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Reperfusion injury following testicular torsion and detorsion in prepubertal rats

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Summary. Acute testicular torsion is a surgical emergency which requires immediate intervention. Although damage to the gonad has been well documented, it remains unknown whether the majority of injury occurs during the period of torsion (ischemia) or following detorsion (reperfusion). The aims of this study were to determine: (1) whether damage following testicular torsion–detorsion has a reperfusion component similar to that described in other tissues, and (2) whether iron-catalyzed oxygen radical formation or altered calcium homeostasis plays a role in this injury. To study this, anesthetized prepubertal rats underwent 720° intravaginal testicular torsion and were divided into groups of torsion only (ischemia) and torsion with reperfusion (ischemia/reperfusion). Reperfusion groups were treated prior to detorsion with either deferoxamine (iron chelator), diltiazem (calcium channel blocker), or saline vehicle. The results indicated that detorsion produces a qualitatively distinct reperfusion injury from that of non-reperfused testicles; however, such damage was not ameliorated by deferoxamine or dilitiazem. Thus, testicular torsion–detorsion appears to have a significant reperfusion component that appears to not be mediated by iron-catalyzed oxygen radical formation or calcium injury.

Key words: Deferoxamine – Diltiazem – Hematoxylin and eosin – Histology – Ischemia – Microscopy

Acute testicular torsion is a surgical emergency which requires immediate intervention to untwist the affected gonad. The incidence of testicular torsion in England has been reported to be 27 cases per 100000 males with a mean age at presentation of 16.7 years [1]. A variety of predisposing factors is associated with the development of testicular torsion. In most instances, there may be a voluminous tunica vaginalis that inserts high on the spermatic cord, thus allowing the testis to rotate freely. Other factors implicated in the development of testicular torsion include sexual or strenuous activity and scrotal trauma [2, 10].

Torsion initially results in obstruction to spermatic cord venous blood flow with secondary hemorrhage and edema. The edema enhances the strangulatory effect of the torsion, resulting in arterial obstruction, ischemia, and necrosis of the gonad [8]. Investigations of the long-term effects of testicular torsion–detorsion have shown profound tissue damage several weeks after 3–6 h of 360–720° cord torsion [7,8]. Other studies have focused on the acute response of the testicle to torsion [3, 9]. Ultrastructural damage has been described as early as 1–3 h following ischemia [9]. Utilizing light microscopy, Cosentino et al. [3] also documented acute testicular injury following 1–3 h of torsion. Moreover, the degree of tissue damage was found to be increased with longer periods of ischemia [3].

Although these previous studies of testicular torsion have focused on histology and function as they relate to acute (i.e., during torsion) [3, 9] or long-term (i.e., weeks after detorsion) [7, 8, 14] sequelae, it remains unknown whether the majority of injury actually occurs during the period of torsion (i.e., ischemia) or immediately following detorsion (i.e., reperfusion). McCord [11] suggests that in tissue ischemia of other organ systems, such as intestine and myocardium, a substantial portion of the injury may involve the production of cytotoxic substances released during reperfusion of the affected tissue.

During ischemia, depletion of intracellular adenosine triphosphate (ATP) results in an increased concentration of adenosine monophosphate (AMP), which is then metabolized to adenosine, inosine, and hypoxanthine [11]. In ischemic tissue, xanthine dehydrogenase undergoes calcium-catalyzed proteolysis to the enzyme xanthine oxidase. Hypoxanthine serves as a substrate for xanthine oxidase and molecular oxygen (supplied at reperfusion) to produce a burst of superoxide radicals, $O_2^{\cdot-}$.

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Table 1. Summary of animal groups

I	Sham animals (operative procedure, no torsion) ($n = 4$)
II	6 h torsion only; no reperfusion ($n = 12$)
III	12 h torsion only; no reperfusion ($n = 8$)
IV	24 h torsion only; no reperfusion ($n = 12$)
V	6 h torsion, 1 h reperfusion ($n = 13$)
VI	6 h torsion, 6 h reperfusion ($n = 12$)
VII	6 h torsion, 24 h reperfusion with saline vehicle injection (0.4 ml IV) 30 min prior to detorsion ($n = 24$)
VIII	6 h torsion, 24 h reperfusion with deferoxamine (50 mg/kg IV) injection 30 min prior to detorsion ($n = 36$)
IX	6 h torsion, 24 h reperfusion with diltiazem (400 μg/kg IV) injection 30 min prior to detorsion ($n = 4$)

IV, Intravenous

Haber and Weiss postulated in 1934 that $O_2^{\cdot -}$ and hydrogen peroxide (H_2O_2), when catalyzed by traces of transitional metals, interact to form the hydroxyl radical ($OH^{\cdot -}$) [5]:

$$O_2^{\cdot -} + H_2O_2 \xrightarrow{\text{Iron salt catalyst}} O_2 + OH + OH^{\cdot -}$$

Hydroxyl radical formation has been inhibited by chelators that bind iron [5]. Deferoxamine, an iron chelator, has been shown to reduce reperfusion-induced injury in the brain, intestine, and heart when administered before reperfusion, presumably by competitively inhibiting the above mechanism [4, 6, 13, 16].

Another proposed pathway of reperfusion injury involves alterations in calcium homeostasis. The calcium pathway has been implicated in reperfusion injuries of the liver and heart [12, 15]. Demonstration of the beneficial effects seen with the administration of calcium channel blockers before reperfusion supports this proposed pathway as a mechanism of reperfusion injury [12, 15].

Thus, the aims of this study were to determine: (1) whether damage following testicular torsion–detorsion has a reperfusion component similar to that described in other tissues, and (2) whether iron-catalyzed Haber-Weiss oxygen radical formation or altered calcium homeostasis plays a role in this injury.

Materials and methods

The following protocol was approved by the All-University Committee on Animal Use and Care, Michigan State University, East Lansing, Michigan.

Experimental animals

Prepubertal (37–45 days old) Sprague-Dawley rats weighing 165–210 g were housed in a temperature-controlled room (22 ± 1 °C) on a 12-h light/dark cycle and allowed rat chow and water ad libitum. On the day of the experiment, the animals were randomly assigned to one of nine groups as detailed in Table 1.

Induction of torsion

All experiments were performed under clean conditions using methoxyflurane inhalation anesthesia and subcutaneous buprenorphine (0.01 mg/kg) for postoperative analgesia. A transscrotal incision was made, and the right tunica vaginalis was dissected from the tunica dartos. After a small incision was made at the base of the tunica vaginalis, the testicle was expressed, twisted 720° clockwise, and returned to the scrotum. The scrotum was closed, fixing the testicle in two places with a running 6-0 Ethilon suture. Torsion was maintained according to the assigned group (Table 1). Sham animals (group I) were prepared, in an identical manner, except that no torsion was induced.

Treatment regimen

Saline vehicle (0.4 ml), deferoxamine (50 mg/kg), or diltiazem (400 μg/kg) was administered by tail vein injection to the appropriate treatment groups (groups VII–IX, respectively) 30 min prior to detorsion.

Detorsion of testicles

Detorsion was accomplished in the appropriate groups (Table 1) by reopening the scrotal incision under methoxyflurane anesthesia, removing the retaining sutures, and untwisting the spermatic cord. The incisions were closed with a running 6-0 suture. The testicle was fixed during closure to prevent subsequent retorsion. Buprenorphine was again administered for analgesia, and the rat was returned to its cage.

Harvesting testicles

The rat was anesthetized with methoxyflurane at the appropriate time (according to group, Table 1), and the scrotal incision was opened. The ipsilateral and contralateral testes were removed, capsules incised, and immediately placed in 10% buffered formalin for histologic examination. The rat was then killed by methoxyflurane overdose and pneumothorax.

Histologic preparation

All specimens were submitted in a masked fashion to a pediatric pathologist who was not present at the time of the experiments. Testes were paraffin embedded, sectioned at 4 μm, stained with hematoxylin and eosin (H&E), and examined by light microscopy. The contralateral testicle served as an internal control.

Histologic grading

A four-level grading scale similar to that of Cosentino et al. [3] was used to quantify histologic injury. Grade 1 (Fig. 1) showed normal testicular architecture with an orderly arrangement of germinal cells. Grade 2 injury (Fig. 2) demonstrated less orderly, noncohesive germinal cells and closely packed seminiferous tubules. Grade 3 injury (Fig. 3) exhibited disordered, sloughed germinal cells with shrunken, pyknotic nuclei and less distinct seminiferous tubule borders. Grade 4 injury (Fig. 4) defined seminiferous tubules that were closely packed with coagulative necrosis of the germinal cells.

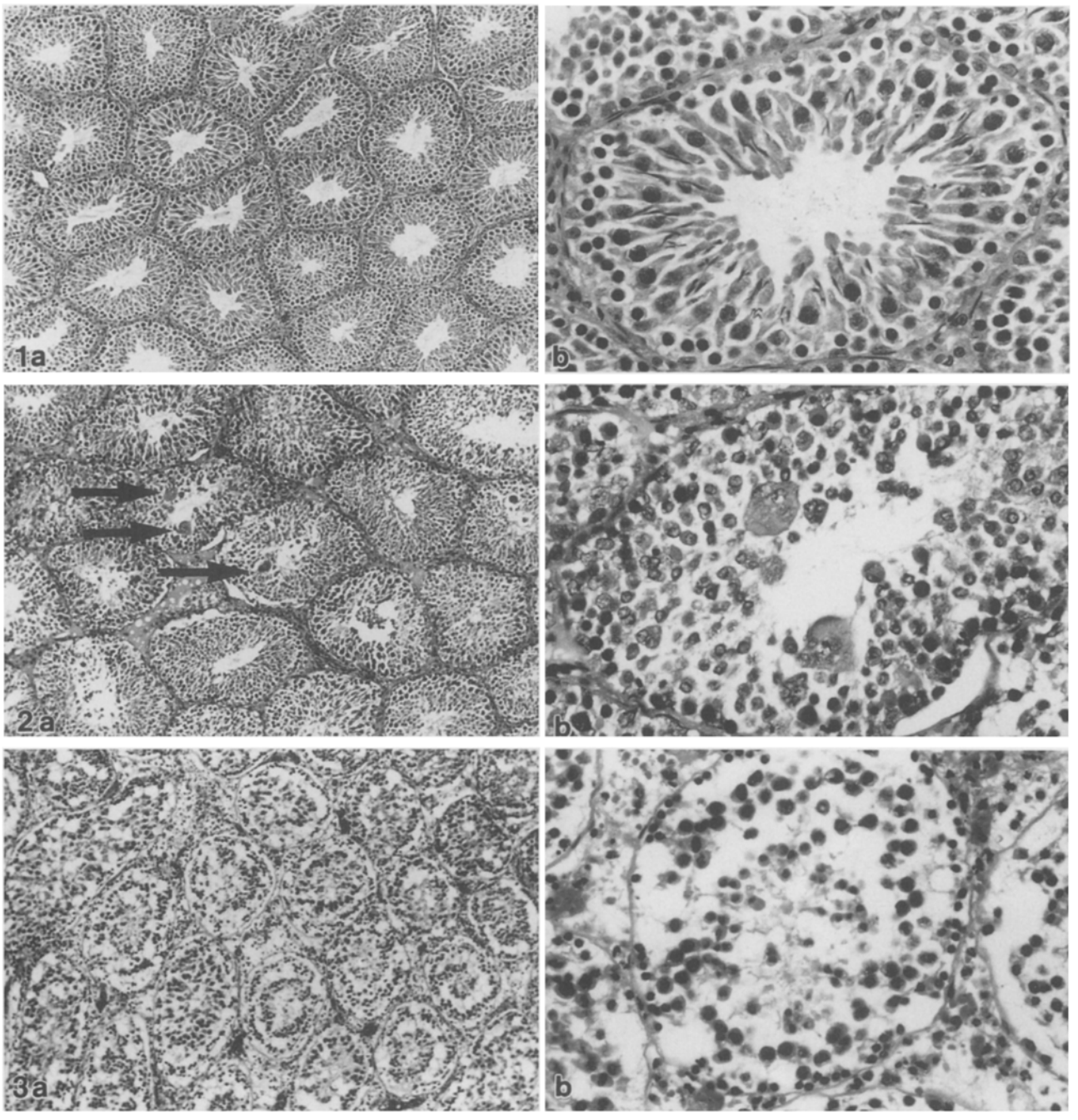

Fig. 1. a Grade 1, normal testis, low power (H&E, ×100). **b** Grade 1, normal testis, high power. Note the orderly arangement of germinal cells. (H&E, ×400)

Fig. 2. a Grade 2 injury, low power. Seminiferous tubules are closely packed. Germinal cells are less orderly. Note the focal "giant cells" (*arrows*). (H&E, ×100). **b** Grade 2 injury, high power. Germinal cells are not cohesive. Two "giant cells" are seen in this field. (H&E, ×400)

Fig. 3. a Grade 3 injury, low power. Borders of seminiferous tubules are less distinct but are still closely packed. Germinal cells have a very disordered arrangement. (H&E, ×100). **b** Grade 3 injury, high power. Cells are sloughed into the lumen and have shrunken, pyknotic nuclei. (H&E, ×400)

Statistical analysis

The histologic data were tested for significance using the Mann-Whitney two-sample *U*-test for non-parametric data.

Results

The majority (83%) of group II (6 h torsion only; no reperfusion) specimens had normal tubular architecture by light microscopy. Two of the 12 specimens showed very mild histologic changes, the most severe specimen having a histologic score of 2. The group II specimens also had prominent peritubular hemorrhage.

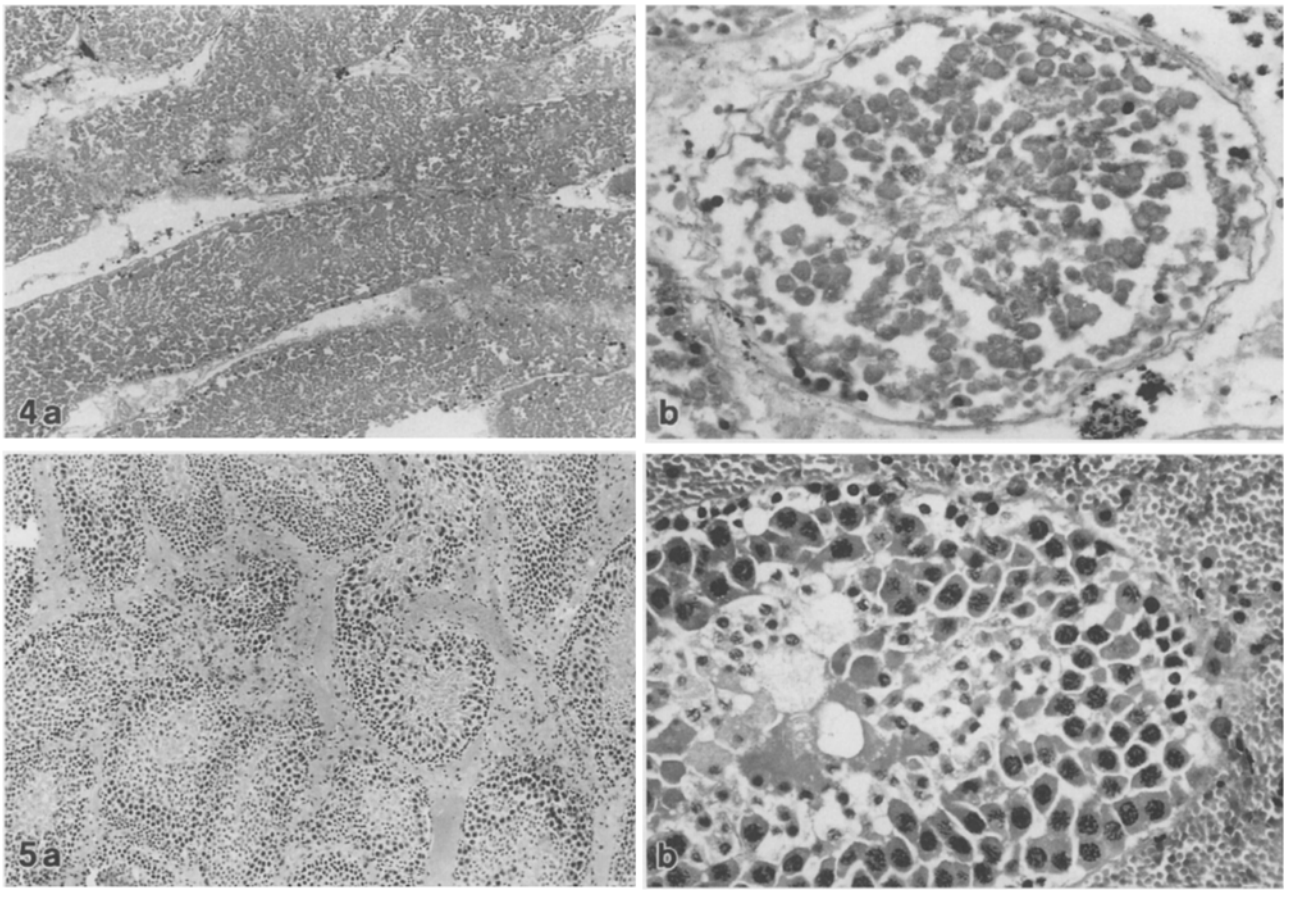

Fig. 4. a Grade 4 injury, low power. Tubules are closely packed with loss of cellular detail. (H&E, ×100). **b** Grade 4 injury, high power. There is cell sloughing with coagulative necrosis and loss of all nuclear detail. (H&E, ×400)

Fig. 5. a Ischemia-only (24 h) pattern of injury, low power. Tubules are separated by interstitial blood, and tubular basement membranes are disrupted. Germinal cells are not cohesive. (H&E, ×100). **b** Ischemia-only (24 h) pattern of injury, high power. Germinal cells are not cohesive but maintain a somewhat more orderly arrangement than in reperfusion injury. The nuclear chromatin appears stellate or serpentine. (H&E, ×400)

Many of the specimens of group V (6 h torsion, 1 h reperfusion) and group VI (6 h torsion, 6 h reperfusion) had features similar to the injuries seen in group II (6 h torsion only). As the time of reperfusion progressed, however, the injury became more severe. Specimens from group VII (6 h torsion, 24 h reperfusion with saline) had a significantly ($P<0.001$) greater histologic injury than group II (6 h torsion only) (2.6 ± 0.9 vs 1.3 ± 0.3, respectively). The vast majority of specimens in group VII demonstrated marked disarray of germinal cells with shrunken, pyknotic nuclei (median score = 3).

Group III (12 h torsion only; no reperfusion) and group IV (24 h torsion only; no reperfusion) specimens had injuries not quantifiable on the histologic grading scale and qualitatively different from the injuries seen in the reperfusion groups (groups VI–IX). The specimens in group IV (Fig. 5) displayed germinal cells which had a more orderly architecture with a stellate pattern of karyorrhexis. The ischemia-only injury was greater in the 24 h torsion-only group (group IV) than in the 12 h torsion-only group (group III).

Table 2 summarizes the degree of histologic damage that occurred in groups VII, VIII, and IX (treatment with saline, deferoxamine, and diltiazem, respectively). Neither drug ameliorated the reperfusion-induced histologic damage.

Discussion

In the present study, 6 h of ischemia alone (group II) did not damage tubular or germinal cell architecture significantly but did produce widespread peritubular hemorrhage. This type of injury is consistent with a torsion injury, since venous occlusion produces increased capillary hydrostatic pressure, thus inducing vascular rupture and interstitial hemorrhage. Both arterial and venous occlusion occur, however, as tissue edema increases. This results in tissue hypoxia and degeneration of cellular architecture [2]. In fact, degeneration of cellular architecture was seen in the 12 and 24 h torsion-only groups (groups III and IV, respectively) and in the previous study by Cosentino et al. [3]. Thus, ischemia alone results in cellular damage due to hypoxia, the inadequate delivery of nutrients, and removal of waste products.

When the component of reperfusion (detorsion) was added, a qualitatively different pattern of injury was observed. The histologic damage seen in group V (6 h torsion, 1 h reperfusion) and group VI (6 h torsion, 6 h

Table 2. Histologic grading of reperfused testes with various treatments

Treatment	Group no.	*n*	Mean (± SD) histologic score	Median histologic score	Significant difference vs control
Control	II	12	1.3 ± 0.3	1	–
Saline	VII	24	2.6 ± 0.9	3	$p < 0.001$
Deferoxamine	VIII	36	2.4 ± 0.9	3	$p < 0.001$
Diltiazem	IX	4	2.8 ± 0.4	3	$p < 0.001$

Control group, 6 h torsion only; no reperfusion. See Table 1 for detailed explanation of the treatment groups. Statistical significance ($p < 0.001$ vs control) was identified by the Mann-Whitney two-sample *U*-test

reperfusion) has features consistent with torsion-only injuries combined with injuries seen in reperfusion (group VII: 6 h torsion, 24 h reperfusion). This suggests that in addition to the damage due to ischemia alone, there is a time-dependent component of further injury related to reperfusion.

The testicular "reperfusion injury" identified by this study may be explained by non- or malperfusion of the testicle over the course of the reperfusion (i.e., the tissue remained persistently ischemic).If this were true, however, one would expect the reperfused testes that were persistently ischemic to display a similar morphologic appearance to that of the torsion (ischemia)-only testes. The appears not to be the case since the histologic injury in the non-reperfused testes at 12 h of ischemia-only (group III) was different from the time-matched, 12 h reperfused testes (group VI: 6 h of ischemia followed by 6 h reperfusion).

To evaluate the role of the iron-catalyzed Haber-Weiss pathway in the reperfusion-induced histologic damage seen with testicular torsion–detorsion, deferoxamine, an iron chelator, was administered prior to detorsion/reperfusion. Deferoxamine had no effect on the degree of histologic damage incurred with testicular torsion–detorsion (Table 2). The role of altered intracellular calcium homeostasis was evaluated by administering a calcium channel blocker, diltiazem, prior to detorsion/reperfusion (Table 2). Again, no significant effect on the histologic injury was demonstrated by the administration of diltiazem.

The inefficacy of iron chelators or calcium channel blockers in ameliorating injury may be due to one of three possibilities. First, the injury following torsion may not be related to reperfusion. The data in this study, however, indicate that a time-dependent, qualitatively different injury occurs with reperfusion. This makes this first possibility unlikely. Secondly, since the testis is an organ with slow arterial inflow with little capacity for post-ischemic flow supplementation (hyperemia) and a high first-pass oxygen extraction, its mechanism of reperfusion injury may differ from that of other organs studied. Third, the ischemia/reperfusion injury in this model may be too severe for amelioration. Further work on each of these explanations is warranted, since torsion-induced injury as a clinical entity could be amenable to treatment if an appropriate pharmacologic agent could be administered at the time of operative detorsion.

In conclusion: (1) the histologic changes produced by ischemia alone versus ischemia with reperfusion indicate that reperfusion produces a qualitatively distinct injury at the light microscopic level in this model, and (2) iron-chelating agents and calcium channel blockers do not ameliorate the histologic damage following testicular reperfusion in this model. In the future, agents other than iron chelators and calcium channel blockers should be used in this model to determine whether the reperfusion-induced injury can be ameliorated following testicular torsion–detorsion.

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