

# Montelukast protects against testes ischemia/reperfusion injury in rats

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## Abstract

**Introduction:** In this study, we investigate the effect of montelukast on histologic damage induced by testicular torsion-detorsion in rats.

**Methods:** Twenty-one male Sprague-Dawley rats were separated into 3 groups, each containing 7 rats. A sham operation was performed in group 1 (control). In group 2 (ischemia-reperfusion [I-R]/untreated), 1-hour detorsion of the testis was performed after 6 hours of unilateral testicular torsion. In group 3 (I-R/dextroamphetamine), after performing the same surgical procedures as in group 2, montelukast was given intraperitoneally. In all experimental rats, ipsilateral orchietomies were performed for histological examination and tissue malondialdehyde (MDA), glutathione and myeloperoxidase assays.

**Results:** Montelukast treatment significantly decreased the I-R-induced elevation in testes tissue MDA and glutathione levels were found to be preserved. The level of myeloperoxidase (MPO) activity was significantly increased in the testes tissue of the I-R/untreated group. However, in I-R/montelukast treatment group significantly decreased testes tissue MPO level. Histopathologically, the in the group 2 rats, edema, congestion, hemorrhage between seminiferous tubules and necrosis of the germinal cells were predominant features in sections. However, most of the specimens in the montelukast treated group 3 showed grades-I and II injury. Additionally, the testicular injury score was lower in group 3 rats compared with group 2.

**Conclusion:** The current findings demonstrate that the montelukast decreased the severity of testicular injury by reversing the oxidative effects of testes I-R.

## Résumé

**Introduction :** Dans cette étude, nous examinons l'effet du montelukast sur les lésions tissulaires provoquées par torsion-détorsion testiculaire chez le rat.

**Méthodologie :** Vingt-et-un rats Sprague-Dawley mâles ont été répartis en trois groupes de 7 rats. Une opération fictive a été réalisée dans le groupe 1 (groupe témoin). Dans le groupe 2 (ischémie-reperfusion / non traité), une détorsion testiculaire d'une heure a été réalisée après 6 heures de torsion testiculaire unilatérale. Dans le groupe 3 (ischémie-reperfusion / dextroamphétamine), on a administré du montelukast par voie intrapéritonéale après les mêmes interventions chirurgicales que pour le groupe 2. Chez tous les rats, une orchidectomie homolatérale a été effectuée en vue d'un examen histologique et de mesures du malondialdéhyde, du glutathion et de la myéloperoxydase dans les tissus.

**Résultats :** Le traitement par montelukast a significativement réduit l'élévation dans les tissus testiculaires provoquée par l'ischémie-reperfusion, mais les taux de malondialdéhyde et de glutathion sont demeurés les mêmes. Le niveau d'activité de la myéloperoxydase (MPO) a significativement augmenté dans les tissus testiculaires du groupe ayant subi l'ischémie-reperfusion sans traitement subséquent. Cependant, dans le groupe ayant subi l'ischémie-reperfusion avec traitement par montelukast, le taux de MPO dans les tissus testiculaires avait significativement diminué. Selon les examens d'histopathologie, dans le groupe 2, de l'œdème, de la congestion, des hémorragies entre les tubules séminifères et une nécrose des cellules germinales étaient les caractéristiques prédominantes observées dans les coupes. Par comparaison, la plupart des échantillons du groupe 3 (traité par montelukast) présentaient des lésions de stade I et II. En outre, le score de lésions testiculaires était plus faible dans le groupe 3 que dans le groupe 2.

**Conclusion :** Les observations actuelles montrent que l'administration de montelukast diminue la gravité des lésions testiculaires en annulant les effets oxydatifs de l'ischémie-reperfusion des testicules.

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## Introduction

Testicular torsion is a common pediatric urologic emergency.<sup>1,2</sup> Late presentation or failure to diagnose can lead to decreased spermatogenesis, altered hormone production and subfertility.<sup>3,4</sup> Mammalian testes are extremely sensitive to oxidative-free radical damage.<sup>5,6</sup> Reperfusion is one of the most important factors in further injury.<sup>7</sup> Ischemia-reperfusion (I-R) injury in the testis induces germ cell-specific apoptosis attributable to increases in neutrophil infiltration and reactive oxygen species (ROS).<sup>8,9</sup> These ROS can lead to lipid peroxidation and loss of cell viability by disrupting membrane integrity and DNA damage.<sup>5,6</sup> Once neutrophils migrate into the ischemic area, they release proteases, elastase, myeloperoxidase (MPO), cytokines and various other mediators,<sup>10</sup> all of which are involved in tissue injury. The ROS are difficult to quantify directly in tissue because of their high reactivity and short half-life. In our experiment, we determined the malondialdehyde (MDA) level, an end product of lipid peroxidation;<sup>11</sup> the glutathione (GSH) level, a key antioxidant;<sup>12</sup>

and the tissue-associated MPO activity, as indirect evidence of neutrophil infiltration.<sup>13</sup>

Anti-leukotriene agents have been shown to be effective to protect against injury in several inflammatory models in rats, such as in ethanol-induced gastric mucosal damage,<sup>14</sup> colitis<sup>15,16</sup> burn- and sepsis-induced multiorgan damage<sup>17,18</sup> and renal I-R injury.<sup>19</sup> Based on these findings, in the present study, we examined the protective effect of the selective LTD<sub>4</sub> receptor antagonist, montelukast (MK-0476), on histologic damage induced by testes I-R in rats.

## Methods

Twenty-one prepubertal (35 days old) male Sprague-Dawley rats, weighing 120 to 140 g, were randomly divided into 3 groups, with each group containing 7 rats. All the animals were housed at a temperature- and light-controlled room with ad libitum access to water and rat food. All the experimental protocols were approved by the Abant Izzet Baysal University School of Medicine Animal Care and Use Committee. Rats were anesthetized during all the surgical procedures.

The surgical procedures were performed under 50 mg/kg ketamine and 20 mg/kg xylazine body weight, intraperitoneal injection (IP). The right femoral vein was cannulated to administer drugs and saline. The testes were exposed through identically opened and closed right-sided ilioinguinal incision. Torsions were created by rotating the ipsilateral testes 720° clockwise for 6 hours and were maintained by fixing the testes medially and laterally to the scrotum using a 5/0 silk suture. The detorsion procedure was performed again under sterile conditions, after 6 hours of testicular torsion. The rats were divided into 3 groups according to the procedure performed.

**Group 1:** In this control group, the testes were brought through the incision and then replaced with a fixation to the scrotum.

**Group 2:** In this torsion group, we performed detorsion of the twisted testis after 6 hours of unilateral testicular torsion. Saline (NaCl at 0.02%, 10 mL/kg/min) was administered during the procedure.

**Group 3:** In the montelukast-treated group, the same surgical procedure (torsion and detorsion) as in group 2 was performed. The rats were treated with montelukast (10 mg kg<sup>-1</sup> IP) (Mustafa Nevzat Drug Company, Istanbul, Turkey) 30 minutes prior to reperfusion period.

In all 3 groups, ipsilateral orchiectomies were performed for the biochemical and histological examinations.

## Tissue malondialdehyde, glutathione and myeloperoxidase assays

Samples of testis were frozen at -70°C until the assay. Tissue samples were homogenized with ice-cold solution (150 mM

KCl) to determine MDA and GSH levels. The MDA levels were assayed for products of lipid peroxidation by monitoring thiobarbituric acid reactive substance formation as previously described.<sup>11</sup> Lipid peroxidation was expressed in terms of MDA equivalents using an extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$  and the results were expressed as nmol MDA/g tissue.

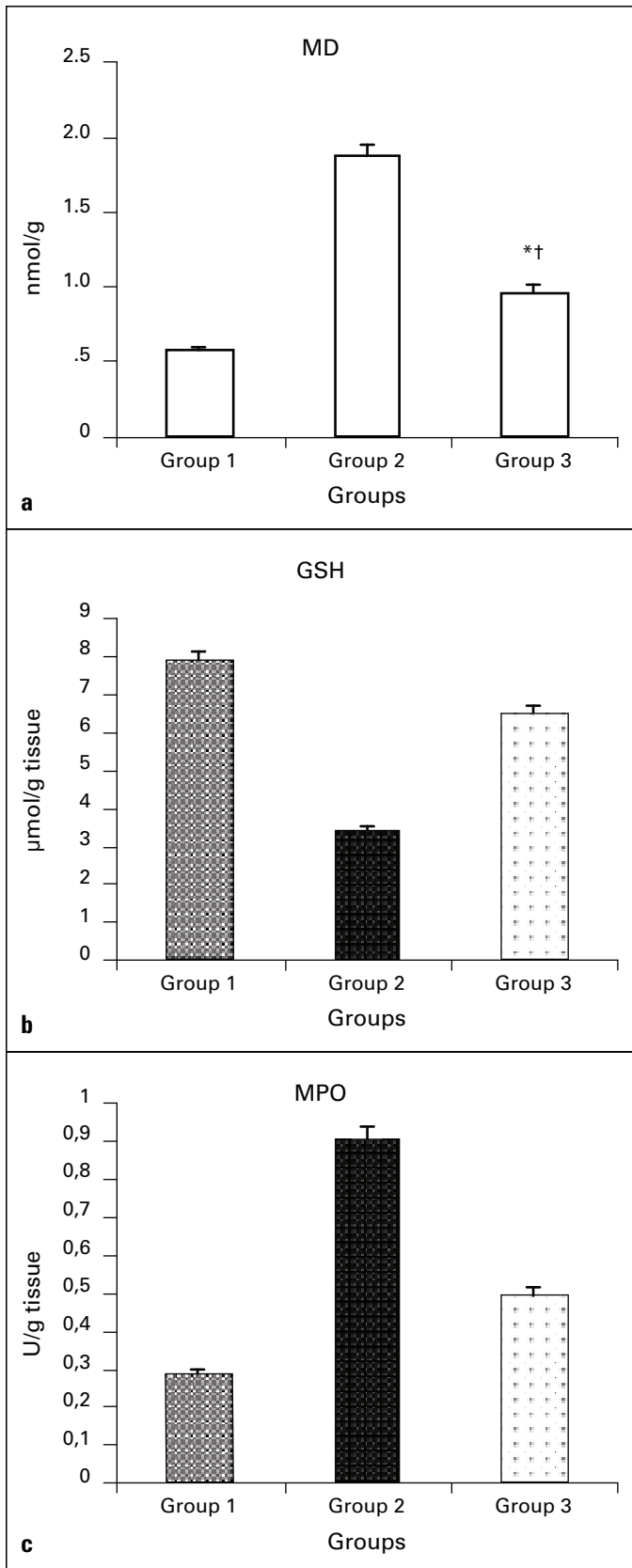
The GSH measurements were performed using a modification of the Ellman procedure.<sup>12</sup> Briefly after centrifugation at 2000 g for 10 minutes, 0.5 mL of supernatant was added to 2 mL of 0.3 mol/L Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O solution. A 0.2-mL solution of dithiobisnitrobenzoate (0.4 mg/mL, 1% sodium citrate) was added and the absorbance at 412 nm was measured immediately after mixing. The GSH levels were calculated using an extinction coefficient of  $1.36 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ . Results were expressed in μmol GSH/g tissue.

Activity of tissue MPO, an enzyme that is found predominantly in the azurophilic granules of polymorphonuclear leukocytes, correlates with the number of polymorphonuclear neutrophils determined histochemically in the inflamed tissues; it is therefore used as an indication of tissue neutrophil accumulation.<sup>13</sup> Testes MPO activity was measured using a procedure similar to that documented previously.<sup>20</sup> Testes samples were homogenized in 50 mM potassium phosphate buffer (IPB), pH 6.0, and centrifuged at 41 400 g (10 minutes); pellets were suspended in 50 mM PB containing 0.5% hexadecyl trimethyl ammonium bromide (HETAB). After 3 freeze and thaw cycles, with sonication between cycles, the samples were centrifuged at 41 400 g for 10 min. Aliquots (0.3 ml) were added to 2.3 ml of reaction mixture containing 50 mM PB, o-dianisidine, and 20 mM H<sub>2</sub>O<sub>2</sub> solution. One unit of enzyme activity was defined as the amount of the MPO present that caused a change in absorbance measured at 460 nm for 3 minutes. The MPO activity was expressed as U/g tissue.

**Table 1. Histological grading system**

Grade I	Showed normal testicular architecture with an orderly, arrangement of germinal cells
Grade II	Injury showed less orderly, noncohesive germinal cells and closely packed seminiferous tubules
Grade III	Injury exhibited disordered sloughed germinal cells with shrunken pyknotic nuclei and less distinct seminiferous tubule borders
Grade IV	Injury defined seminiferous tubules that were closely packed with coagulative necrosis of the germinal cells

This system is developed by Cosentino and colleagues.<sup>21</sup>



**Fig. 1.** (a) Malondialdehyde (MDA) and (b) glutathione (GSH) levels and (c) myeloperoxidase (MPO) activity in the testes tissue of sham-operated control groups, ischemia-reperfusion (I-R)/untreated groups and I-R/ montelukast-treated groups. Each group consists of seven animals. \* =  $p < 0.05$ , compared to control group. † =  $p < 0.05$ , compared to I-R/Untreated group. Group 1: sham-operated control; Group 2: I-R/untreated; Group 3: I-R/montelukast-treated

**Histological examination**

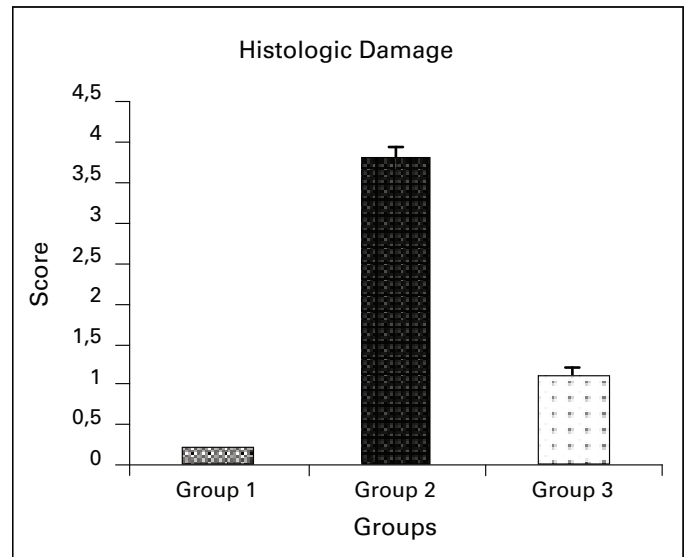
The extracted testes were immediately placed into 10% formalin solution. The tissue specimens were placed in paraffin blocks, sectioned at 5 µm, and stained with hematoxyline & eosine. The sections were blindly examined under light microscope by 2 investigators. The histological parameters was scored according to Cosentino and colleagues’s classification (Table 1).<sup>21</sup>

**Statistical analysis**

All values were expressed as mean standard deviation. The significance of the data obtained was evaluated by using analysis of variance (ANOVA). Differences between means were analyzed by using the post-ANOVA (Tukey’s b) test. Significance was determined as  $p$  values less than 0.05.

**Results**

The MDA, GSH and MPO values for the different groups are shown in Fig. 1. The testes MDA levels in the control group were elevated by I-R injury ( $p < 0.05$ ); however, mon-teelukast treatment significantly decreased the I-R-induced



**Fig. 2.** Comparative histologic score measurements at the the groups. \* =  $p < 0.05$  compared with group 1. † =  $p < 0.05$  compared with group 3. Vales are mean ±SEM. Group 1: sham-operated control ; Group 2: ischemia-reperfusion (I-R)/untreated; Group 3: I-R/montelukast-treated.

elevation in the testes MDA level ( $p < 0.05$ ). The I-R caused a significant decrease in testes GSH level ( $p < 0.05$ ) when compared with the control group, while in the montelukast-treated I-R group, the testes GSH level was found to be preserved ( $p < 0.05$ ) and not significantly different from that of the control group. The level of MPO activity was significantly increased in the testes tissue of the I-R/untreated group ( $p < 0.001$ ) compared with the control group. However, in the I-R/montelukast treatment group, there was significantly decreased testes tissue MPO level ( $p < 0.5$ ); this was found to be similar to the control group.

The testicular injury score increased significantly in the I-R/untreated and I-R/montelukast groups compared with the control group ( $p < 0.05$ ,  $p < 0.05$ , respectively). On the other hand, this score was decreased in the I-R/montelukast group compared with the I-R/untreated ( $p < 0.05$ ) (Fig. 2).

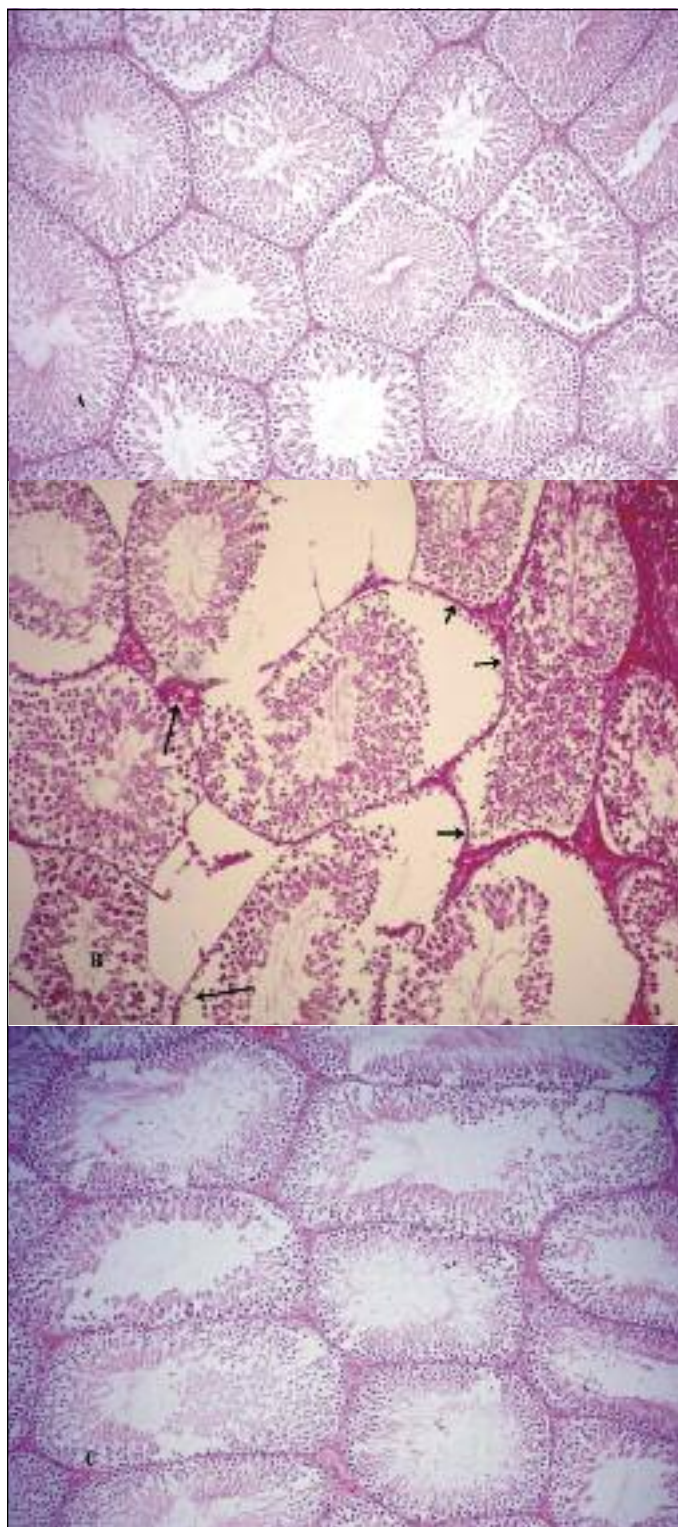
Histopathologically, the rats in the control group had essentially normal seminiferous tubule morphology (Fig. 3, part a). In the I-R/untreated group (group 2), the lesions varied between grade-III and grade-IV. In this group, edema, congestion, hemorrhage between seminiferous tubules and necrosis of the germinal cells were predominant features in sections (Fig. 3b, part b). However, in the montelukast-treated group (group 3), 6 rats had grade-I and II injury and histopathological features which were significantly lower than the I-R/untreated group. In group 3, interstitial edema, congestion and hemorrhage between seminiferous tubules were reduced (Fig. 3c, part c).

## Discussion

The underlying pathophysiologic mechanisms in testicular I-R damage are most likely multifactorial, and interdependent involving hypoxia, inflammatory responses and oxidative stress, which is characterized by an imbalance between ROS and the antioxidative defense system.<sup>22,23</sup> The present study demonstrates that unilateral testicular I-R causes testicular damage in testes, as evidenced by biochemical and histologic changes. Montelukast prevented the biochemical changes and protected the morphology in ipsilateral testes after unilateral testicular I-R.

The pathophysiologic mechanism in testicular damage owing to testicular torsion is an ischemic process for the testis. The ROS can oxidize cell membrane lipids, proteins and DNA, which leads to cellular dysfunction and, sometimes, cell death. This cascade of events is known as reperfusion injury.<sup>23,24</sup> In addition, more neutrophils accumulated in the testis after testicular torsion-detorsion and generated excess ROS; this caused spermatogenic injury in the ipsilateral testis. The elimination of ROS has been shown to be beneficial in treating ischemia-reperfusion injury.<sup>16,25,26</sup>

The ROS are difficult to quantify directly in tissue because of their high reactivity and short half-life. Malondialdehyde,



**Fig. 3.** Photomicrographs of testes tissues in the (A) control group showing normal seminiferous tubule morphology (hematoxylin and eosin [H&E],  $\times 200$ ), (B) ischemia-reperfusion (I-R)/untreated group, presenting with interstitial edema, congestion and evident hemorrhage between seminiferous tubules (arrows) (H&E,  $\times 200$ ), (C) montelukast-treated I-R group, most of the specimens showed grade II injury and reduced interstitial edema, congestion and hemorrhage between seminiferous tubules (arrows) (H&E,  $\times 200$ ).

a stable end-product of lipid peroxidation generated by ROS, is usually used as a good indicator of the degree of lipid peroxidation.<sup>27</sup> In our study, we demonstrated that the levels of tissue MDA were significantly increased in the I-R/untreated group. The increase of MDA levels indicated the presence of oxidative damage I-R injury induced on ipsilateral testes. Furthermore, MDA activity was preserved by montelukast treatment. Glutathione is a key component in cell growth, differentiation and protection.<sup>25</sup> It is a tripeptide, L-g-glutamyl-L-cysteinyl-glycine, with a molecular weight of 307,<sup>28</sup> and is an important cellular thiol "redox buffer" participating actively in the maintenance of the thiol/disulfide redox potential.<sup>29</sup> As reported by Ross and colleagues,<sup>30</sup> cell injury is related to the efflux of GSH precursors and decreases GSH biosynthesis. In this sense, GSH and other antioxidants play a critical role in limiting the propagation of free-radical reactions, which would otherwise result in extensive lipid peroxidation. In the present study, we found that the concentration of GSH decreased in the I-R/untreated group compared to control group. Decreased tissue GSH activities might have occurred as a result of consumption of the enzyme by severe oxidative stress. However, treatment with montelukast enhanced higher antioxidant capacity because the concentrations of testicular GSH were significantly higher in the montelukast-treated animals when compared with the GSH testicular concentrations found in I-R/untreated rats. The infiltration of polymorphonuclear leukocytes in a tissue is characteristic of acute inflammation and indicates the collective action of chemotactic mediators.<sup>31</sup> Various chemokines and a lipid mediator, cysteinyl leukotrienes (CysLTs), the 5-lipoxygenase metabolites of arachidonic acid) are potent inflammatory mediators that are associated with I-R-induced tissue injury.<sup>10</sup> Testicular neutrophil content was determined by MPO assay in the present study. Myeloperoxidase is stored in the primary granules of neutrophils and the enzyme activity is a common measure of neutrophil accumulation.<sup>32</sup> We have found a significant decrease in testis tissue MPO activity after montelukast administration. These data suggest that the anti-inflammatory effects of Montelukast contribute to its protective role in testicular torsion. Although the results of our study are promising, there are several limitations. We did not study the effect of montelukast on testicular I-R injury at different doses or different administration times. Additional studies are required to examine these factors on the effect of montelukast.

## Conclusion

These data collectively support that testes I-R injury causes oxidative response as evidenced by alterations in testes MPO, MDA and GSH levels. The results also demonstrate that montelukast attenuates the I-R-induced testes damage through

the mechanisms that involve an inhibitory action on tissue neutrophil infiltration, release of reactive oxygen species and activation of inflammatory cytokines. This may provide a potential therapeutic approach to post-ischemic testicular damage. Further studies will clarify the role of the selective LTD<sub>4</sub> receptor antagonist, montelukast (MK-0476), in decreasing long-term sequelae responsible for subfertility.

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**Competing interests:** None declared.

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## References

- Romeo C, Antonuccio P, Esposito M, et al. Raxofelast, a hydrophilic vitamin E-like antioxidant, reduces testicular ischemia-reperfusion injury. *Urol Res* 2004;32:367-71.
- Bozlu M, Co kun B, Cayan S, et al. Inhibition of poly (adenosine diphosphate-ribose) polymerase decreases long-term histologic damage in testicular ischemia-reperfusion injury. *Urology* 2004;63:791-5.
- Fisch H, Laor E, Reid RE, et al. Gonadal dysfunction after testicular torsion: luteinizing hormone and follicle-stimulating hormone response to gonadotropin releasing hormone. *J Urol* 1988;139:961-4.
- Turner TT, Tung KS, Tomomasa H, et al. Acute testicular ischemia results in germ cell-specific apoptosis in the rat. *Biol Reprod* 1997;57:1267-74.
- Filho DW, Torres MA, Bordin AL, et al. Spermatic cord torsion, reactive oxygen and nitrogen species and ischemia-reperfusion injury. *Mol Aspects Med* 2004;25:199-210.
- Turner TT, Brown KJ. Spermatic cord torsion: loss of spermatogenesis despite return of blood flow. *Biol Reprod* 1993;49:401-7.
- Akgür FM, Kilingç K, Aktug T. Reperfusion injury after detorsion of unilateral testicular torsion. *Urol Res* 1993;21:395-399.
- Lysiak JJ, Nguyen QA, Turner TT. Peptide and nonpeptide reactive oxygen scavengers provide partial rescue of the testis after torsion. *J Androl* 2002;23:400-9.
- Moon C, Kim JS, Jang H, et al. Activation of Akt/protein kinase B and extracellular signal-regulated kinase in rats with acute experimental testicular torsion. *J Vet Med Sci* 2008;70:337-41.
- Kelly KJ, Williams WW Jr, Colvin RB, et al. Interleukin-1-deficient mice are protected against ischemic renal injury. *J Clin Invest* 1996;97:1056-63.
- Buege JA, Aust SD. Microsomal lipid peroxidation. *Methods Enzymol* 1978;52:302-10.
- Beutler E. Glutathione in red blood cell metabolism. *A manual of biochemical methods*. New York, NY: Grune & Stratton; 1975:112-4.
- Bradley PP, Priebat DA, Christensen RD, et al. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. *J Invest Dermatol* 1982;78:206-9.
- Peskar BM. Leukotrienes in mucosal damage and protection. *J Physiol Pharmacol* 1991;42:135-45.
- Wallace JL, MacNaughton WK, Morris GP, et al. Inhibition of leukotriene synthesis markedly accelerates healing in a rat model of inflammatory bowel disease. *Gastroenterology* 1989;96:29-36.
- Holma R, Salmenperä P, Riutta A, et al. Acute effects of the cys-leukotriene-1 receptor antagonist, montelukast, on experimental colitis in rats. *Eur J Pharmacol* 2001;429:309-18.
- Sener G, Kabasakal L, Cetinel S, et al. Leukotriene receptor blocker montelukast protects against burn-induced oxidative injury of the skin and remote organs. *Burns* 2005;31:587-96.
- Sener G, Sehirli O, Cetinel S, et al. Amelioration of sepsis-induced hepatic and ileal injury in rats by the leukotriene receptor blocker montelukast. *Prostaglandins Leukot Essent Fatty Acids* 2005;73:453-62.
- Sener G, Sehirli O, Velio lu-O ünc A, et al. Montelukast protects against renal ischemia/reperfusion injury in rats. *Pharmacol Res* 2006;54:65-71.

20. Hillegass LM, Griswold DE, Brickson B, et al. Assessment of myeloperoxidase activity in whole rat kidney. *J Pharmacol Methods* 1990;24:285-95.
21. Cosentino MJ, Nishida M, Rabinowitz R, et al. Histological changes occurring in the contralateral testes of prepubertal rats subjected to various durations of unilateral spermatic cord torsion. *J Urol* 1985;133:906-11
22. Williams P, Lopez H, Britt D, et al. Characterization of renal ischemia-reperfusion injury in rats. *J Pharmacol Toxicol Methods* 1997;37:1-7.
23. Carden DL, Granger DN. Pathophysiology of ischaemia-reperfusion injury. *J Pathol* 2000;190:255-66.
24. Avlan D, Erdou an K, Cimen B, et al. The protective effect of selenium on ipsilateral and contralateral testes in testicular reperfusion injury. *Pediatr Surg Int* 2005;21:274-8.
25. Milei J, Ferreira R, Llesuy S, et al. Reduction of reperfusion injury with preoperative rapid intravenous infusion of taurine during myocardial revascularization. *Am Heart J* 1992;123:339-45.
26. Tong L, Li J, Qiao H, et al. Taurine protects against ischemia-reperfusion injury in rabbit livers. *Transplant Proc* 2006;38:1575-9.
27. Cherubini A, Ruggiero C, Polidori MC, et al. Potential markers of oxidative stress in stroke. *Free Radic Biol Med* 2005;39:841-52.
28. Meister A, Anderson ME. Glutathione. *Ann Rev Biochem* 1983;52:711-60.
29. Sies H. Glutathione and its role in cellular functions. *Free Rad Biol Med* 1999; 27: 916-21.
30. Ross D. Glutathione, free radicals and chemotherapeutic agents. *Pharmacol Ther* 1988;37:231-49.
31. Ishikawa F, Miyazaki S. New biodefense strategies by neutrophils. *Arch Immunol Ther Exp (Warsz)* 2005;53: 226-33.
32. Grisham MB, Benoit JN, Granger DN. Assessment of leukocyte involvement during ischemia and reperfusion of intestine. *Methods Enzymol* 1990;186:729-42.

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