

Protective Effects of Colchicine on Testicular Torsion/Detorsion-Induced Ischemia/Reperfusion Injury in Rats

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Purpose: To evaluate the short-term use of colchicine on preventing ischemia-reperfusion injury after surgery in an experimental animal model.

Materials and Methods: A total of 40 rats were divided into five groups (n = 8). Sham (Sh), ischemia-reperfusion (I/R), I/R and colchicine-treated for once per-operatively (I/Rc1), I/R and colchicine-treated for 5 days postoperatively (I/Rc5), and I/R and placebo given for 5 days (I/Rp) groups. Testicular torsion was created by rotating the testicle 720° in clockwise direction and held for 3 hours. In group I/Rc1 30 minutes before detorsion, p.o. 1 mg/kg mL infusion of colchicine was given only once. In group I/Rc5, colchicine continued p.o. once daily for five days. Tissue malonyldialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) were measured for evaluating the oxidative stress. Apoptosis levels shown with Caspase-3 staining and mean seminiferous tubular diameter (MSTD), germinal epithelial cell thickness (GECT), and mean testicular biopsy score (MTBS) were used to evaluate the germ cell damage.

Results: Decreased protein MDA levels therewithal increased SOD, CAT and GPx levels achieved in I/Rc5 group when compared to I/R group and did not differ from the I/Rp group ($p < 0.05$). MSTD, GECT, and JS were better in I/Rc5 than I/Rp which showed the natural course of I/R damage in testis ($p < 0.005$). Caspase 3 positivity, as an apoptosis indicator, were significantly lower ($p < 0.05$) in I/Rc5 group in comparison with I/R, I/Rc1, and I/Rp groups.

Conclusion: The usage of colchicine as a complementary treatment after definitive surgery reduce early-onset ischemia-reperfusion damage and diminishes apoptosis.

Keywords: colchicine; perfusion; injury; rat; torsion

INTRODUCTION

As a urologic emergency, testicular torsion is seen in 4,5/100000 males aged 1-25.⁽¹⁾ This situation may result in testicular atrophy in the absence of definitive surgery within hours. Detorsion of the testis causes ischemia/reperfusion (I/R) injury. The primary indicators of I/R injury are lipid peroxidation and apoptosis arisen from neutrophil recruitment, reactive oxygen species (ROS) and proinflammatory cytokines.⁽²⁾ Low concentrations of ROS promotes sperm capacitation but excessive production acts quite the contrary.⁽³⁾ High levels of ROS degrade polyunsaturated lipids which are found in the plasma membrane of spermatozoa, forming malondialdehyde (MDA) that is an indicator of oxidative stress. On the other side, several protective anti-oxidant scavenger enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) neutralize free radicals. In the presence of excessive ROS production cellular dysfunction and related apoptosis begin.

Apoptosis is a physiologic mechanism used to eliminate nonfunctioning and undifferentiated cells.⁽⁴⁾ Apoptosis occurs in two important pathways, intrinsic and extrinsic whom the caspases are the unique markers of entire processes. The cell death cascade was initiated either with the death receptor-mediated namely extrinsic way or with the mitochondrion-mediated procaspase-activation pathway known as the intrinsic way.⁽⁵⁾ In caspase family, caspase-3 is a significant mediator of both apoptotic and necrotic cell death by inhibiting proteins that are vital for repairing DNA injury, intercellular signal transmission, cell cytoskeleton and continuation of the cell cycle.⁽⁶⁾

Colchicine is a tricyclic alkaloid of the Colchicum Autumnale plant which is a member of Colchicaceae plant family, one of the oldest therapeutic substances known to mankind. It has been used for Gout disease, Familial Mediterranean Fever, acute pericarditis, acute arthritis, and Behçet's Disease.⁽⁷⁾ The suppressing effect of colchicine on inflammation has not been enlightened precisely yet, but the prevailing opinion is inhibition of

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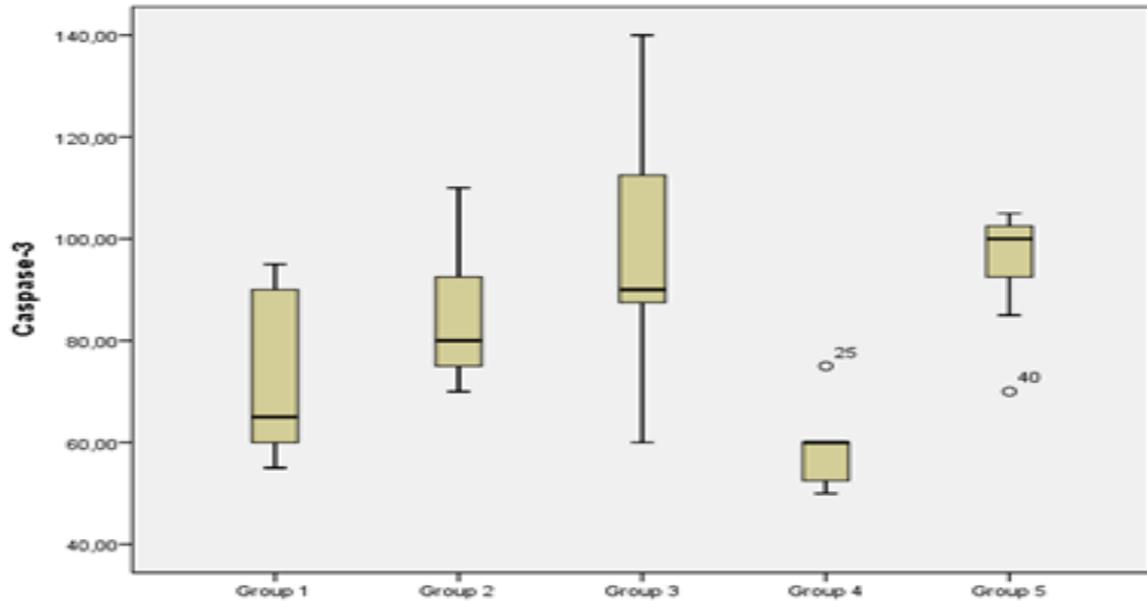


Figure 1. Caspase-3 staining in groups

the neutrophils and endothelial adhesion molecules by disrupting microtubule polymerization in leucocytes.⁽⁸⁾ We hypothesize that the anti-inflammatory effect of colchicine may be beneficial in I/R injury which is closely related to leucocyte accumulation, apoptosis, and increased tissue ROS levels. Therefore, we aimed to evaluate the protective effect of colchicine treatment on ischemia-reperfusion damage in a testicular torsion rat model by Caspase-3 staining.

MATERIALS AND METHODS

Experimental Design

The study was conducted in Mustafa Kemal University School of Medicine Animal Laboratory by local ethical committee approval (Date and decision number:

2015.02.19 / 8). A total of 40 Wistar albino adult male rats weighing 320-440 g were included in the study. Rats were caged individually in a controlled environment at 20°C to 22°C room temperature, 50% to 55% relative humidity, and light/dark cycles of 12 hours and were fed ad libitum. They were acclimatized for ten days. In all procedures that were applied to the animals according to local ethical committee laboratory rules and rules of Guidelines for the Care and Use of Laboratory Animals of the US National Institutes of Health (Washington, DC) was obeyed.

Rats were randomly divided into five groups (n:8). Intramuscular ketamine hydrochloride (50 mg/kg Ketalar; Eczacıbasi, Istanbul, Turkey) and xylazine hydrochloride were used for anesthesia. Rats were placed in a

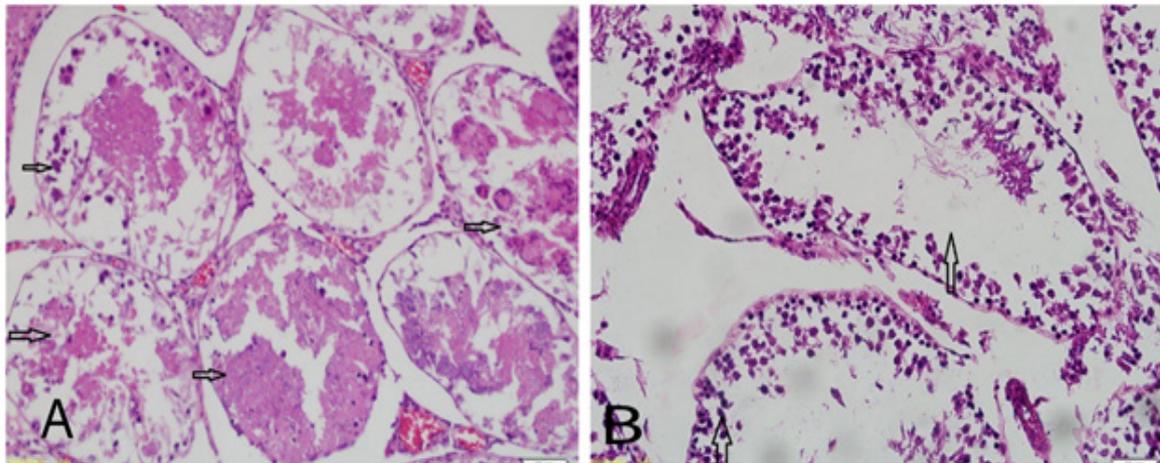


Figure 2. Morphology and spermatogenesis in Group 4, 5. A: Impaired and aborted spermatogenesis (→) with disorganised epithelium, some germ cells absent, necrotic or degenerated in Group 5 (Hematoxylen&Eosinx200). B: Preservation of spermatogenesis in some of the seminifer tubules with regular and compact germ cells (→) in Group 4 (Hematoxylen&Eosinx200).

Table 1. The body and testicular weights of the rats for all 5 groups

	n	Weight g	Testis Weight g
Sham	8	350.5 ± 26	0.72 ± 0.17 ^a
I/R	8	379.37 ± 29	0.58 ± 0.11
I/Rc1	8	350.5 ± 26	0.57 ± 0.09
I/Rc5	8	376.63 ± 30	0.49 ± 0.15
I/Rp	8	367.37 ± 28	0.49 ± 0.08
P values		> 0.005	.009

Findings of the parameters were expressed as mean ± standart deviation. Only statistically significant groups were marked with the letter of the alphabet

^ap = .012 Sham vs I/Rc5; p = .014 Sham vs I/Rp

dorsal recumbent position in a sterile condition. Under anesthesia the scrotal area was shaved and cleaned, disinfected by povidone-iodine solution and a scrotal mid-line incision was done. Torsion was performed by rotating the spermatic cord 720 degrees clockwise along the longitudinal axis and fixed in the scrotal pouch by 5/0 nontraumatic absorbable suture.⁽⁹⁾ The scrotal incision was covered with a moist, warm sterile cover for 3 hours and the surgical technique was the same for all groups. In the Sham group, only a scrotal incision was performed. In I/R group, after 3 hours of testicular torsion, testicle was detorsioned and reperfusioned for 3 hours and then orchietomy was done. In I/Rc1, following 3 hours of ischemia; p.o. 1mg/kg mL colchicine (Recordati Pharmaceutical Industry and Trade Inc., Esenyurt, Istanbul) was given 30 minutes prior to detorsion, and then the testicle was removed after 3 hours of reperfusion. In I/Rc5 group, p.o. 1mg/kg mL colchicine was given 30 minutes before detorsion and continued for five days postoperatively, and the testicle was removed. In I/Rp, all procedures were followed the same as the I/Rc5 group; only placebo (0,2 mL of serum physiologic; %0.9 p.o.) was given instead of colchicine. At the end of the experiment, under the general anesthesia, for histologic and biochemical evaluation 5-6 cc

blood was taken from the heart of the rats and euthanasia was applied by exsanguination. The biochemical examination was performed for determining the oxidative stress levels and studying antioxidant agents in the blood and tissue specimens over MDA, SOD, CAT, and GPx.

Pathology methods

A pathologist -blinded to the study protocol- evaluated prepared slides, the mean seminiferous tubular diameter (MSTD), germinal epithelial cell thickness (GECT), and mean testicular biopsy score (MTBS) were used to evaluate in 20 seminiferous tubules of each section.⁽¹⁰⁾ The MSTD was calculated using an eyepiece micrometer (ZA3262, U-OCMC, 24 mm cross, 10/100X) mounted within one of the eyepiece objectives. At 400X power the field is 0.44 mm x 0.44 mm, yielding an area of approximately 0.19 mm². The MSTD of each testis was determined in microns. GECT was determined by counting the number of epithelial cells from the basement membrane to the lumen at 90°, 180°, 270°, and 360°, and averaged. The MTBS was graded using Johnsen's score.⁽¹¹⁾ A score of 1 to 10 was given to each tubule according to epithelial maturation: 10, complete spermatogenesis, with many spermatozoa, and germinal epithelium organized with a regular thickness, leaving an open lumen; 9, many spermatozoa present but germinal epithelium disorganized with marked sloughing or obliteration of the lumen; 8, only a few spermatozoa (fewer than 5 to 10) present; 7, no spermatozoa but many spermatids present; 6, no spermatozoa and only a few spermatids (fewer than 5 to 10) present; 5, no spermatozoa and no spermatids but several or many spermatocytes present; 4, only a few spermatocytes (fewer than 5) and no spermatids or spermatozoa present; 3, spermatogonia the only germ cells present; 2, no germ cells, but Sertoli cells present; and 1, no cells present in tubular section.

Table 2. Biochemical results of all 5 groups

	n	MDAprot nmol/g	SODprot U/g	CATprot k/mg protein	GPXprot U/ g protein
Sham	8	113.4 ± 22,6 ^a	1401.5 ± 189,5 ^e	0.12 ± 0.017 ^h	277.6 ± 31.6 ^l
I/R	8	336.4 ± 62.8 ^b	790.9 ± 177.2 ^f	0.067 ± 0.011 ⁱ	141.37 ± 26.1 ^m
I/Rc1	8	364.1 ± 65.2 ^c	910.5 ± 136.4 ^g	0.069 ± 0.011 ^j	141.8 ± 31.6 ⁿ
I/Rc5	8	188.1 ± 74.9	1246.4 ± 232.1	0.11 ± 0.019 ^k	215.8 ± 43
I/Rp	8	359.6 ± 87.3 ^d	978 ± 171.1	0.069 ± 0.026	151.4 ± 36.4
P values		< 0.001	< 0.001	< 0.001	< 0.001

MDAprot, Tissue Malonyldialdehyde; SODprot, Tissue Superoxide Dismutase; CATprot, Tissue Catalase; GPXprot, Tissue Glutathione Peroxidase.

Findings of the parameters were expressed as mean ± standart deviation. Only statistically significant groups were marked with the letter of the alphabet.

^ap < 0.001 Sham vs I/R; p < 0.001 Sham vs I/Rc1; p = .001 sham vs IRp

^bp = .007 IR vs IRc5

^cp = .002 I/Rc1 vs I/Rc5

^dp = .008 I/Rp vs I/Rc5

^ep < 0.001 Sham vs I/R; p < 0.001 Sham vs I/Rc1; p = .003 sham vs IRp

^fp = .006 IR vs IRc5

^gp = .04 I/Rc1 vs I/Rc5

^hp < 0.001 Sham vs I/R; p < 0.001 sham vs I/Rc1; p < 0.001 sham vs IRp

ⁱp = .004 IR vs IRc5

^jp = .005 I/Rc1 vs I/Rc5

^kp = .006 I/Rp vs I/Rc5

^lp < 0.001 Sham vs I/R; p < 0.001 Sham vs I/Rc1; p < 0.001 Sham vs IRp

^mp = .01 IR vs IRc5

ⁿp = .0016 I/Rc1 vs I/Rc5

Table 3. Histopathologic results of all 5 groups

	n	Johnsen Score	GECT	MSTD
Sham	8	9.6 ± 0.52 ^b	8.5 ± 0.53 ^f	12.5 ± 1.93 ^j
I/R	8	7 ± 0.54 ^c	7.1 ± 0.84 ^g	9.1 ± 1.36 ^k
I/Rc1	8	6.6 ± 0.52 ^d	6.9 ± 0.64 ^h	9.4 ± 0.91 ^l
I/Rc5	8	6.4 ± 0.74 ^e	6.6 ± 0.74 ⁱ	7.1 ± 0.84 ^m
I/Rp	8	4.3 ± 0.52	4.5 ± 1.07	4.9 ± 0.84
P values		< 0.001	< 0.001	< 0.001

GECT, Germinal Epithelial Cell Thickness; MSTD, Mean Seminiferous Tubular Diameter

Findings of the parameters were expressed as mean ± standard deviation. Only statistically significant groups ($p=0.005$) were marked with the letter of the alphabet

^b $p < 0.001$ Sham vs I/R; Sham vs I/Rc1; Sham vs I/Rc5; Sham vs IRp

^c $p < 0.001$ I/R vs I/Rp

^d $p < 0.001$ I/Rc1 vs I/Rp

^e $p < 0.001$ I/Rc5 vs I/Rp

^f $p = .018$ Sham vs I/R; $p = .001$ Sham vs I/Rc5; $p < 0.001$ Sham vs IRp

^g $p = .001$ I/R vs I/Rp

^h $p < 0.001$ I/Rc1 vs I/Rp

ⁱ $p = .005$ I/Rc5 vs I/Rp

^j $p = .013$ Sham vs I/R; $p = .017$ sham vs I/Rc1; $p < 0.001$ sham vs I/Rc5; $p < 0.001$ Sham vs IRp

^k $p = .036$ I/R vs I/Rc5; $p < 0.001$ I/R vs I/Rp

^l $p = .001$ I/Rc1 vs I/Rc5; $p < 0.001$ I/Rc1 vs I/Rp

^m $p = .001$ I/Rc5 vs I/Rp

Immunohistochemistry

Section of 3-4 mm thickness was cut from the paraffin blocks of these preparations and then was de-paraffinized and rehydrated through a graded series of alcohol, microwave antigen retrieval method was used, endogenous peroxidase was blocked in 5% H₂O₂ at room temperature for 8 minutes, followed by washing with PBS three times. The slices were then incubated with Caspase 3 (CPP32-Ab-4) (Prediluted Polyclonal Rabbit primer antibody, Thermo Scientific, Fremont, CA, USA.) Immunohistologic staining (IHS) was applied, followed by washing with PBS three times. Subsequently, the slices were incubated with biotinylated goat anti-polyvalent/labvision secondary antibodies at room temperature for one h, washed with PBS three times, followed by incubation with DAB reagent for 8 min at room temperature. Nuclear staining in tonsil tissue was accepted as the positive control. Caspase-3-positive cells were count in one mm² of the tissue modified from the study of Mosadegh et al.⁽¹²⁾

Biochemical methods

Testis tissues were weighed and homogenized in ice-cold phosphate-buffered saline at pH 7.4 (10% w / v). After centrifugation at 10 000 rpm for 20 minutes, all supernatants were removed for biochemical analysis. Protein levels of supernatants and homogenates were measured by the Bradford method using bovine serum albumin as a standard.⁽¹³⁾

The MDA levels of homogenates were measured by the double heating method of Draper and Hadley.⁽¹⁴⁾ MDA equivalents (1,1,3,3-tetramethoxypropane, Lot no, MK-BP9901V, Sigma-Aldrich) were used as standards, and MDA results were expressed as nmol / g-protein.

Catalase activities were assayed by the Aebi method.⁽¹⁵⁾ The decomposition of the substrate H₂O₂ was spectrophotometrically monitored at 240 nm (Shimadzu UV 1601, Japan). Absorption reduction was measured and expressed as k / mg protein.

GSH-Px activity was measured by Paglia and Valentine method.⁽¹⁶⁾ The enzymatic reaction was initiated by the addition of H₂O₂ to the reaction mixture containing reduced glutathione, reduced nicotinamide adenine

dinucleotide phosphate and glutathione reductase. A spectrophotometer monitored the absorbance change at 340 nm. One unit of GSH-Px is defined as NADPH micromoles oxidized per minute. The activity was given as units per g protein.

Total (Cu-Zn and Mn) SOD activity values were obtained from Sun et al.⁽¹⁷⁾ and Durak et al.⁽¹⁸⁾ A unit of SOD, nitro blue tetrazolium (NBT), was defined as the amount of enzyme causing 50% inhibition at the reduction rate. The results are expressed in units per g protein.

Statistical Analysis

Kolmogorov-Smirnov test was performed to determine whether the distribution of the data obtained was normal or not. Weight, testicular weight GECT, MSTD, Caspase 3 staining, MDAprot, SODprot, CATprot and GPXprot values were analysed with one-way analysis of variance and as a post-hoc test Tukey's was used to determine the group that caused statistical difference between groups. Kruskal Wall Test was used to analyse Johnsen score values. Bonferonni corrected Mann-Whitney U test was used to compare two by two groups regarding to Johnsen scores to determine statistically different groups.

Each test group was compared with the appropriate control groups and P values less than 0.05 were considered as significant except Johnsen groups where $p = 0.005$ were considered as significant. The results were expressed as mean + standard error mean or median and 25-75 percentile values. SPSS v21.0 program was used for statistical analysis.

RESULTS

The mean body and testicle weights of the rats were not statistically different between groups. (Table 1) The biochemical and histopathological findings were given in Table 2, and 3.

In the 5-day colchicine given the group, the protein MDA levels were significantly lower than I/R and I/Rp groups ($p < 0.002$) which were reached the significantly highest levels.

GPx, CAT, and SOD levels were higher in Sh and I/Rc5 groups ($p < 0.05$). Also, antioxidant levels (SOD, CAT) in I/Rc5 group did not differ from the Sh group statistically ($p > 0.05$).

As a positive indicator of apoptosis, Caspase-3 positivity was higher in group I/R, I/Rc1, and I/Rp than group Sh and I/Rc5. I/Rc5 group had lower Caspase-3 positivity than group I/R ($p < 0.05$). (Figure 2)

When comparing Johnsen's scores in I/Rc5 group with I/R and I/Rc1 groups, there was no statistical difference ($p = .211$ and $p = .905$, respectively) that implies to an alleviation in tissue deterioration. Also, I/Rp group's Johnsen's scores were significantly lower than the others ($P < 0.001$).

The mean seminiferous tubular diameter and germinal epithelial cell thickness were affected negatively in all groups but group Sh. With the use of colchicine in the postoperative period, in I/Rc5 group, MSTD and GECT measurements were significantly better than group I/Rp ($P < 0.005$) which showed the natural course of ischemic events and not statistically differed from the group I/R. (Figure 1)

DISCUSSION

Testicular torsion is a urological urgency that's incidence peaks in 2 periods of life (neonatal and prepubertal), resulting in testicular infarct if not immediately treated. The first-line treatment is surgery⁽¹⁹⁾, but testicular salvage and postoperative changes do not only depend on operation. Because atrophy can be seen in one-fourth of the torsion cases that are treated in time.⁽²⁰⁾ Logically an additional medical salvage therapy should be considered in the postoperative period. In this respect, numerous substances like vardenafil, sildenafil, rosuvastatin, Coenzyme Q10, different surgical, and interventional techniques showed positive results on preserving testis from ischemia-reperfusion damage.⁽²²¹⁻²³⁾ However, none of these studies discuss their subjects for postoperative usage. From this point of view, our study revealed that oxidative stress was reduced in rats which colchicine usage was started preoperatively and continued for five days after the operation. Additionally, the levels of anti-oxidative agents such as SOD, CAT, and GPx kept their levels in 5-day colchicine group postoperatively. Total antioxidant capacity and total oxidative stress findings were also significantly better with the short term colchicine usage ($p < 0.005$). This favorable effect of colchicine may be explained with leucocyte interaction. Chappey et al.⁽²⁴⁾ determined the colchicine deposition dynamics in leucocytes and revealed that colchicine rises to the plasma peak level at 1-hour after application and accumulates mostly in leukocytes. Mitsui et al.⁽²⁵⁾ showed increased major proinflammatory cytokines which lead to leucocyte accumulation in the tissues in the first hours of the torsion. So colchicine could be easily accumulated in migrated leucocytes. After this, the main anti-inflammatory effect of colchicine occurs by inhibiting the assembly and polymerization of microtubules which are the keystones in cell migration, secretion of cytokines, maintenance of the cytoskeleton and cell shape.⁽²⁶⁾

Additionally, colchicine suppresses the TNF-alpha production from macrophages that generated after tissue necrosis induced by lipopolysaccharides.^(27,28) In the present study, short term colchicine treatment group; SOD, CAT, and GPx molecules which are endogenous

antioxidants commissioned to protect the steady-state of the cell against oxidative stress were found significantly higher as the sham group ($p < 0.005$). The high levels of endogenous anti-oxidants can be explained by the suppression of inflammation, which is triggered by many stress pathways, rather than increasing the generation of these molecules.

The other possible protective mechanism of colchicine may be related to lipid peroxidation, cytosolic Ca+2, and oxidative stress. Testicular injury increases linearly with the degree and duration of torsion.⁽²⁹⁾ Due to ischemia, new acidic environment induces Ca+2 influx into cells in various ways. Increased intracellular Ca+2 levels trigger the activation of inflammatory markers and cell death especially over mitochondria.^(30,31) At this point, Korkmaz et al.⁽³²⁾ reported that colchicine has a reducing effect on cytoplasmic Ca+2 release in neutrophils. In this way, colchicine reduces the oxygen radicals generated by mitochondria which are most damaging cellular macromolecules, lipids in particular. One of the most critical indicators of lipid peroxidation and the secondary index of oxidative stress is MDA.⁽³³⁾ MDA interacts with DNA and proteins per the aldehyde component, which is toxic to the structure at high levels and causes irreversible damage linked to DNA fragmentation, protein denaturation. We found that MDA levels were significantly lower in the colchicine-treated group compared to other untreated groups ($p < 0.005$).

Caspase is one of the cysteine endoprotease families and plays a regulatory role in cell death and inflammation.⁽⁶⁾ They have classified into two groups; apoptotic and inflammatory, and apoptotic caspases work as initiator or executioners. Caspase 3 is a well-known executioner apoptotic caspase and proteolytically processed to the active form that is essential for apoptosis.⁽³⁴⁻³⁶⁾ The active form of caspase 3 causes cell death by DNA damage and protein degradation.⁽³⁷⁾ In our study, we found that Caspase 3 staining in the 5-day colchicine-treated group was significantly lower ($P < 0.005$) when compared to groups without colchicine treatment and this score was not statistically different from the sham group, and even lower scores were obtained. This finding also emphasizes the less DNA fragmentation and cell death with short term colchicine usage like forementioned MDA. Colchicine may trigger controlled apoptosis by stopping the cell cycle in the G2/M phase and contribute to planned cell death by microtubule depolymerization.⁽²⁶⁾

Because of this feature, it has been tried for some cancer treatments⁽²⁶⁾ but not preferred due to its high side-effect profile. However, with the secondary necrosis or apoptosis which is seen in testicle torsion, the inflammatory, and immunogenic activity of colchicine become prominent; thus uncontrolled apoptosis is precluded⁽³⁸⁾.

To our knowledge, long term usage of colchicine harms sperm cell maturation.⁽³⁹⁾ However, the sustained benefit of short-term colchicine treatment on torsioned-detorsioned testis survival is unknown and side effects arising from short term usage are still presumptive. In our study, when compared with the placebo group the Johnsen score was better with 5-day colchicine treatment group. Similar results were found with the GECT and MTBS scores. The most significant damage to testicular tissue was created with the activation of inflammatory and apoptotic cascades.⁽⁴⁰⁾ These results have allowed us to concentrate on the potent an-

ti-inflammatory effect of colchicine. In an overall perspective, the benefits outweigh the possible danger of short-term colchicine treatment in patients with torsion. Experimental design and relatively small sample size are the major limitations of our study. However, this is one of the few studies related to colchicine treatment on testicular torsion.

CONCLUSIONS

Colchicine is a highly active and fruitful molecule on ischemia-reperfusion injury in testicle torsion animal model with short-term usage and may be a convenient option for this patient group over its noticeable anti-inflammatory effect. But further experimental animal studies are required to determine the drug dose, duration, and way of administration of colchicine in patients with testicular torsion.

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