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The ameliorative effects of *Ginkgo biloba* on apoptosis, LH-R expression and sperm morphology anomaly in testicular torsion and detorsion

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Summary

Torsion/detorsion (T/D) induces testicular damages in both germinal epithelial and interstitial tissues. *Ginkgo biloba* extract (GbE) exerts antioxidant and free radical scavenger. We investigated the effect of GbE on testicular tissues, Leydig and sperm cells in rats injured with T/D. Twenty-eight Wistar albino rats were randomly assigned into four groups (Control, GbE, Treatment: T/D+GbE, T/D). T/D performed to the rats in torsion, treatment received GbE (50 mg/kg) 1 hr before T/D, GbE group received only GbE (50 mg/kg) and control was defined as sham group. After T/D, the testes along with epididymis were removed and processed. LH-R expression, apoptosis, sperm morphology and histopathological damage scores were determined for each group. Testicular T/D caused significant increases in apoptosis and sperm morphology anomaly, and a significant decrease in Johnsen's testicular biopsy scores, LH-R expression of Leydig cell and normal sperm cell count. GbE ameliorated testicular histopathology and caused significant increases in LH-R expression, normal sperm cell count in the treated and particularly GbE group. Consequently, GbE may prevent testicular injury and enhance Leydig and sperm cell activity following both T/D and normal situation owing to its antioxidant, anti-apoptotic, free radical scavenger and anti-inflammatory effects.

KEYWORDS
Ginkgo biloba extract, leydig, sperm, testis, torsion

1 | INTRODUCTION

Testis torsion (TT) is a case that is known worldwide as one of the reasons for male infertility which requires urgent surgical intervention. In TT, as a result of the spermatic cord turning vertically around its axis, blood flow is obstructed firstly by venous obstruction and then by stoppage of blood flow, and testes become ischaemic (Sekerci et al., 2017; Zhao, Lautz, Meeks, & Maizels, 2011). If TT is not immediately intervened with a surgical operation and treated, it may lead to the loss of the affected testis (Mellick, 2012) and a set of andrological issues, especially infertility, in connection with the ischaemia of germ cells (Kostakis et al., 2017; Ringdahl & Teague, 2006). TT is usually seen in children and more frequently in adolescents. Annual incidence of spermatic cord torsion is 3.8–4.5 in 100,000 males 1-25 years of age (Pogorelić, Mrklić, & Jurić, 2013). If treated within 6 hr of the presenting pain, there is a good chance of saving the affected testicle, as 90%–100% testicles will be saved. If treated within 6 hr-12 hr, depending on degree of the torsion, 20%–50% testicles will be saved and if treated within 12 hr-24 hr 0%–10% testicles will be saved (Pogorelić et al., 2016).

T/D is in fact an ischaemia-reperfusion issue. During ischaemia, low levels of oxygen in comparison with the needed amounts, reduction in cellular energy storage and accumulation of toxic metabolites lead to germ cell deaths (Kostakis et al., 2017; Mogilner et al., 2006).

With progression of this process, disruption of spermatogenesis is followed by toxic molecules joining the circulation. Serious ischaemic testis damage occurs in 4–8 hr after torsion and while possibility of saving the testis decreases as the time between T/D increases, it decreases to 90% after the 6th hr, to 50% after the 12th hr and to 10% after the 24th hr (Kostakis et al., 2017; Reyes et al., 2012).

Reperfusion also leads to the production of a set of proinflammatory cytokines such as interleukin-1β and tumour necrosis factor-α that lead to reinforcement of neutrophils and macrophages that infiltrate into the testicular parenchyma (Kostakis et al., 2017). Reperfusion, in connection to the increase in reactive oxygen species (ROS) in the tissue, leads to DNA damage in testicular cells, disruption of membrane permeability and integrity as a result of mitochondria and cell membrane lipid peroxidation, and protein damages (Shimizu et al., 2016). These pathophysiological changes lead to testicular atrophy, germ cell apoptosis and Sertoli cell function damage (Kostakis et al., 2017; Lysiak, 2004) and may lead to disruption of normal sperm production and functions at the end. It was also reported that autoantibodies that are produced against damaged sperm cells and tubular basal membrane in the ischaemic testis lead to damages in the other testis (Ringdahl & Teague, 2006).

While spermatogenic cells are sensitive for ischaemia, Leydig cells are more resistant. These cells are the main source of the androgenic hormone, testosterone, which is essential for male sexual differentiation, gamete production and maturation, and development of secondary sexual characteristics. Leydig cells described by the German histologist Franz Leydig in 1850 (Dong & Hardy, 2004) are located in the interstitial compartment of the testis. Foetal, neonatal and adult-type Leydig cell generations are responsible in testicular steroidogenesis in these periods of life. Foetal Leydig cells start to be seen among undifferentiated mesenchymal cells in the 8th week. Neonatal Leydig cells start in the first year of life and continue until they are replaced by adult Leydig cells at the ages of 10 to 13. Adult Leydig cells increase in puberty and reach maximum in the age of 20. They are in a certain balance in the ages of 20 to 60 (Dong & Hardy, 2004; Huhtaniemi & Pelliniemi, 1992; Teerds, de Boer-Brouwer, Dorrington, Balvers, & Ivell, 1999). Leydig cells need gonadotropins (Lutein hormone (LH) and follicular stimulant hormone (FSH)) to be able to differentiate and continue normal development and functions. LH stimulates Leydig cells via LH receptor for production of testosterone that is needed for the development of normal spermatogenic serial cells (Dong & Hardy, 2004). In addition to cell damage, TT may lead to a set of androgenic issues by causing irregularities in this hormonal axis.

Although detorsion is necessary to return blood flow, reperfusion to the ischaemic tissues may exaggerate the injury. Therefore, several studies have examined ischaemia-reperfusion injury and efficiency of treatment with potential therapeutic agents in T/D with free radical scavengers, antioxidant drugs, anti-inflammatory drugs, nonsteroidal anti-inflammatory drugs. *Ginkgo biloba* extract (GbE) is a standardised mixture that contains quercetin, flavonoid and terpenoid substance (Wang et al., 2008). GbE, due to its high antioxidant, anti-apoptotic and free radical scavenging activity (Yeh et al., 2009), is considered to protective and be therapeutic against ischaemia/reperfusion injuries and other oxidative stress in various tissues and organs (Akgul et al., 2008).

Thus, this study aimed to evaluate the protective and therapeutic role of GbE against testicular tissues, Leydig and sperm cells in rats injured ischaemia/reperfusion after testicular T/D.

2 | MATERIALS AND METHODS

In the light of these knowledge, we can hypothesis that GbE is a natural antioxidant molecule and it can be protective and curable against testicular injury and related abnormalities. T/D is ischaemia and reperfusion that can cause serious damage to reproductive system tissue structure and activity. Testicular tissues, sperm production and Leydig cell activity may negatively affect from T/D. The combination of all these negativities can also result in infertility. Primary outcome measurement of this study is to ameliorate these hazardous effects with GbE administration. Secondary outcome of our study is to treat T/D with natural antioxidant molecules like GbE instead of chemical agent and drugs.

2.1 | Animals

After animal experimental protocol had been approved by the Animal Ethics Committee of Gaziosmanpasa University, this study was started. Twenty-eight adult male Wistar rats (each weighing 300–400 g) were randomly divided into four groups (Control, GbE, Test and Torsion) seven rats in each. The rats were housed at 22–24°C with a 50%–65% relative humidity under a 12-hr light/dark cycle and had free access to food and water. The experiments were conducted in strict accordance with the Guide of the National Institutes of Health for the Care and Use of Laboratory Animals.

2.2 | Experimental design

Control group rats were not subjected to any experimental process. The GbE group rats were only given *Ginkgo biloba* as in the GbE group without any additional treatment. The left testes of the T/D and treated (T/D+GbE) group rats were rotated clockwise 720° for TT. In the T/D+GbE group, GbE was given one hour before torsion (50 mg/kg, orally) and then TT was made. The test and torsion groups were exposed to 3 hr of torsion followed by two hours of detorsion. The control and T/D groups were given isotonic NaCl (saline) instead of GbE in the same amount and with the same method. At the end of the experiment, all rats were sacrificed by removing their testes along with their epididymis. The epididymis was removed from the testes at the point of attachment. After the testes were left for two days in Bouin fixation solution, they were embedded in paraffin blocks. Epididymal semen content was diluted one-to-one by physiological saline solution and sperm smear preparation was created. About 5 μm-thick sections were obtained from the blocked testes using a rotary microtome (LeicaRM2135, Germany). The sections were used in the histopathological, immunohistochemical and apoptotic analyses.

Dispersed sperm samples were used in morphological analyses. To avoid observer's bias, an independent person coded the slides before subjecting them to evaluations.

2.3 | Histological evaluation

Thin sections were stained with haematoxylin-eosin and analysed for the general architecture, spermatogenesis, seminiferous tubules epithelial tissues and interstitial connective tissues. Seven rats from each group and ten sections and twenty-five seminiferous tubules in each testis were evaluated, and the mean Johnsen score was calculated. For seminiferous tubules, histopathological analyses modified Johnsen scoring criteria system was used (Johnsen, 1970). Histological criteria for the modified Johnsen Scoring are stated in Table 1.

2.4 | Immunohistochemistry

Five-μm-thick sections, placed on slides with adhesive, were deparaffinized with xylene and hydrated with ethanol from 99, 96%–70% and distilled water. Then, the sections were boiled in a 10-mM sodium citrate buffer at a pH of 6.0 and maintained at a sub-boiling temperature for 5 min for antigen unmasking; then, the sections were cooled immediately with distilled water, incubated for 15 min in 3% hydrogen peroxide and then washed with phosphate-buffered saline (PBS, 3 × 5 min). The sections were encircled with a hydrophobic pap pen. After incubation in blocking serum (nonimmune) at room temperature (15 min), primary antibodies anti-hCG-R (LH-R) (1:500, Abcam) were added to each section and incubated overnight at 4°C in a moist, dark environment. Next, the sample was stabilized at room temperature (30 min) and washed with PBS (3 × 5 min). The sections were then incubated for 30 min with the biotinylated secondary antibodies and washed with PBS (3 × 5 min). Then, streptavidin-marked secondary antibody was added to each section. Sections were incubated for 30 min in a dark humidified atmosphere and then washed with PBS (3 × 5 min). DAB chromogen was applied to each section then washed in water and counterstained with haematoxylin and washed again. Finally, slides were mounted with the sections with coverslips. For the negative control, PBS was added to the sections instead of a primary antibody; the other steps were the same and no immunostaining was observed (Figure 1e).

TABLE 1 Histological criteria for the modified Johnsen Scoring system (Johnsen, 1970)

Score	Histological criteria
1	No cells within seminiferous tubules.
2	No germ cells, there are only Sertoli cells.
3	Only spermatogonia as germ cells.
4	No spermatozoa and spermatids, there are a few spermatocytes.
5	No spermatozoa and spermatids, there are many spermatocytes.
6	No spermatozoa, and there are a few (less than 10) spermatids.
7	No spermatozoa, there are no late spermatids but many early spermatids
8	Late spermatids without any mature spermatozoa.
9	Slightly impaired spermatogenesis, many late spermatids, disorganised epithelium.
10	Full spermatogenesis.

2.5 | Immunohistochemistry analysis

Six randomly selected fields were evaluated on each slide using a 40× objective. Two blinded histologists completed the immunostaining intensity score quantification, and the average of the scores was used for subsequent analysis. For quantitative evaluation of the immunohistochemical data, H-Score analysis was used as previously described. LH-R Immunoreactivity was scored according to a scoring scale (Table 2).The H-Score was calculated as the sum of proportion of cells in each staining intensity category multiplied by the weighed intensity score as follows: H-Score $=\Sigma Pi(i+1)$, where i: intensity score and Pi: proportion of cells (Cheon, Lee, Parhar, & Kang, 2001; Gevrek, Öncü, Gülle, Bayram, & Karaöz, 2016; Godbole, Modi, & Puri, 2007).

2.6 | TUNEL staining

To evaluate the extent of apoptosis in the testicular cells, staining was performed using a TUNEL staining assay kit (in situ cell death detection kit, AP; Roche) according to the manufacturer's instructions. Briefly, five-μm-thick sections from paraffin-embedded tissues were deparaffinized in xylene and rehydrated. They were subjected to 750W microwave irradiation (1 min) in 0.1M citrate buffer (pH6.0), cooled rapidly, transferred to PBS, immersed for 30 min at room temperature in Tris-HCL. After being rinsed with PBS and allowing the excess fluid to drain off, 50-μl of the TUNEL reaction mixture was added to the sections. For the negative control, a 50-μl label solution was added, and slices were incubated for 60 min at 37°C in a humidified atmosphere in the dark. After being rinsed with PBS (3 × 5 min) three times and dried, 50 μl Converter-AP (vial-3) was added to the samples. They were incubated in a humidified chamber for 30 min at 37°C and then rinsed with PBS (3 × 5 min); a 50–100 μl substrate solution (Fast Red) was added, and the slices were incubated for 10 min at 15–20°C in the dark. After being rinsed with PBS (3 × 5 min), the slides were counterstained with haematoxylin and mounted under a glass coverslip. For the TUNEL reaction, the haematoxylin-stained cells served as controls, and red-stained nuclei cells were apoptotic (Figure 2). Five slides from each group were used to count apoptotic cells. The apoptotic index was evaluated as a percentage of the apoptotic and control cells (Soyalıç, Gevrek, & Karaman, 2017; Soyalıç et al., 2016).

2.7 | Sperm analysis

Epididymal semen smears were stained using the Diff-quick staining method. For this, the slides were left for 1-2 min in 4%-buffered neutral formalin. Then, they were kept in the fixative of the Diff-quick staining set for 10 min, and they were then left in eosin and azure

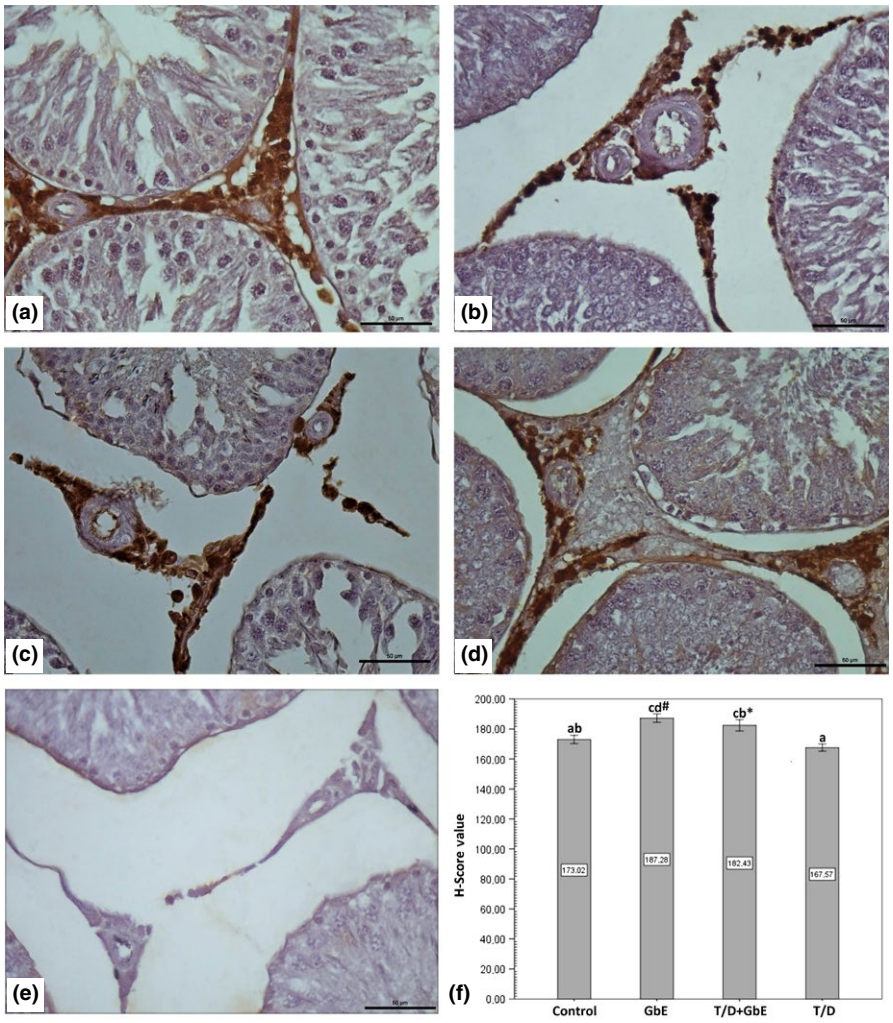

FIGURE 1 Immunohistochemistry of LH-R in rat testes. (a): Control group with strong expression; (b): GbE and (c): T/D+GbE groups with increased strong expression respectively; treated group showing a significant increase in LH-R immunostaining; (d): T/D group showing a slightly decrease in LH-R immunostaining; (e): Negative control section for immunohistochemical analyses, no immunostaining cells were seen in the sections (DAB, Scale bars: 50 μm). (f): Comparison of the H-Score values of LH-R in groups. The data are represented as mean ±*SEM*. * and #: $p < .05$, versus control and T/D (Tamhane test). The same letter on the bars shows statistical similarities

TABLE 2 Criteria for scoring the immunoreactivity of anti-hCG-R (LH-R) in the rat testis

Score	Immunoreactivity
0+	Negative staining
1+	Weak staining
2+	Moderate staining
3+	Strong staining

solutions for 10 min each and passed through distilled water. They were dried, treated with entellan and covered with cover glass. In these slides, the sperms were analysed in terms of head, neck and tail anomalies under 100× magnification rate with a microscope based on the modified Kruger strict morphology analysis criteria and taking the 2010 decisions of the World Health Organization (WHO) as a reference. For each animal, two different counts with at least 200 cells in each were made, and the average of these was taken.

2.8 | Statistical analysis

All statistical analyses were conducted using the Statistical Package for the Social Science (SPSS) for Windows, version 20 (SPSS, IBM Co., Somers, NY, US). Distribution normality was confirmed by the Kolmogorov–Smirnov test. According to normality test results, One-way ANOVA and the Kruskal–Wallis *H* test were used for statistical analysis as appropriate. All data were represented as mean ± standard error. Sperm morphology analysis results were compared using Tukey HSD test; Johnsen scores were compared using LSD; Apoptotic indices were compared using Tukey HSD test. H-Score values of LH-R were compared using Tamhane test. $p < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Histopathological results

In the control group, seminiferous, tubular and interstitial tissues had a normal appearance (Figure 3a). Therefore, the Johnsen scores' weighted group average was naturally high. The testicular tissue histology of the GbE group was similar to the control group. The seminiferous tubules in both groups were on normal levels, Sertoli cells and spermatogenic serial cells were all present in the epithelium; there were multiple spermatozoa in the lumen of the tubules. The interstitial tissues also had a normal histological order (Figure 3b). The weighted

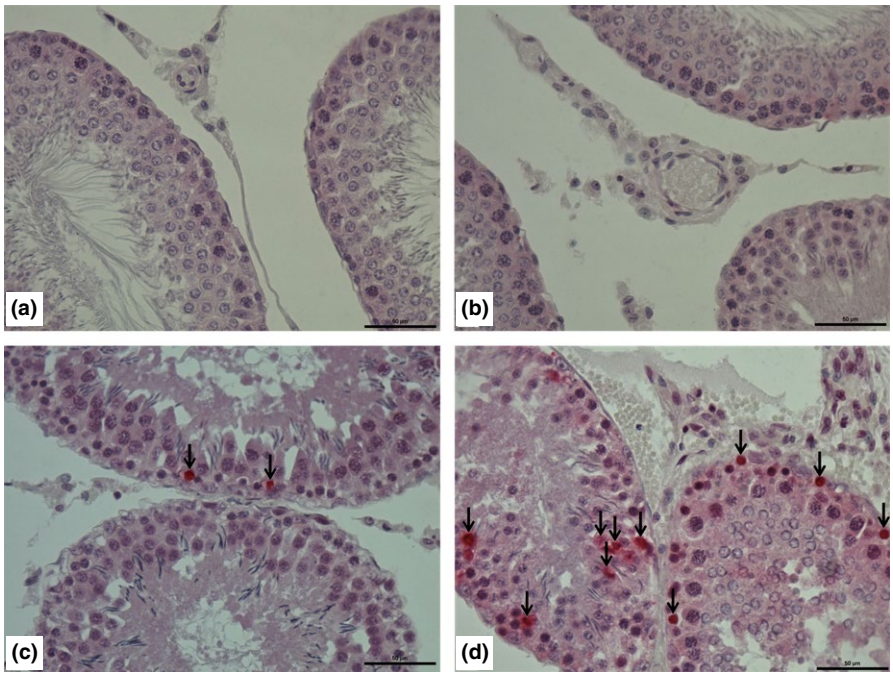

FIGURE 2 Representative photomicrographs showing apoptotic cells from each group. Arrows point apoptotic cells. (a): Control, (b): GbE, (c): Treated, (d): Torsion (TUNEL, Scale bars: 50 μm)

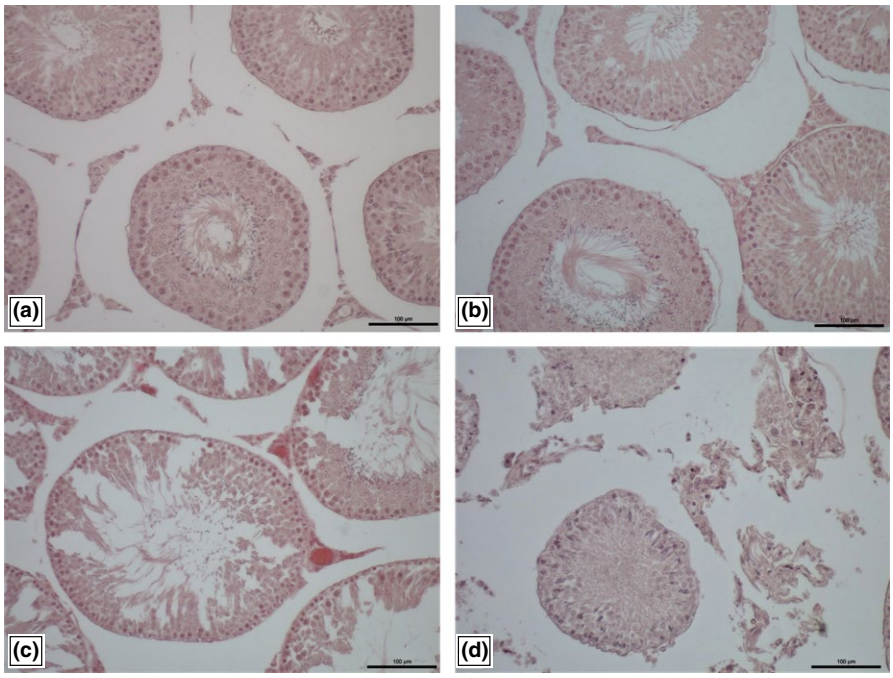

FIGURE 3 Representative photomicrographs showing histological injury of testicular tissues from: (a) Control and (b) GbE groups showing normal histology of testicular tissues; (c) Treatment (T/D+GbE) group showing marked improvement with minimal damage; (d) Torsion detorsion (T/D) group showing significant histological damage of testicular tissues (H&E, Scale bars: 100 μm)

Johnsen score average of the group was similar to the control group ($p > .05$) (Table 3A).

In the T/D group, there were irregularities, degeneration and losses in seminiferous tubule epithelium cells. Most seminiferous tubules' integrity was compromised, some of them were completely eroded, and some of them were emptied or left with very few cells. Additionally, there were highly deflated tubules, tubules containing no or almost no spermatozoa in their lumen and coagulative necrosis in the tubule lumen. Moreover, basal lamina detachments were observed around the tubules. In the interstitial area among the tubules, there were tissue dilatations and occasional severe oedema, gathered blood cells on the floor, vascular dilatations and congestions in veins (Figure 3d). In connection to these findings, the Johnsen score average of the group was significantly lower than those of all the others ($p < .05$) (Table 3A).

The histological damage observed in the T/D group was reduced in the T/D+GbE group. While there were occasional distortions in the seminiferous tubules and interstitial tissue integrities, the damage was not as severe as the one in the T/D (Figure 3c). In addition to these histopathological findings, the weighted Johnsen score average of

TABLE 3 (A) Mean Johnsen score for the different groups. (B) Apoptotic cell index group mean values (±: SEM, API: Apoptotic index, Sc: score.)

		Control	GbE	T/D+GbE	T/D
(A)	JHONSEN Sc. (min-max)	9.67 ± 0.01^{a} (9.74-9.56)	9.36 ± 0.02^{a} (9.54-9.20)	$8.85 \pm 0.13^{b,*}$ (9.28-7.66)	8.19 ± 0.07^{c} (8.57-7.68)
(B)	API (min-max)	0.82 ± 0.07^{A} (1.00-0.60)	1.22 ± 0.07^{A} (1.60-1.00)	$1.34 \pm 0.12^{AB*}$ (1.68-1.00)	3.36 ± 0.16^{C} (4.40-2.64)

Lowercase superscript represents differences in A line (LSD test) and uppercase superscript represents differences in B line (Tukey HSD test). No significant differences between groups having same superscripts.
*$p < .05$.

TABLE 4 Mean values of Kruger strict morphology analysis in groups (±: SEM)

		Sperm Anomaly			
	Normal	Head	Neck	Tail	Total
Control	108.00 ± 3.54^{A}	16.00 ± 1.93^{A}	20.86 ± 1.58^{A}	55.43 ± 2.25^{A}	92.29 ± 3.56^{A}
GbE	$123.88 \pm 3.32^{B\#}$	16.63 ± 1.21^{BC}	16.13 ± 1.81^{BA}	$43.25 \pm 3.40^{B\#}$	$76.13 \pm 3.32^{B\#}$
T/D+GbE	$94.17 \pm 2.39^{C*}$	17.33 ± 3.30^{BC}	32.33 ± 3.03^{CD}	$56.50 \pm 2.40^{A*}$	$106.17 \pm 2.63^{C*}$
T/D	46.13 ± 1.96^{D}	23.88 ± 1.88^{C}	37.25 ± 2.20^{D}	92.88 ± 2.09^{C}	154.00 ± 1.98^{D}

Uppercase superscript represents differences and similarities of colons for each parameter.
No significant differences between groups having same superscripts.
*$p < .05$ versus torsion.
#$p < .05$ versus control (Tukey HSD test).

the group was lower than the control, but higher than the T/D. It was significantly different from the control and from the T/D towards the favour of the control ($p < .05$) (Table 3A).

3.2 | Tunel assay results

Table 3B shows the mean results of the groups' apoptotic cell indices. As seen in these results and in a way that corresponds to the histopathological results, the apoptotic cell indices were the highest in the T/D group. It was found to be significantly different from all the other groups ($p < .05$).

The control group had the lowest apoptotic cell index. While the apoptotic cell index seemed a little bit higher in the GbE group, it was statistically similar to the control group ($p > .05$). In the T/D+GbE group, apoptotic index was higher than those of the control group and lower in comparison with the torsion group. It was significantly different from T/D ($P > 0.05$), but similar to the other groups ($p > 0.05$) (Table 3B and Figure 2).

3.3 | Immunohistochemistry results

As expected, it was observed that immune reactivity was in the interstitial tissue Leydig cells (Figure 1). Immune staining degree H-Score weighted group average results are given in Figure 1f. The H-Scores were lower in the torsion group but not significantly different from the control group ($p > .05$). Immune reactivity was higher in the treatment group and similar to the control group ($p > .05$) while the treatment group differed from torsion significantly ($p < .05$). The immune reactivity in the GbE group was the highest, while it was similar to the treatment group ($p > .05$) and significantly different from the others ($p < .05$). Therefore, its immune staining degree H-Score average was also high. It was statistically similar to the treatment group, but different from the other groups (Figure 1a–d,f).

3.4 | Sperm morphology results

Table 4 shows the results of the microscopic morphology analysis, and Figure 4 shows the representative microscopic image of the groups. Normal spermatozoa were significantly lowered in the torsion group in comparison with other groups (p: .001), and increased in order in the treatment, control and GbE groups. The increase in the GbE group was statistically significant based on control (p: .003). While there was a reduction in the treatment group in comparison with the control (p: .019), there was a significant increase in comparison with the torsion group (p: .000).

Spermatozoa with head anomaly increased only in the torsion group in comparison with the control (p: .050), and there was no significant difference in other groups (Table 4). The number of spermatozoa with neck anomaly significantly increased in the torsion group in comparison with the control and GbE groups (p: .001). There was a statistically insignificant decrease in the treatment group in comparison with torsion (p: .410). The control and GbE groups were similar (p: .406). The number of spermatozoa with tail anomaly increased significantly in torsion in comparison with the others (p: .000). In the treatment group, the values were significantly lower than the torsion group (p: .001) and slightly and insignificantly lower than the control (p: .993). The lowest level of tail anomaly was in the GbE group (Table 4). The groups were statistically different from each other, considering the anomalies in total. Total anomaly was significantly

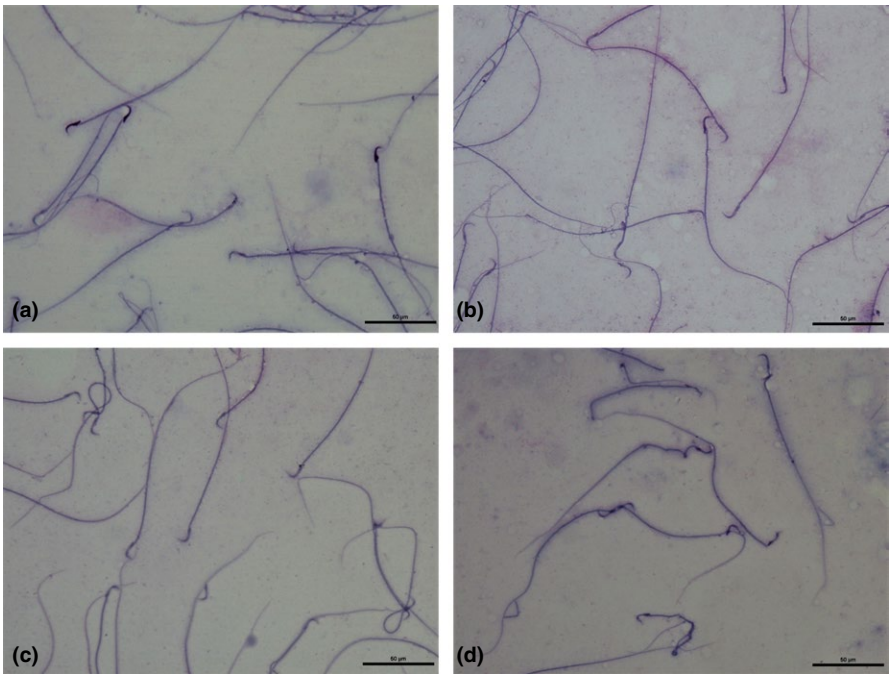

FIGURE 4 Microscopic images of sperm cells from each group. (a): Control and (b): GbE groups showing normal sperm smear, (c): T/D+GbE group with slightly increased abnormal sperm cells. (d): T/D group showing a significant increase in abnormal shaped sperm cells (Diff-quick, Scale bars: 50 μm)

higher in torsion in comparison with the other groups (*p*: .001). In the T/D+GbE group, it was lower than torsion (*p*: .001) and higher than control (*p*: .021). The highest reduction was seen in the GbE group, even significantly different than the control group (*p*: .003) (Table 4, Figure 4a–d).

4 | DISCUSSION

Testes begin to develop during embryonic period and continue until adolescence. Towards the end of the second month, testis begins to descend towards the inguinal ring and they reach the scrotum by 33 weeks (Sadler, 2011). During this descent of the testes or any other period in life, testicular ischaemia and tissue damages may take place due to torsion that occurs out of various reasons (Matfin, 2005). The damage in the testis related to the degree of torsion and its duration is a surgical case that requires urgent intervention. Early intervention increases the probability of saving the testis. However, only 32% of testes may be saved (Krarup, 1978). After Cosentino et al. investigated testes following torsion applied in different durations, they reported that testicular blood flow did not come back following three hours of 720° torsion as in our study, and serious changes took place in the tissue and these progresses up to necrosis in the tissue (Cosentino, Nishida, Rabinowitz, & Cockett, 1985).

TT leads to tissue hypoxia and damages that reach up to germ cell necrosis and infertility (Meštrović et al., 2014). Some studies found that testis necrosis occurred in two hours in arterial blockage and six hours in venous blockage (Melekos, Asbach, & Markou, 1988). It was reported that irreversible damages took place in testes not treated in the first 12 hr in ischaemic cases, and testes may be lost (Prater & Overdorf, 1991). Studies reported that spermatogenic serial damages in an hour-long TT in rats occur firstly in seminiferous tubule Sertoli cells (Harrison & Weiner, 1949). The continuation of testis blood flow is absolutely necessary for the survival, function and therefore fertility of the testis. Experimental TT firstly decreases the blood flow of the testis, and then, it leads to apoptosis in germ cells, testicular atrophy and disruption of spermatogenesis (Turner, Caplis, & Rhoades, 1996).

Our TT study is in the form of ischaemia-reperfusion. The damage during TT develops based on ischaemia and tissue hypoxia. In ischaemic tissues, firstly, the vascular response that is seen in the acute period takes place. As we observed in our study, vascular permeability increases and oedema arises in line with the extravasation in parallel to this. The oxidative metabolism in the ischaemic tissue continues with hypoxic metabolism. Damage creation in the cell is not only dependent on the duration of ischaemia, the time of reperfusion is also critical (Zar, Tanigawa, Kim, & Lancaster, 1998). As seen in the results of our study in relation to creation of free radicals based on ischaemia and reperfusion, there is an increase in cell apoptosis.

The ROS that emerges after reperfusion creates a negative effect in the cell, especially on cell membranes. They lead to peroxidation by reacting with membrane phospholipids. As a result, they lead to damage in the tissues by starting cell damages (Altavilla et al., 2012; Turner, Bang, & Lysiak, 2004). The degree of the damage in the testis may be determined by histopathological examinations. As Johnsen grading reveals the histopathological states of the germ and Sertoli cells in tubules, it presents the level of influence by ischaemia-reperfusion on spermatogenesis on a semi-quantitative basis. In our study, a general judgement was made on spermatogenesis by obtaining data regarding seminiferous tubular epithelium damage and spermatogenic serial cells via Johnsen grading. Our study shows that Johnsen scores increased in the torsion group and increased closer to the control group in the treatment group; tissue damage increased with torsion as in agreement with the literature, and therefore, spermatogenesis was affected negatively. In previous studies, sperm samples have been taken

from epididymal region. The effect of torsion and detorsion on epididymal sperm parameters is not clear. However, it can be thought that epididymal spermatozoon may reflect testicular sperm production. We think that spermatic cord torsion is inhibited epididymal blood supply likewise testis. Therefore, ischaemia and reperfusion induced oxidative stress and hypoxic conditions effects sperm cells both indirectly at production stage in testicle and directly at maturating stage from seminifer tubule lumen to epididymal tracts and epididymal luminal content.

The finding in our study that germ cells were influenced more suggests that highly toxic lipid peroxidation products emerge more in germ cells than Leydig and Sertoli cells. In our results and other studies, it was found that the cells that received the first damage in ischaemia were spermatogonia and primary spermatocytes rather than Sertoli cells (Lysiak et al., 2001; Markey, Jequier, Meyer, & Martin, 1994; Somuncu et al., 2006).

Testes exposed to ischaemia or hypoxia respond in and out of the cells with oedema due to increased vascular permeability. The vascular permeability in the testis increases as a result of an hour of torsion and 24 hr of reperfusion (Turner et al., 1996). As a result of reperfusion, levels of toxic aldehyde, hydroperoxide and proxylipid are increased based on increased vascular permeability and decreased levels of antioxidants. This situation leads to damage resulting in apoptosis in germ cells (Gonzalez-Flecha, Cutrin, & Boveris, 1993; Zar et al., 1998) like our results. The findings in the histological analysis of our study such as increased vascular dilatations, congestion, oedema and germ cell apoptosis agree with the literature.

Reperfusion involves O_2 and nutrients reaching the tissue again. In the beginning of 1980s, McCord et al. showed that O_2 increased the damage that took place in the tissues during its reperfusion (McCord, 1985). Imbalance between peroxidants and antioxidants leads to the potential damage named oxidative stress. Spermatozoa are highly sensitive against oxidative stress. This process induces peroxidative stress-based damages in sperm plasma membrane, DNA disintegration and both nuclear and mitochondrial damages (Aitken & Baker, 2006).

Studies stated that testicular ischaemia also damages the blood-testis barrier and as a result of this, it leads to autoimmune potential risk against spermatogonia or contralateral testicular damage (Arda & Ozyaylali, 2001). In their study, Turner et al. proposed after one hour of torsion followed by 4 hours of reperfusion that apoptosis was triggered, and antioxidant treatment reduced the reperfusion damage (Turner et al., 1996).

ROS formation to an extent is a normal physiological process in spermatozoon, while especially immotile or morphologically abnormal sperm cells are produced by leucocytes and morphologically normal but functionally abnormal spermatozoa (Koksal et al., 2012). Insufficient function of spermatozoa is a widespread reason for male infertility and it is difficult to treat. In such cases, abnormalities in sperm morphology, motility and numerical parameters, and therefore, ROS production increase. Our data obtained from the numerical and morphological analyses of sperm cells are consistent with the literature.

Previously, the positive effect of antioxidants on testicular tissues has been revealed in T/D-induced experimental rat models (Meštrović et al., 2017). Antioxidant molecules have various biological activities and different pharmacological effects including anti-inflammatory effects and immune response regulation (Fouad, Qutub, & Jresat, 2017). Nifedipine, a calcium channel blocker, is used principally as a vasodilator has potential antioxidant effects. In this context, calcium channel blockers have been reported to improve I/R injury in heart. Mestrovic et al. observed that administration of nifedipine before detorsion prevents ischaemia/reperfusion cellular damage in the testicular tissue. The rats treated with nifedipine had a significant decrease in malondialdehyde level and apoptosis like our study results and had significant increases in superoxide dismutase and glutathione peroxidase activities in testes compared with those of the T/D group (Meštrović et al., 2014). This result suggests that biochemical and histological T/D injury has been improved with administration of this antioxidant molecule.

Apocynin has the effect of an NADPH oxidase inhibitor. Apocynin is one of the most promising selective NADPH oxidases, and it has a role in the production of superoxide inhibitors. Ozbek et al. determined the protective effect of apocynin on testicular I/R damage induced by testicular torsion. They showed that a significantly increase in the number of giant, degenerated and desquamated cells in the ischaemia-reperfusion group. Similar to our antioxidant administration, they observed that apocynin significantly improved these histological alterations (Ozbek et al., 2015).

In another similar study, it was explored that apocynin treatment reversed both all biochemical indices and histopathological alterations that were induced by I/R. According to their data, high dose of apocynin provides better protection than lower dose of apocynin (Şener et al., 2015).

Urapidil is a sympatholytic antihypertensive drug. It acts as an α1-adrenoceptor antagonist and as an (5-hydroxytryptamine, 5-HT) receptor agonist. In their study, Meštrović et al. showed that urapidil has a protective effect on testicular T/D injury in rats. Urapidil can significantly increase the SOD and GPx activity and reduce the MDA level in ipsilateral testes (Meštrović et al., 2017). Similarly, in our study, an impaired blood supply to the testis resulted in increased male germ cells apoptosis.

GbE is used in treatment of nervous system diseases with high inflammatory mediator relations, acute pancreatitis, myocardial and intestinal ischaemia-reperfusion damage (Mesquita et al., 2017; Zhang, Wang, Zhang, Han, & Yang, 2017). In vivo and in vitro studies stated that GbE is a broad-spectrum free radical scavenger due to the GbE flavonoids scavenge free radicals, decrease lipid peroxidation and increase antioxidant activities (Mohamed & El-Moneim, 2017; Tredici, 1991). In another study, it was reported that GbE has superoxide radical anion scavenging capabilities and activities like superoxide dismutase and that it increased glutathione levels (Wang et al., 2008). In an experimental animal and clinic study, Wesnes et al. reported that GbE may increase memory performance in both young and elderly men (Wesnes, Ward, McGinty, & Petrini, 2000). Another study reported that GbE is a protector against ischaemic damage in the male genital system (Akgul et al., 2008); however, we could not find a study on the effect of GbE on Leydig and sperm cell activity in the literature.

It was found in our study that GbE was therapeutic and protective against TT tissue damage, especially the LH-R expressions of Leydig and sperm morphology qualities of the rats given only GbE were even better than the control. In summary, GbE alleviated the testicular tissue damage induced by ischaemia and reperfusion, regulated the disrupted spermatogenesis, increased sperm quality and the activities of Leydig cells. However, it would be better to take precise decisions that are supported by advanced-level future studies.

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REFERENCES

Aitken, R. J., & Baker, M. A. (2006). Oxidative stress, sperm survival and fertility control. *Molecular and Cellular Endocrinology*, *250*, 66–69. https://doi.org/10.1016/j.mce.2005.12.026

Akgul, T., Ayyildiz, A., Nuhoglu, B., Karaguzel, E., Ogus, E., Yagmurdur, H., … Germiyanoglu, C. (2008). *Ginkgo biloba* (EGb 761) usage attenuates testicular injury induced by testicular ischemia/reperfusion in rats. *International Urology and Nephrology*, *40*, 685–690. https://doi.org/10.1007/s11255-007-9296-5

Altavilla, D., Romeo, C., Squadrito, F., Marini, H., Morgia, G., Antonuccio, P., & Minutoli, L. (2012). Molecular pathways involved in the early and late damage induced by testis ischemia: Evidence for a rational pharmacological modulation. *Current Medicinal Chemistry*, *19*, 1219–1224.

Arda, I. S., & Ozyaylali, I. (2001). Testicular tissue bleeding as an indicator of gonadal salvageability in testicular torsion surgery. *BJU International*, *87*, 89–92. https://doi.org/10.1046/j.1464-410x.2001.00021.x

Cheon, K. W., Lee, H. S., Parhar, I. S., & Kang, I. S. (2001). Expression of the second isoform of gonadotrophin-releasing hormone (GnRH-II) in human endometrium throughout the menstrual cycle. *Molecular Human Reproduction*, *7*, 447–452. https://doi.org/10.1093/molehr/7.5.447

Cosentino, M. J., Nishida, M., Rabinowitz, R., & Cockett, A. T. K. (1985). Histological-changes occurring in the contralateral testes of prepubertal rats subjected to various durations of unilateral spermatic cord torsion. *The Journal of Urology*, *133*, 906–911. https://doi.org/10.1016/S0022-5347(17)49278-0

Dong, Q., & Hardy, M. P. (2004) Leydig cell function in man. In Winters, S. J. (Eds.), *Male hypogonadism* (pp 23–43). Totowa, New Jersey: Springer. https://doi.org/10.1007/978-1-59259-727-7

Fouad, A. A., Qutub, H. O., & Jresat, I. (2017) Dose-dependent protective effect of baicalin against testicular torsion-detorsion in rats. *Andrologia*, *49*, e12580. https://doi.org/10.1111/and.12580

Gevrek, F., Öncü, M., Gülle, K., Bayram, D., & Karaöz, E. (2016). Immunohistochemical expression of leptin (Ob-protein) in experimentally hypertensive rat kidney tissues. *Journal of Contemporary Medical Sciences*, *6*, 255–265.

Godbole, G. B., Modi, D. N., & Puri, C. P. (2007). Regulation of homeobox A10 expression in the primate endometrium by progesterone and embryonic stimuli. *Reproduction*, *134*, 513–523. https://doi.org/10.1530/REP-07-0234

Gonzalez-Flecha, B., Cutrin, J. C., & Boveris, A. (1993). Time course and mechanism of oxidative stress and tissue damage in rat liver subjected to in vivo ischemia-reperfusion. *The Journal of clinical investigation*, *91*, 456–464. https://doi.org/10.1172/JCI116223

Harrison, R., & Weiner, J. (1949). Vascular patterns of the mammalian testis and their functional significance. *Journal of Experimental Biology*, *26*, 304–316.

Huhtaniemi, I., & Pelliniemi, L. J. (1992). Fetal Leydig cells: Cellular origin, morphology, life span, and special functional features. *Proceedings of the Society for Experimental Biology and Medicine*, *201*, 125–140. https://doi.org/10.3181/00379727-201-43493

Johnsen, S. G. (1970). Testicular biopsy score count–a method for registration of spermatogenesis in human testes: Normal values and results in 335 hypogonadal males. *Hormones*, *1*, 2–25.

Koksal, M., Oguz, E., Baba, F., Eren, M. A., Ciftci, H., Demir, M. E., … Ulas, T. (2012). Effects of melatonin on testis histology, oxidative stress and spermatogenesis after experimental testis ischemia-reperfusion in rats. *European Review for Medical and Pharmacological Sciences*, *16*, 582–588.

Kostakis, I. D., Zavras, N., Damaskos, C., Sakellariou, S., Korkolopoulou, P., Misiakos, E. P., … Karatzas, T. (2017). Erythropoietin and sildenafil protect against ischemia/reperfusion injury following testicular torsion in adult rats. *Experimental and Therapeutic Medicine*, *13*, 3341–3347. https://doi.org/10.3892/etm.2017.4441

Krarup, T. (1978). The testes after torsion. *British journal of urology*, *50*, 43–46. https://doi.org/10.1111/j.1464-410X.1978.tb02764.x

Lysiak, J. J. (2004). The role of tumor necrosis factor-alpha and interleukin-1 in the mammalian testis and their involvement in testicular torsion and autoimmune orchitis. *Reproductive Biology and Endocrinology : RB&E.*, *2*, 9. https://doi.org/10.1186/1477-7827-2-9

Lysiak, J. J., Turner, S. D., Nguyen, Q. A. T., Singbartl, K., Ley, K., & Turner, T. T. (2001). Essential role of neutrophils in germ cell-specific apoptosis following ischemia/reperfusion injury of the mouse testis. *Biology of reproduction*, *65*, 718–725. https://doi.org/10.1095/biolreprod65.3.718

Markey, C. M., Jequier, A. M., Meyer, G. T., & Martin, G. B. (1994). Testicular morphology and androgen profiles following testicular ischaemia in rams. *Journal of Reproduction and Fertility*, *101*, 643–650. https://doi.org/10.1530/jrf.0.1010643

Matfin, G. (2005) *Disorders of the male genitourinary system. Essentials of Pathophysiology* (pp. 901–923), 2nd ednPhiladelphia, PA: Lippincott Williams & Wilkins.

McCord, J. M. (1985). Oxygen-derived free radicals in postischemic tissue injury. *The New England Journal of Medicine*, *312*, 159–163.

Melekos, M. D., Asbach, H. W., & Markou, S. A. (1988). Etiology of acute scrotum in 100 boys with regard to age distribution. *Journal of Urology*, *139*, 1023–1025. https://doi.org/10.1016/S0022-5347(17)42756-X

Mellick, L. B. (2012). Torsion of the testicle: It is time to stop tossing the dice. *Pediatric Emergency Care*, *28*, 80–86. https://doi.org/10.1097/PEC.0b013e31823f5ed9

Mesquita, T. R. R., de Jesus, I. C. G., Dos Santos, J. F., de Almeida, G. K. M., de Vasconcelos, C. M. L., Guatimosim, S., … Lauton-Santos, S. (2017). Cardioprotective Action of *Ginkgo biloba* Extract against Sustained beta-Adrenergic Stimulation Occurs via Activation of M2/NO Pathway. *Frontiers in Pharmacology*, *8*, 220. https://doi.org/10.3389/fphar.2017.00220

Meštrović, J., Drmić-Hofman, I., Pogorelić, Z., Vilović, K., Šupe-Domić, D., Šešelja-Perišin, A., & Čapkun, V. (2014). Beneficial effect of nifedipine on testicular torsion-detorsion injury in rats. *Urology*, *84*, 1194–1198.

Meštrović, J., Pogorelić, Z., Drmić-Hofman, I., Vilović, K., Todorić, D., & Popović, M. (2017). Protective effect of urapidil on testicular torsion–detorsion injury in rats. *Surgery today*, *47*, 393–398.

Mogilner, J. G., Lurie, M., Coran, A. G., Nativ, O., Shiloni, E., & Sukhotnik, I. (2006). Effect of diclofenac on germ cell apoptosis following testicular ischemia-reperfusion injury in a rat. *Pediatric Surgery International*, *22*, 99–105. https://doi.org/10.1007/s00383-005-1580-9

Mohamed, N. E.-S., & El-Moneim, A. E. A. (2017). *Ginkgo biloba* extract alleviates oxidative stress and some neurotransmitters changes induced by aluminum chloride in rats. *Nutrition*, *35*, 93–99. https://doi.org/10.1016/j.nut.2016.10.012

Ozbek, O., Altintas, R., Polat, A., Vardi, N., Parlakpinar, H., Sagir, M., … Yildiz, A. (2015). The protective effect of apocynin on testicular ischemia-reperfusion injury. *The Journal of Urology*, *193*, 1417–1422. https://doi.org/10.1016/j.juro.2014.11.086

Pogorelić, Z., Mrklić, I., & Jurić, I. (2013). Do not forget to include testicular torsion in differential diagnosis of lower acute abdominal pain in young males. *Journal of Pediatric Urology*, *9*, 1161–1165. https://doi.org/10.1016/j.jpurol.2013.04.018

Pogorelić, Z., Mustapić, K., Jukić, M., Todorić, J., Mrklić, I., Mešštrović, J., … Furlan, D. (2016). Management of acute scrotum in children: A 25-year single center experience on 558 pediatric patients. *The Canadian Journal of Urology*, *23*, 8594–8601.

Prater, J. M., & Overdorf, B. S. (1991). Testicular torsion: A surgical emergency. *American Family Physician*, *44*, 834–840.

Reyes, J. G., Farias, J. G., Henríquez-Olavarrieta, S., Madrid, E., Parraga, M., Zepeda, A. B., & Moreno, R. D. (2012) The hypoxic testicle: Physiology and pathophysiology. *Oxidative Medicine and Cellular Longevity*, *2012*, 929285.

Ringdahl, E., & Teague, L. (2006). Testicular torsion. *American Family Physician*, *74*, 1739–1743.

Sadler, T. W. (2011) *Langman's medical embryology*. Philadelphia: Lippincott Williams & Wilkins.

Sekerci, C. A., Tanidir, Y., Sener, T. E., Sener, G., Cevik, O., Yarat, A., … Akbal, C. (2017) Effects of platelet-rich plasma against experimental ischemia/reperfusion injury in rat testis. *Journal of Pediatric Urology*, *13*, 317 e311-317 e319

Şener, T. E., Yüksel, M., Özyılmaz-Yay, N., Ercan, F., Akbal, C., Şimşek, F., & Şener, G. (2015). Apocynin attenuates testicular ischemia–reperfusion injury in rats. *Journal of pediatric surgery*, *50*, 1382–1387.

Shimizu, S., Tsounapi, P., Dimitriadis, F., Higashi, Y., Shimizu, T., & Saito, M. (2016). Testicular torsion-detorsion and potential therapeutic treatments: A possible role for ischemic postconditioning. *International Journal of Urology: Official Journal of the Japanese Urological Association*, *23*, 454–463. https://doi.org/10.1111/iju.13110

Somuncu, S., Cakmak, M., Erdogan, S., Caglayan, O., Akman, H., & Kaya, M. (2006). Protective effects of trapidil in ischemia-reperfusion injury due to testicular torsion and detorsion: An experimental study. *International Journal of Urology: Official Journal of the Japanese Urological Association*, *13*, 601–605. https://doi.org/10.1111/j.1442-2042.2006.01355.x

Soyalıç, H., Gevrek, F., & Karaman, S. (2017). Curcumin protects against acoustic trauma in the rat cochlea. *International Journal of Pediatric Otorhinolaryngology*, *99*, 100–106.

Soyalıç, H., Gevrek, F., Koç, S., Avcu, M., Metin, M., & Aladağ, İ. (2016). Intraperitoneal curcumin and vitamin E combination for the treatment of cisplatin-induced ototoxicity in rats. *International Journal of Pediatric Otorhinolaryngology*, *89*, 173–178.

Teerds, K. J., de Boer-Brouwer, M., Dorrington, J. H., Balvers, M., & Ivell, R. (1999). Identification of markers for precursor and leydig cell differentiation in the adult rat testis following ethane dimethyl sulphonate administration. *Biology of reproduction*, *60*, 1437–1445. https://doi.org/10.1095/biolreprod60.6.1437

Tredici, P. (1991). Ginkgos and people-a thousand years of interaction. *Arnoldia (Boston)*, *51*, 2–15.

Turner, T. T., Bang, H. J., & Lysiak, J. L. (2004). The molecular pathology of experimental testicular torsion suggests adjunct therapy to surgical repair. *Journal of Urology*, *172*, 2574–2578. https://doi.org/10.1097/01.ju.0000144203.30718.19

Turner, T. T., Caplis, L. A., & Rhoades, C. P. (1996). Testicular vascular permeability: Effects of experimental lesions associated with impaired testis function. *Journal of Urology*, *155*, 1078–1082.

Wang, W., Zhong, X. H., Ma, A., Shi, W., Zhang, X. S., & Liu, Y. (2008). Effects of *Ginkgo biloba* on testicle injury induced by diethylstilbestrol in mice. *American Journal of Chinese Medicine*, *36*, 1135–1144. https://doi.org/10.1142/S0192415X08006478

Wesnes, K. A., Ward, T., McGinty, A., & Petrini, O. (2000). The memory enhancing effects of a *Ginkgo biloba*/*Panax ginseng* combination in healthy middle-aged volunteers. *Psychopharmacology (Berl)*, *152*, 353–361. https://doi.org/10.1007/s002130000533

Yeh, Y. C., Liu, T. J., Wang, L. C., Lee, H. W., Ting, C. T., Lee, W. L., … Lai, H. C. (2009). A standardized extract of *Ginkgo biloba* suppresses doxorubicin-induced oxidative stress and p53-mediated mitochondrial apoptosis in rat testes. *British Journal of Pharmacology*, *156*, 48–61. https://doi.org/10.1111/j.1476-5381.2008.00042.x

Zar, H. A., Tanigawa, K., Kim, Y. M., & Lancaster, J. R. Jr (1998). Rat liver postischemic lipid peroxidation and vasoconstriction depend on ischemia time. *Free radical biology & medicine*, *25*, 255–264. https://doi.org/10.1016/S0891-5849(98)00032-X

Zhang, C., Wang, D. F., Zhang, Z., Han, D., & Yang, K. (2017). EGb 761 protects cardiac microvascular endothelial cells against hypoxia/reoxygenation injury and exerts inhibitory effect on the ATM pathway. *Journal of Microbiology and Biotechnology*, *27*, 584–590. https://doi.org/10.4014/jmb.1611.11024

Zhao, L. C., Lautz, T. B., Meeks, J. J., & Maizels, M. (2011). Pediatric testicular torsion epidemiology using a national database: Incidence, risk of orchiectomy and possible measures toward improving the quality of care. *Journal of Urology*, *186*, 2009–2013. https://doi.org/10.1016/j.juro.2011.07.024

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