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Research Article

Protective Role of Doxofylline against Cyclophosphamide-induced Testicular Toxicity in Rats

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Abstract

Background: Cyclophosphamide (CP) is a widely used anticancer and immunosuppressant drug. However, CP's significant toxicities and adverse effects restrict its clinical use. Doxofylline (DF) is a phosphodiesterase inhibitor that has antioxidant and anti-inflammatory properties. ***Objective***: To determine the protective effect of doxofylline on rats exposed to a single dose of CP. ***Methods***: We randomly assigned thirty Sprague-Dawley male rats to three groups. On the sixth day, the apparently healthy control group (AH) received normal saline; the induction group (IN) received normal saline and a single dose of CP (100 mg/kg); and the DF group received DF (50 mg/kg) and a single dose of CP (100 mg/kg). Treatment in all groups was given once daily and intraperitoneally. At the end of treatment, caudal fluid is used to determine sperm parameters. A blood sample was taken to determine the testosterone levels. We determined the levels of testicular glutathione peroxidase and malondialdehyde. The testes were harvested, allowing for histological examination. ***Results***: Sperm parameters, testosterone and oxidative stress markers were notably declining in testicular tissue among the IN compared with AH, although these measures can be markedly improved with DF. Histopathological analysis revealed a substantial change in the testicular tissue structure in the CP group, although doxofylline co-treatment significantly attenuated and even reversed these changes. ***Conclusions***: Doxofylline may function as a protective agent, mitigating the testicular damage caused by CP therapy in rats. The proposed process may be attributed to its phosphodiesterase inhibitory and antioxidant properties.

Keywords: Cyclophosphamide, Doxofylline, Sperm parameters, Testicular damage, Testosterone.

دورالدوكسوفيلين كعامل وقائي ضد تسمم الخصية التي يسببها السيكلوفوسفاميد في الجرذان

الخلاصة

الخلفية: السايكلوفوسفاميد دواء مضاد للسرطان ومثبط للمناعة يستخدم على نطاق واسع. إن السمية الكبيرة والآثار الضارة تحد من استخدامه السريري. دوكسوفيلين هو مثبط فوسفوديستراز له خصائص مضادة للأكسدة ومضادة للالتهابات. **الهدف:** تحديد ما إذا كان دوكسوفيلين يحمي خصيتي الفئران الذكور بعد جرعة واحدة من السايكلوفوسفاميد. **الطريقة:** تم تقسيم ثلاثين من ذكور الفئران عشوائيا الى ثلاث مجموعات. المجموعة الاولى: الاصحاء ظاهريا (المجموعة الضابطة): تلقوا محلول ملحي مرة واحدة يوميا. المجموعة المستحثة غير المعالجة: تلقوا تلقوا محلول ملحي مرة واحدة يوميا قبل وبعد الجرعة الوحيدة من سيكلوفوسفاميد (100 ملغ/كغ) التي أعطيت في اليوم السادس. مجموعة الدوكسوفيلين: تلقى دوكسوفيلين (50 ملغ/كغ) مرة واحدة يوميا قبل وبعد الجرعة الوحيدة من سيكلوفوسفاميد (100 ملغ/كغ) التي أعطيت في اليوم السادس. تم إعطاء العلاج في جميع المجموعات داخل الصفاق. في نهاية العلاج، تم التضحية بالفئران، واستخدم سائل البربخ الذيلي لتحديد تركيز الحيوانات المنوية وحركتها وقابليتها للحياة. تم أخذ عينة دم لتحديد مستويات هرمون تستوستيرون. تم تحديد مستويات الجلوتاثيون بيروكسيديز ومالونداليهيد عن طريق جمع عينات الخصية.استخدم نسيج الخصيتين للفحص النسيجي. **النتائج**: كانت مستويات تركيز الحيوانات المنوية وحركتها وقابليتها للحياة وتركيز هرمون التستوستيرون والمستويات الكيميائية منخفظة في انسجة الخصية في المجموعة المستحثة مقارنة بمجموعة الاصحاء. وقد تم حدوث تغير كبير في هذا المستويات عند استخدام عقار الدوكسوفلين الذي خفف الى حد كبير بل وعكس هذه التغيرات. **الاستنتاج**: قد يعمل الدوكسوفلين كعامل وقائي من تلف خصية الفئران الناجم عن السايكلوفوسفاميد وذلك عن طريق تعزيز معايير الحيونات المنوية ومستوى التستوستيرون والمستويات الكيميائية وقد تعزى آلية عمل العقار الى خاصيته في تثبيط انزيم الفوسفوداياستريز و مضاد للاكسدة و الالتهابات.

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INTRODUCTION

Infertility is commonly described as a couple's failure to conceive despite one year of unprotected, regular sexual intercourse. The male is entirely responsible for approximately 20% of all cases of infertility and substantially contributes to about 50% [1]. Numerous medications have been reported to be associated with male infertility, including chemotherapies. Chemotherapy was administered to more than half of the male patients as an antineoplastic drug to treat a variety of cancers and as an immunosuppressive medication for multiple sclerosis, systemic lupus erythematosus, organ transplantation, and other benign conditions [2]. Because many of these patients receive chemotherapy treatments both before and during their reproductive years, and because many tumors can be cured, treatment-related sterility is a serious problem [3]. One of the main causes of toxicity due to the use of cyclophosphamide (CP) and other chemotherapy medications is their interaction with DNA, which can result in damaged DNA, abnormal cell function, and cell death [4]. Despite the beneficial therapeutic effect of CP in clinical usage, it also adversely affects healthy tissues like the testicles, liver, and bladder in humans and experimental animals [5,6]. The precise mechanism via which CP causes organ damage, such as in testis tissue and epididymis, remains unclear, but research has revealed that highly oxidative stress, increased reactive oxygen species (ROS) supply and low antioxidant ability have been implicated in the pathophysiology of CP toxicity [7]. While low amounts of ROS in semen are crucial for maintaining normal sperm activity, elevated levels of ROS can have a detrimental impact on sperm generation in testes and sperm maturation in the epididymis [8]. Patients treated with this drug for four months or more will have impairment in spermatogenesis; most patients suffered from both short-term and long-term azoospermic or oligozoospermic infertility [3]. Consequently, compounds that protect the tissues from CP's harmful side effects are required. As a result, the current study aimed to investigate the protective effects of doxofylline against CP-related toxicity in rat testicular tissue. Doxofylline is a novel methylxanthine derivative known for inhibiting phosphodiesterase and increasing cyclic adenosine monophosphate (cAMP) [9]. cAMP is a second messenger involved in most of the critical biochemical processes that lead to the maturation of both male and female gametes. Overall, cAMP is an ideal therapeutic target for a wide variety of human disorders affecting many organs and tissues because of its prominent involvement in intracellular signal transduction pathways [10]. Doxofylline and other xanthine derivatives were known to exhibit immune regulation, anti-inflammatory properties of cancer cells, and a reduced oxidative stress response in patients undergoing radical resection of esophageal cancer, as well as improving wound healing [11–13]. The current study aims to assess the impact of doxofylline as a protective agent against CP-induced testicular damage in rats.

METHODS

Chemicals and drugs

The origins of all chemicals and reagents used in the current study were as follows: cyclophosphamide, doxofylline (Hangzhou Jinlan Pharmaceutical Technology, China), testosterone, and malondialdehyde (MDA) (Cloud-Clone Corp., USA). Glutathione peroxidase (GPX) (Elabscience, USA). All measurements were dependent on the enzyme-linked immunosorbent assay technique.

Animals

Thirty apparently healthy Sprague-Dawley male rats weighing 250–360 gm were purchased and housed in the animal house of the Biotechnology Centre/AL-Nahrain University, where the *in vivo* study was conducted. The animals were kept for one-week acclimatization before starting the work. The current study was started after receiving approval from the department of pharmacology's scientific and animal ethical committee (approval No. 20240508, approval date: September 17, 2023). Animals were treated in accordance with the institutional guidelines for the care and use of experimental animals, and approval for the study protocol was obtained from the local ethics committee. The animals were randomly divided into three groups, with ten rats in each (n=10) (Figure 1).

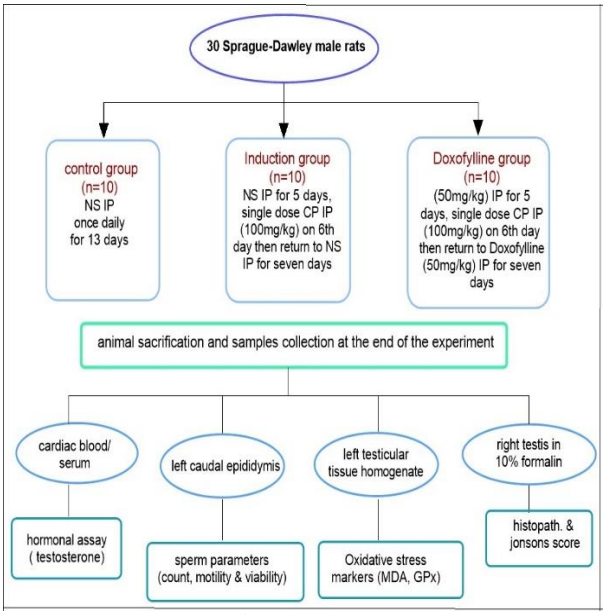

Figure 1: Flow chart of the study. NS: normal saline, CP: cyclophosphamide, IP: intraperitoneal, MDA: Malondialdehyde, GPx: Glutathione peroxidase.

The apparently healthy-control group (AH) received 0.9% normal saline for 13 days. Induction non-treated (IN): given 0.9% normal saline for five days, then one dosage of cyclophosphamide (100 mg/kg) on day six,

and finally, normal saline for seven days. Doxofylline group (DF): given doxofylline (50 mg/kg) for five days, one dosage of cyclophosphamide (100 mg/kg) on day six, and then another round of doxofylline (50 mg/kg) for seven days. All treatments were given once daily via the intraperitoneal (IP) route. At the end of the experiment, the animals were anesthetized by intramuscular injection of a mixture of ketamine (10%) and xylazine (10%). Blood samples were obtained via cardiac puncture using a 5 ml syringe [14], and the serum was used to determine the testosterone level. After laparotomy, the testes and epididymis of the rats were quickly excised. Testicular tissue homogenization was performed according to Hamzeh *et al*. 2017, and the homogenate was used to measure glutathione peroxidase (GPx) and malondialdehyde (MDA) levels [15]. In order to conduct histological examination, the right testis was immersed in a formalin-phosphate buffered saline (PBS) solution at a ratio of 1:9. A certified pathologist assessed the severity of testicular damage using Jonson's scoring system, which uses a scale from 10 (maximum spermatogenesis activity) to 1 (total absence of germ cells) [16] (Figure 1).

Measurement of sperm parameters

The left epididymis of each rat was harvested immediately after sacrifice and transferred to a petri dish containing 1.0 ml of prewarmed flushing media. They were incubated at 37 °C for 5 minutes to allow the dispersal of spermatozoa. The sperm analysis was conducted in accordance with the standards set by the World Health Organization (WHO). To determine the sperm count, a hemocytometer slide was utilized. Under a light microscope set at x40 magnification, only sperm cells identified as having a head, middle, and tail were counted. Each sample was counted twice, and the mean was reported. The results were presented as the concentration of sperm per milliliter [17]. To measure sperm motility, 10 µL of diluted sperm suspension was added to each counting chamber of the hemocytometer. The current study followed the recommendations of the World Health Organization to assess the percentage of sperm motility after counting 200 sperm cells [18]. Sperm viability was examined under a light microscope at a magnification of x10 using eosin-nigrosine staining [19].

Statistical analysis

The data was analyzed using SPSS version 26. Using normality tests showed that data was non-normally distributed; thus, the Mann-Whitney test and Kruskal-Wallis test were used instead of the independent t test and one-way ANOVA for continuous variables to determine mean differences between groups (20). The results were considered statistically significant at $p<0.05$.

RESULTS

Table 1 shows that the IN group has a significant increase in (sluggish sperm motility, immotile sperms, and dead sperms) and a significant decrease in (sperm concentration, progressive sperm motility, and viable sperms). When compared to the IN group, DF shows a significant increase in (sperm concentration, progressive sperm motility, and viable sperms) and a significant decrease in (sluggish sperm motility, immotile sperms, and dead sperms) ($p<0.05$).

Table 1: Comparison between apparently healthy control group (AH), induced non-treated group (IN) and doxofylline group (DF) in relation to sperm parameter

Variables		Groups		
		AH	IN	DF
Sperm Concentration *10⁶/ml		66.14±24.10	18.00±12.22*[a]	51.43±10.77*[b]
Sperm motility (%)	Progressive	96.00±1.15	7.00±2.83*[a]	90.57±4.08*[b]
	Sluggish	0.57±0.53	58.43±8.70*[a]	3.00±1.83*[b]
	Immotile	3.43±0.79	33.14±7.90*[a]	6.43±2.37*[b]
Sperm viability (%)	Viable	96.00±1.29	28.00±8.41*[a]	91.86±2.48*[b]
	Dead	4.00±1.29	72.00±8.41*[a]	8.14±2.48*[b]

Values were expressed as mean±SD. * Significantly different compared to AH group ($p<0.05$). Values with different superscripts (a,b) among IN and DF groups are significantly different ($p<0.05$).

The IN group shows a significant decrease in the level of serum testosterone when compared to the AH group ($p=0.002$). Table 2 reveals that the DF group significantly outperforms the IN group in testosterone levels ($p=0.002$). Table 3 reveals that the IN group significantly increases MDA levels and decreases GPx levels compared to the AH group ($p=0.002$). However, when compared to the IN group, the DF group shows a significant increase in GPx levels and a significant decrease in MDA levels ($p=0.002$).

The rats in the AH group had normal testicular tissue characteristics, with whole spermatogenic cells, epithelial layers, and the structure of the seminiferous tube, as shown in Figure 2 (A and B) at two different magnifications).

Table 2: Comparison between AH, IN and DF groups in relation to testosterone level

Variables	Groups		
	AH	IN	DF
Testosterone (pg/ml)	147.93±8.04	11.09±1.93*[a]	63.74±9.35*[b]

Values were expressed as mean±SD. * Significantly different compared to AH group ($p<0.05$). Values with different superscripts (a,b) among IN and DF groups are significantly different ($p<0.05$).

Table 3: Comparison between AH, IN and DF groups in relation to testicular oxidative stress markers

Variables	Groups		
	AH	IN	DF
MDA (ng/ml)	8.80±0.52	123.23±7.88*[a]	43.64±3.08*[b]
GPx (pg/ml)	967.68±16.77	322.89±16.42*[a]	902.05±89.02*[b]

Values were expressed as mean±SD. * Significantly different compared to AH group (p<0.05). Values with different superscripts (a,b) among IN and DF groups are significantly different (p<0.05).

Conversely, the IN group exhibited significant damage to Leydig cells, resulting in impaired production of spermatids and mature sperm. This is evident in Figure 2 (C and D) at two different magnifications.

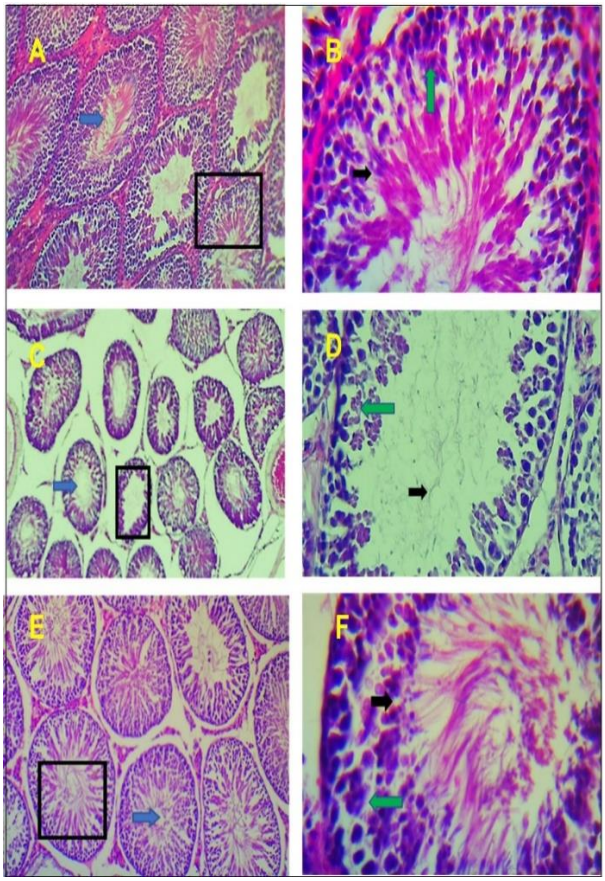

Figure 2: The histological section of testicular tissue in rats stained with H&E. Black arrows denote the presence of sperm, blue arrows the tubular lumen, and green arrows the epithelial layers of the lumen. A and B: AH group; C and D: IN group; E and F: DF group. A, C, and E, 10X magnification; B, D and F: 40X magnification.

Meanwhile, rats treated with doxofylline demonstrated a significant decrease in the harmful effects of CP on spermatogenic cells (Leydig and Sertoli cells) and a near return to normal tubular architecture, as indicated in Figure 2 (E and F) at different magnifications. The IN group shows a significant decrease in Jonson's score when compared to the AH group (p=0.001); whereas the DF group shows a highly significant increase in Jonson's score when compared to the IN group (p<0.05), as shown in Table 4.

Table 4: comparison between AH, IN and DF groups in relation to Jonson's score

Variable	Groups		
	AH	IN	DF
Jonson's score	10.0±0.0	8.0±0.82*[a]	10.0±0.0[b]

Values were expressed as mean±SD. * Significantly different compared to AH group (p<0.05). Values with different superscripts (a,b) among IN and DF groups are significantly different (p<0.05).

DISCUSSION

When the testicular cells are permanently destroyed by chemotherapy, they are unable to create healthy, mature sperm again. This might lead to permanent infertility. A variety of neoplastic and autoimmune diseases can be treated with cyclophosphamide, an immunosuppressive and anti-cancer chemotherapeutic drug [7]. World Health Organization (WHO) recommendations state that seminal fluid examination can predict male infertility by evaluating sperm parameters such as concentration, motility, and viability [21]. In this study, giving CP at a dose of 100 mg/kg significantly decreased the number, concentration, motility, and viability of sperm in the epididymis compared to the control group. These results support what Razak et al. found, which is that CP is harmful to reproduction, which is why the sperm parameter went down [22]. Reportedly, CP exposure reduces sperm motility and viability. This is likely because it disrupts the function of the sperm flagellum, which is critical for sperm cell movement, by rapidly losing intracellular adenosine triphosphate (ATP) and impairing energy metabolism [23]. According to Freitas et al. (2017), the common source of energy for cellular metabolism is ATP, which is required for both sperm motility and fertilization [24]. The process of oxidative phosphorylation in the mitochondrial membrane produces ATP, which is then transported to the microtubules in order to propel sperm motility [25]. Therefore, decreased sperm motility may result from oxidative damage to mitochondrial DNA. Another thing that can affect sperm movement and survival is when ROS attacks reduce the fluidity of the sperm membrane, which can mess up the function of the membrane pump and the balance of calcium ions in the cells [26]. Meistrich (2013) said that CP therapy leads to spermatogonial stem cell loss, which in turn causes long-term or permanent azoospermia. Because these quickly dividing, differentiating spermatogonia are more likely to be hurt by cytotoxic chemotherapeutic agents than the later-stage germ cells, spermatogenesis fails and sperm production drops [27]. Singh et al. (2015) say that CP can stop spermatogenesis by messing up the regulation of the pituitary luteinizing hormone. This stops Leydig cells from making testosterone, which leads to low testosterone levels in the blood. On top of that, CP could hurt the spermatogenic compartment directly and change how the antioxidant enzymes in the testes work [23]. The antioxidant enzymes, which are present at a low level in semen, are essential for the proper differentiation and development of spermatogonia cells into mature spermatozoa by

preserving these cells from damage caused by ROS (28). So, stopping these antioxidant enzymes from working can lead to structural problems in sperm and the production of sperm that can't live. Doxofylline treatment, in the current study, significantly improved the damage caused by CP on sperm parameters. Drobnis *et al*. (2017) showed that methylxanthine, particularly pentoxifylline (PTX), may be related to the inhibition of phosphodiesterase activity; thus, increasing the levels of cAMP and Ca^{2+} stimulates sperm motility and enhances capacitation, permitting the acrosome reaction [29]. This agrees with Tesarik and Mendoza-Tesarik (2022), who suggested that cAMP, a second messenger, represents a key regulator of sperm development [10]. The elevated concentration of cAMP is required for protein kinase A (PKA) activation. Then, PKA goes to the nucleus and turns on cAMP response element binding protein (CREB). This changes how well the Sertoli cell can help germ cells form. An *in vitro* study using testicular tissue biopsies from men with obstructive azoospermia and normal spermatogenesis revealed that raising intracellular cAMP concentrations with phosphodiesterase inhibitors like pentoxifylline could emulate the two key effects of FSH on promoting germ cell differentiation and preventing apoptosis [30]. Additionally, CREB activation triggers the synthesis of pro-resolving mediators and the transcription of anti-inflammatory cytokines. Doxofylline may also help reduce inflammation through PKA activation, a protein that stops the release of pro-inflammatory cytokines and pro-survival signals. In addition to the antioxidant property of doxofylline, increasing the antioxidant enzymes and ROS scavenging activity [31] may play a role in protecting the remaining spermatogonial stem cells from cytotoxic damage and ensuring their survival. This provides an additional explanation for the results of the current study on the protection of sperm cells in animals treated with CP. As a diagnostic tool for male infertility and a predictive biomarker for testicular function, serum testosterone levels can be measured [32]. According to the results of this study, there was a notable and statistically significant decrease in serum testosterone in the group that was treated with CP. This result matched what Bakhtiary et al. (2020) found: that higher oxidative stress causes serious damage at the Leydig cell level, making them less responsive to luteinizing hormone and directly stopping the production of testosterone [33]. Testosterone plays a crucial role in the spermatogenesis process, including the division of germ cells, meiosis, and the formation of the blood-testis barrier [34]. In the current study, the CP impact on testosterone was notably reversed by IP administration of 50 mg/kg doxofylline. The results were similar to those of Fallahzadeh et al. (2017), which found that the antioxidant properties of pentoxifylline can protect testicles from damage and raise testosterone levels in rats' serum [35]. Give additional clarification on the protective effect of doxofylline on CP-treated rats. Compared to histology alterations, biochemical markers of oxidative stress are considerably more sensitive and can be used as early indications of tissue damage [36]. The concentration of lipid peroxidation was assessed by measuring malondialdehyde (MDA). In this study, it was found that CP exposure increased the amount of MDA and decreased the amount of glutathione peroxidase (GPx) in the testes. The results were similar to those of Shabaan et al. (2021), who said that CP caused an imbalance in oxidant and antioxidant levels by raising MDA levels and lowering those of antioxidant enzymes like GPx [37]. Lipid peroxidation is the main mechanism by which ROS attack lipids in cell membranes. Lipid peroxidation and ROS assault are known to cause severe damage to testicular tissues and spermatozoa. Sperm membranes have a high concentration of polyunsaturated fatty acids (PUFA), which makes testicular cells vulnerable to oxidation [38]. Free radical damage can be prevented by the presence of many endogenous scavengers found in various tissues, such as SOD, CAT, GPx, ascorbic acid, and β-tocopherol [39]. According to Sikka *et al*. (1995), sufficient antioxidant levels such as glutathione peroxidase and reductase maintain the scavenging potential in gonads and seminal fluids, which is referred to as oxidative stress status [40]. In the current study, GPx levels significantly increased and MDA levels significantly decreased in doxofylline-treated animals. The results relating to GPx and MDA activity levels indicated that doxofylline may have a preventive effect against testicular injuries. These findings, along with earlier research, demonstrate that pentoxifylline has a strong antioxidant capability. Pentoxifylline has both antioxidative activities, which prevent oxidation, and ROS-scavenging properties, which include inhibition of ROS generation and scavenging the generated one [41]. Research like this lends credence to the idea that doxofylline could be useful for treating testicular damage since it belongs to the same family as pentoxifylline. Consistent with the results of Iqubal *et al*. (2020), this study found that CP significantly damaged seminiferous tubules and spermatozoa, leading to a reduction in the thickness of the tubular layers, the severe destruction of Leydig cells, and finally, a defect in the formation of spermatids and mature sperms [42]. The co-administration of doxofylline with CP resulted in a significant restoration of the testicular histomorphologic integrity and Jonson's score. These findings were consistent with a previous study conducted by Dhulqarnain *et al*. (2021) that employed pentoxifylline to mitigate the histopathological damage caused by testicular torsion-detorsion [43].

Conclusion

The testicular damage in rats induced by CP can be ameliorated with doxofylline, as represented by the improvement of sperm parameters, testosterone, oxidative stress markers, and histopathological morphology. The proposed protective mechanism of doxofylline may be attributed to its antioxidant activity.

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Conflict of interests

No conflict of interests was declared by the authors.

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Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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