular protection²⁶. A recent research by Oliveira et al.²⁶ showed tramadol (an opioid substance) had a protective effect against renal I/R oxidative injury.

A study by Guimarães et al.²⁷ involving testicular ischemia in rats, also showed no statistical difference between the groups regarding plasma antioxidant
capacity, suggesting that the torsion of the spermatic cord does not affect the systemic antioxidant system, at least under the conditions of the present research. In addition, Costa et al. observed in a previous study that the antioxidant capacity presented an increase of this capacity at 10 minutes, not being observed a significant increase in the time of 60 minutes, which would explain such result.

Furthermore, high mortality was observed in the animals in which NAC was used. Five animals died in the NAC group and in previous studies it has been observed that such substance promotes a decrease in prothrombin time, anaphylactic reactions and deaths. Besides that, Oliveira et al. demonstrated that, for the myocardium, NAC actually significantly inhibits the protective effects of ischemic conditions and could explain the difference found in TBARS levels between the conditioning group and those who were treated with NAC.

Further studies are necessary to evaluate the effects of perconditioning on reducing I/R injury, exploring neuro-humoral pathways.

■ Conclusion

The perconditioning method was successful in reducing lipid peroxidation; however, NAC alone or combined to ischemic perconditioning was not effective to reduce the effects of oxidative stress.

■ References

1. Pentyala S, Lee J, Yalamanchili P, Vitkun S, Khan SA. Testicular torsion: a review. J Low Genit Tract Dis. 2001;5(1):38-47. doi: 10.1046/j.1526-0976.2001.51008.x.
2. Percário S. Prevenção do estresse oxidativo na síndrome de isquemia e reperfusão renal em ratos com suplementação nutricional com antioxidantes. Rev Nutr. 2010;23(2):259-67. doi: 10.1590/S1415-527320100000200009.
3. Cuckow PM, Frank JD. Torsion of the testis. BJU International. 2000;86:349-53. doi: 10.1046/j.1464-410x.2000.00106.x.
4. Dajusta D, Granberg CF, Villanueva C, Baker L. Contemporary review of testicular torsion: new concepts, emerging technologies and potential therapeutics. J Pediatr Urol. 2013;9(6):723-30. doi: 10.1016/j.jpurol.2012.08.012.
5. Takhtfooladi MA, Jahanshahi A, Jahanshahi G, Sotoudeh A, Takhtfooladi HA, Khansari M. Protective effect of N-acetylcysteine on kidney as a remote organ after skeletal muscle ischemia-reperfusion. Acta Cir Bras. 2012;27(9):611 doi: 10.1590/S0102-86502012000900004.
6. Chi KK, Zhang WH, Chen Z, Cui Y, He W, Wang SG, Zhang C, Chen J, Wang GC. Comparison of quercetin and resveratrol in the prevention of injury due to testicular torsion/detorsion in rats. Asian J Androl. 2016;18:1-5. doi: 10.4103/1008-682X.167720.
7. Shimizu S, Tsounapli P, Dimitriadis F, Higashi Y, Shimizu T, Saito M. Testicular torsion-detorsion and potential therapeutic treatments: A possible role for ischemic postconditioning. Int J Urol. 2016;23(6):454-63. doi: 10.1111/iju.13110.
8. Costa FL, Teixeira RK, Yamaki VN, Valente AL, Silva AM, Brito MVH, Percário S. Remote ischemic conditioning temporarily improves antioxidant defense. J Surg Res. 2016;202(1):105-9. doi: 10.1016/j.jss.2015.07.031.
9. Wang SY, Cui XL, Xue FS, Duan R, Li RP, Liu GP, Yang GZ, Sun C. Combined morphine and limb remote ischemic perconditioning provides an enhanced protection against myocardial ischemia/reperfusion injury by antiapoptosis. J Surg Res. 2016;202(1):13-25. doi: 10.1016/j.jss.2015.12.007.
10. Yamaki VN, Gonçalves TB, Coelho JVB, Pontes RVS, Costa FLS, Brito MVH. Efeito protetor do percondicionamento isquêmico remoto nas lesões da síndrome de isquemia e reperfusão renal em ratos. Rev Col Bras Cir. 2012;39(6):529-33. doi: 10.1590/S0100-69912012000000014.
11. Ren C, Wang P, Wang B, Li N, Li W, Zhang C, Jin K, Ji X. Limb remote ischemic per-conditioning in combination with post-conditioning reduces brain damage and promotes neuroglobin expression in the rat brain after ischemic stroke. Restor Neurol Neurosci. 2015;33(3):369-79. doi: 10.3233/RNN-140413.
12. Wang S, Wang C, Yan F, Wang T, He Y, Li H, Xia Z, Zhang Z. N-Acetylcysteine attenuates diabetic myocardial ischemia reperfusion injury through inhibiting excessive autophagy. Mediators Inflamm. 2017;2017:9257291. doi: 10.1155/2017/9257291.
13. Yasojima EY, Ribeiro Júnior RFG, Pessôa TCP, Cavalcante LCC, Ramos SR, Gouveia EHH, Galvão LN, Serruya YAA, Moraes MM. Effects of nitrofurazone on correction of abdominal wall defect. Treated with polypropylene mesh envolved by fibrous tissue. Acta Cir Bras. 2015;30(10):686-90. doi: 10.1590/S0102-86502015100000006.
14. Kabay S, Ozden H, Guven G, Burukoglu D, Ustuner MC, Topal F, Gunes HV, Ustuner D, Ozbayar C. Protective effects of the nuclear actor kappa b inhibitor pyrroline dithiocarbamate on experimental testicular torsion and detorsion injury. Korean J Physiol Pharmacol. 2014;18:321-6. doi: 10.4196/kjpp.2014.18.4.321.
15. Takhtfooladi HA, Hesaraki S, Khansari M, Takhtfooladi MA, Hajizadeh H. Effects of N-acetylcysteine and pentoxifylline on remote lung injury in a rat model of hind-limb ischemia/reperfusion injury. J Bras Pneumol. 2016;42(1):9-14. doi: 10.1590/S1808-37562016000000183.
16. Kohn HI, Liversedge M. On a new aerobic metabolite whose production by brain is inhibited by apomorphine, emetine, ergotamine, epinephrine, and menadione. J Pharmacol Experimen Ther. 1944;82:292–300.
17. Percário S, Vital A, Jablonka F. Dosagem do malondialdeído. Experimen Ther. 1944;82:292–300.
18. Re R, Pellegrini R, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Rad Biol Med. 1999;26:1231-7. doi: 10.1016/s0891-5849(98)00315-3.
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19. Ayres M, Ayres Junior M, Ayres DL, Santos AS. BioEstat 5.0: aplicações estatísticas nas áreas das ciências biológicas e médicas. Sed. Instituto de Desenvolvimento Sustentável Mamirauá-IDS/ MCT/ CNPq, Belém; 2007.
20. Donato M, Buchholz B, Rodríguez M, Pérez V, Inserte J, García-Dorado D, Gelpi RJ. Role of the parasympathetic nervous system in cardioprotection by remote hindlimb ischaemic preconditioning. Exp Physiol. 2013;98(2):425-34. doi: 10.1113/expphysiol.2012.066217.
21. Lim SY, Yellon DM, Hausenloy DJ. The neural and humoral pathways in remote limb ischemic preconditioning. Basic Res Cardiol. 2010;105(5):651-5. doi: 10.1007/s00395-010-0099-y.
22. Szijártó A, Czigány Z, Turóczi Z, Harsányi L. Remote ischemic perconditioning – a simple, low-risk method to decrease ischemic reperfusion injury: models, protocols and mechanistic background. A review. J Surg Res. 2012;178(2):797-806. doi: 10.1016/j.jss.2012.06.067.
23. Schulte G, Sommerschild H, Yang J, Tokuno S, Goiny M, Lövdahl C, Johansson B, Fredholm BB, Valen G. Adenosine A1 receptors are necessary for protection of the murine heart by remote, delayed adaptation to ischaemia.
24. Schoemaker RG, Van Heijningen CL. Bradykinin mediates cardiac preconditioning at a distance. Am J Physiol. 2000;278:. H1571. doi: 10.1152/ajpheart.2000.278.5.H1571.
25. Patel HH, Moore J, Hsu AK, Gross GJ. Cardioprotection at a distance: mesenteric artery occlusion protects the myocardium via an opioid sensitive mechanism. J Mol Cell Cardiol. 2002;34:1317. doi: 10.1006/jmcc.2002.2072.
26. Oliveira RCS, Brito MVH, Ribeiro Júnior RFG Oliveira LOD, Monteiro AM, Brandão FMV, Cavalcante LCC, Gouveia EHH, Henriques HYB. Influence of remote ischemic conditioning and tramadol hydrochloride on oxidative stress in kidney ischemia/reperfusion injury in rats. Acta Cir Bras. 2017;32(3):229-35. doi: 10.1590/s0102-86502017003000007.
27. Guimarães SB, Aragão AA, Santos JM, Kimura Ode S, Barbosa PH, Vasconcelos PR. Oxidative stress induced by torsion of the spermatic cord in young rats. Acta Cir Bras. 2007;(1):30-3. doi: 10.1590/S0102-86502007000100005.

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Received: Oct 02, 2019
Review: Dec 05, 2019
Accepted: Jan 03, 2020

Conflict of interest: none
Financial source: none

Research performed at Laboratory of Experimental Surgery, Universidade Estadual do Pará (UEPA), Belem-PA, Brazil.

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