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ORIGINAL ARTICLE

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Effect of diclofenac on germ cell apoptosis following testicular ischemia-reperfusion injury in a rat

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Abstract Recent evidence suggests that enhanced cell apoptosis is responsible for germ cell loss following testicular ischemia-reperfusion (IR) injury. A nonsteroidal anti-inflammatory drug diclofenac sodium (Voltaren) is a prostaglandin-synthesis inhibitor, which is widely used in many testicular disorders. The purpose of the present study was to examine the effect of diclofenac (DIC) on germ cell apoptosis in the ischemic and contralateral testes following testicular IR in a rat. Forty rats were divided randomly into four experimental groups of ten rats each: group A (Sham)—Sham operated animals; group B (Sham-DIC)—Sham operated rats that were treated with DIC given subcutaneously at a dose of 10 mg/kg, once daily, 24, 48 and 72 h following operation; group C (IR) underwent 90 min of unilateral testicular IR; group D (IR-DIC)—rats underwent 90 min of unilateral testicular IR and were treated with DIC similarly to group B. Ninety-six hours following operation, the rats were sacrificed and testes were harvested. Johnsen's criteria and the number of germinal cell layers were used to categorize the spermatogenesis. TUNEL assay was used to determine germ cell apoptosis in both the ischemic and contralateral testes. Statistical analysis was performed using the nonparametric Kruskal–Wallis ANOVA test, with *P* less than 0.05 considered statistically significant. Testicular ischemia in rats led to histological damage in the ipsilateral testis. In the contralateral testis, minimal damage was observed. Germ cell apoptosis in both the ischemic and the contralateral testes increased significantly after IR. Treatment with DIC did not change histologic parameters of spermatogenesis in both the ischemic and contralateral testes, but decreased germ cell apoptosis in both testes following testicular IR. We conclude that testicular ischemia causes an increase in germ cell apoptosis in the contralateral testis. Diclofenac may be beneficial for spermatogenesis following testicular IR by decreasing germ cell apoptosis.

Keywords Testis · Ischemia-reperfusion · Germ cell apoptosis · Diclofenac

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Introduction

Testicular torsion constitutes a common surgical emergency affecting newborns, children and adolescents. The mechanisms of testicular injury following torsion include nonspecific damage induced by ischemia per se and damage caused by reperfusion. Testicular ischemia induces germ cell death, which is attributed mainly to a reduction in oxygen supply relative to metabolic demands, depletion of the stored cellular energy and accumulation of toxic metabolites. The reperfusion phase may significantly exacerbate ischemia-induced germ cell injury via the formation of reactive oxygen and nitrogen species [6]. Despite improvement in early diagnosis and changes in clinical management, infertility remains the most serious long-term complication of testicular torsion [2]. The degree of fertility loss in an individual with testicular

torsion depends on the extent of the ischemia and the subsequent damage to the contralateral testis. In addition to injury to ipsilateral (ischemic) testis, testicular IR may affect spermatogenesis in the contralateral testis [17]. The mechanisms of this effect remain unclear. There is growing evidence that testicular ischemia impairs the blood-testis barrier and exposes the contralateral testis to the potential risk of autoimmunization against its own spermatogonia [7]. Reperfusion time appears to be determinant of contralateral testis damage due to the consequent oxidative insult that accompanies the rise in reactive oxygen species following the IR event [6]. Recent evidence suggests that accelerated apoptosis of the primary spermatocytes might partially account for the mechanism of germ cell loss in the ischemic testis [22]. In a rat model of testicular IR, we have recently shown that 24 h after IR, germ cell apoptosis in the contralateral testis increased significantly and that the extent of apoptosis increases with the duration of the ischemia [19].

The identification of factors that prevent germ cell injury during IR as well as factors that improve testicular recovery might suggest new therapeutic strategies in preventing infertility following testicular torsion. Nonsteroidal anti-inflammatory drugs (NSAIDs) are frequently used in the treatment of many testicular disorders because of their analgesic and anti-inflammatory activities. A major mechanism of the action of NSAIDs is generally thought to be the inhibition of cyclooxygenase (COX), which is a key enzyme in catalyzing the conversion of arachidonic acid to prostaglandins PGG_2 and PGH_2 [23]. The major effect of NSAIDs administration in certain disorders is related to the ability of NSAIDs to induce apoptosis in a variety of cells.

The purpose of the present study was to evaluate the effect of the NSAID diclofenac sodium (Voltaren) on spermatogenesis and germ cell apoptosis in both the ischemic and the contralateral testes after testicular IR in rats.

Materials and methods

Animals

Animal facilities and protocols were approved by Rappaport Faculty of Medicine (Technion, Haifa, Israel) Institutional Animal Care and Use Committee. Male Sprague-Dawley rats weighing 250–300 g were acclimatized at 21°C on 12 h day and night cycles for 3–5 days before the experiment. The rats had free access to water and were pair fed with standard chow. Rats were fasted for 24 h before the experiment but were allowed free access to water.

Experimental design

Forty rats were divided randomly into four experimental groups of ten rats each: group A (Sham)—Sham rats underwent laparotomy; group B (Sham-DIC)—rats underwent laparotomy and were treated with diclofenac sodium given subcutaneously at a dose of 10 mg/kg, once daily, for 3 days following operation; group C (IR) rats underwent laparotomy and 90 min of unilateral testicular ischemia-reperfusion; group D (IR-DIC)—rats underwent laparotomy, 90 min of unilateral testicular ischemia-reperfusion and were treated with diclofenac similar to group B.

Surgical procedure

Following overnight fasting, the rats were anesthetized with intraperitoneal pentobarbital (40 mg per kg). The abdomen was opened through a midline incision. In rats undergoing a Sham operation (groups A and B), the left testis was exteriorized through an incision with simple mobilization of the testicular vessels without their clamping. Then, the testis was replaced immediately into the scrotum and the incision of the abdominal wall was covered with a warm moist dressing to prevent hypothermia for 90 min. In groups C and D, after mobilization of the left testis, its vascular was occluded by an atraumatic microvascular clamp for 90 min, during which time it remained in the abdomen with a closed incision to avoid hypothermia. After 90 min, the incision was reopened, the vascular clamp was removed, and the testis was reinserted into the scrotum via the inguinal canal. For all operations, the abdominal cavity was closed in two layers with a running suture of Dexon ‘‘S’’ Polyglycolic Acid 3/0. Rats were allowed free access to water and food. Ninety-six hours later, the rats were anesthetized with an overdose of intraperitoneal pentobarbital (75 mg/kg) and were sacrificed. Both testes were quickly removed and weighed. The samples of testicular tissues were fixed in a buffered 4% formaldehyde solution and then embedded in paraffin wax using standard techniques. Sections (5 μm each) were cut and stained with hematoxylin and eosin. Histological alterations were studied using a graded eye piece at ten times magnification.

Histopathologic evaluation of spermatogenesis

The number of germinal cell layers and Johnsen’s score were used to categorize the spermatogenesis in both ischemic and contralateral testes. The number of germinal epithelial layers was counted in ten seminiferous tubules as described by Miller et al [14] and the mean number was calculated. Each tubular section was given a score ranging from 10 to 1 according to the presence or absence of the main cell types arranged in the order of maturity as described by Johnsen [9]: 10—complete spermatogenesis and normally organized tubules; 9—many spermatozoa present but germinal epithelium disorganized; 8—only a few spermatozoa present in the section; 7—no spermatozoa but many spermatids

present; 6—only a few spermatids present; 5—no spermatozoa or spermatids but many spermatocytes present; 4—only a few spermatocytes present; 3—only spermatogonia present; 2—no germ cells but only Sertoli cells present; 1—no germ cells and no Sertoli cells present.

Evaluation of germ cell apoptosis

Germ cell apoptosis was evaluated by in situ TUNNEL assay for apoptotic cell detection using the I.S. Cell Death Detection kit (Boehringer Mannheim GmbH, Mannheim, Germany). Briefly, serial 5 μm thick paraffin-embedded sections were deparaffinized, rehydrated in graded alcohol, and microwave-pretreated in 10 mM citrate buffer (pH 6.0) for 10 min. After washing in phosphate-buffered saline (PBS), the specimens were incubated with fluorescein-labeled deoxy-UTP and TdT at 37°C for 60 min. After washing, the slides were incubated with blocking solution (3% H_2O_2 in methanol) for 10 min and were stained with anti-fluorescein antibody, fab fragment from sheep and conjugated with horse-radish peroxidase (converter-POD) at 37°C for 30 min. AES substrate (Zymed Laboratories) was applied for color development. For each group, the number of stained cells was counted in ten tubules in the areas without necrosis. For each group, the number of stained germ cells was counted. The apoptotic index AI-1 was defined as the number of apoptotic TUNEL-positive cells per 100 tubules and AI-2 as the number of tubules containing apoptotic cells per 100 tubules. Pathologists blinded to the source of testicular tissue performed all measurements.

Statistical analysis

The data are expressed as the mean ± SEM. A nonparametric Kruskal–Wallis ANOVA test was used for statistical analysis with P less than 0.05 considered statistically significant.

Results

Testicular parameters of spermatogenesis in the ischemic testis

Table 1 compares the weight of testes and histologic changes among the four experimental groups. As expected, manipulation of the ipsilateral testis in Sham rats (group A) led to a decrease in testis weight, mean testicular score (Johnsen criteria) (8.0 ± 0.7 vs 9.3 ± 0.2, $P < 0.05$) and a trend toward a decrease in the number of germ cell layers compared to the contralateral testis. Treatment with DIC (group B) did not change the ipsilateral testis weight significantly but resulted in a mild decrease in the number of germ cell layers (14%, $P < 0.05$) compared to Sham-nontreated animals (group A). Testicular ischemia (group C) resulted in a severe damage to the ischemic testis and was accompanied by a significant decrease in testicular weight, in germ cell layer count (threefold, $P < 0.05$) and in the mean testicular score (Johnsen's criteria) (3.5-fold, $P < 0.05$) compared to Sham animals (group A). Treatment with DIC (group D) did not significantly change testicular parameters of spermatogenesis following testicular IR. IR-DIC rats (group D) demonstrated a mean score and germ cell layer count similar to that of IR-rats (group C) and lower values compared to Sham (group A) and Sham-DIC (group B) rats.

Testicular parameters of spermatogenesis in the contralateral testis

The Sham operated animals (group A) demonstrated a normal architecture of the seminiferous tubules and interstitium and had intact germinal epithelium in the contralateral testis with an average thickness of about four to five cell layers. Treatment with DIC (group B) resulted in a mild but significant decrease in mean score (6.5%, $P < 0.05$) and germ cell layer count (7%, $P < 0.05$) change compared to Sham animals (group A). Ninety-six hours after IR (group C), there were minor changes in histopathologic parameters of spermatogenesis in the contralateral testis (vs. Sham animals, group A) that did not achieve statistical significance. Administration of diclodenac (groups D) did not affect histologic parameters of spermatogenesis (mean testicular score and the number of germ cell layers) in the contralateral testis compared to IR-nontreated rats (group C).

Evaluation of germ cell apoptosis

Table 2 represents data concerning germ cell apoptosis in both ipsilateral and contralateral testes in all experimental groups. As expected, Sham rats (group A) exhibited a low apoptotic index in both testes. Treatment with DIC (group B) resulted in a significant increase in programmed germ cell death in the ipsilateral testis, expressed as the number of apoptotic cells per 100 tubules (AI-1, twofold increase, $P = 0.08$) and the number of positive tubules per 100 tubules (AI-2, threefold increase, $P < 0.05$) and a trend toward an increase in germ cell apoptosis in the contralateral testis compared to Sham animals (group A); however, this trend did not achieve statistical significance. Testicular IR (group C) resulted in a marked increase in germ cell apoptosis in the ischemic testis (11-fold increase in AI-1 and 20-fold increase in AI-2, $P < 0.05$), compared to Sham animals (group A). Treatment with DIC (group D) resulted in a significant decrease in germ cell apoptosis in both the ischemic (2.5-fold decrease in AI-1 and 2.4-fold decrease in AI-2, $P < 0.05$) and contralateral (3.5-fold decrease in AI-1 and threefold decrease in AI-1, $P < 0.05$) testes compared to IR-untreated animals (group C) (Fig. 1).

Table 1 Effect of testicular ischemia-reperfusion and treatment with DIC on testis weight and spermatogenesis in ischemic and contralateral testes

Groups	Testis weight (g)		Ipsilateral testis		Contralateral testis	
	Ipsilateral testis	Contralateral testis	Johnsen's criteria	Number of germ cell layers	Johnsen's criteria	Number of germ cell layers
Sham	1,635 ± 46	1,797 ± 43	8.5 ± 0.5	4.2 ± 0.2	9.2 ± 0.2	4.4 ± 0.1
Sham-DIC	1,717 ± 31	1,772 ± 30	7.6 ± 0.4	3.6 ± 0.2*	8.6 ± 0.2*	4.0 ± 0.1*
IR	1,181 ± 74*, **	1,716 ± 65	2.5 ± 1.0*, **	1.4 ± 0.7*, **	9.2 ± 0.4	4.1 ± 0.2
IR-DIC	1,099 ± 10*, **	1,707 ± 49	3.1 ± 0.5*, **	1.0 ± 0.4*, **	8.8 ± 0.2	4.0 ± 0.1*

Values are mean ± SEM *IR* ischemia-reperfusion, *DIC* diclofenac
*$P < 0.05$ versus Sham operated animals
**versus Sham-DIC rats

Table 2 Effect of testicular ischemia-reperfusion and treatment with DIC on germ cell apoptosis in ischemic and contralateral testes

Experimental groups	Ipsilateral testis		Contralateral testis	
	AI-1	AI-2	AI-1	AI-2
Sham	2.1 ± 1.0	1.2 ± 0.6	3.0 ± 1.3	1.9 ± 0.8
Sham-DIC	4.4 ± 1.2	4.0 ± 1.4*	4.2 ± 1.5	2.8 ± 0.5
IR	41.1 ± 9.5*, **	13.4 ± 5.4*, **	7.6 ± 1.4*	5.2 ± 1.1*, **
IR-DIC	16.6 ± 3.0*, **, ***	5.6 ± 0.6*, ***	2.1 ± 0.5***	1.7 ± 0.4***

Values are mean ± SEM *IR* ischemia-reperfusion, *DIC* diclofenac, *AI-1* apoptotic index 1, the number of apoptotic cells per 100 tubules, *AI-2* apoptotic index 2, the number of positive tubules per 100 tubules
*$P < 0.05$ versus Sham operated animals
**versus Sham-DIC rats
***IR-DIC versus IR rats

Discussion

Germ cell apoptosis has been reported by several investigators to play an important role in the normal testicular physiology. Programmed germ cell death is crucial during embryonic development of the human gonads [16]. Apoptosis control is important for regulation of the population of germ cells in the adult testis [2]. Recent evidence suggests that enhanced germ cell apoptosis is related to a decrease in germ cell mass in the aged testis with impaired spermatogenesis [11]. Besides its role in normal testicular physiology, apoptosis has been identified as important in the development of a variety of testicular disorders. Apoptosis of germ cells has been reported recently as a mechanism responsible for infertility in undescended testis. Hikim et al. [8] have recently shown that exposure of the rat testis to heat results in stage- and cell-specific activation of germ cell apoptosis. Tomomasa et al. [21] observed an increased germ cell apoptosis and impaired spermatogenesis in rats with experimental cryptorchidism. Varicocele is a common cause of male infertility. Recent experiments have demonstrated an increased apoptosis among developing germ cells in patients with varicocele, which may be the cause of oligospermia. Baccetti et al. observed that apoptosis was abnormally frequent in the sperm cells of the ejaculate of sterile men suffering from varicocele. The authors concluded that the natural presence of apoptosis, which starts in the testis and is revealed in the ejaculate, may explain many abnormal ultrastructural sperm patterns hitherto unexplained in fertile and infertile individuals [1]. Recent evidence suggests that apoptotic cell death plays an important role in limiting testicular germ cell population following testicular IR and its dysregulation may be associated with male infertility. Lysiak et al. [13] have recently demonstrated that neutrophil recruitment to the testis after IR in mice stimulates secretion of the pro-inflammatory cytokines, TNF-alpha and IL-1beta, which trigger germ cell apoptosis and decrease germ cell mass. Turner and colleagues have observed the first evidence for torsion-induced apoptosis at 4 h after repair of 1-h torsion. The authors hypothesized that apoptosis had been triggered by reactive oxygen species arising from reperfusing leukocytes [22]. Although the effect of testicular IR on germ cell apoptosis in the ischemic testis has been reported previously, an understanding of the mechanisms that regulate this pathway in the contralateral testis is lacking. In a recent experiment, we have shown that 24 h after testicular IR, there are minimal histologic changes in the contralateral testis which include a decrease in the number of germ cell layers, mild disorganization, and single cell apoptosis. However, germ cell apoptosis in the contralateral testis increased significantly after 2, 3, and 24 h of ischemia and showed direct, time-related correlation with the duration of

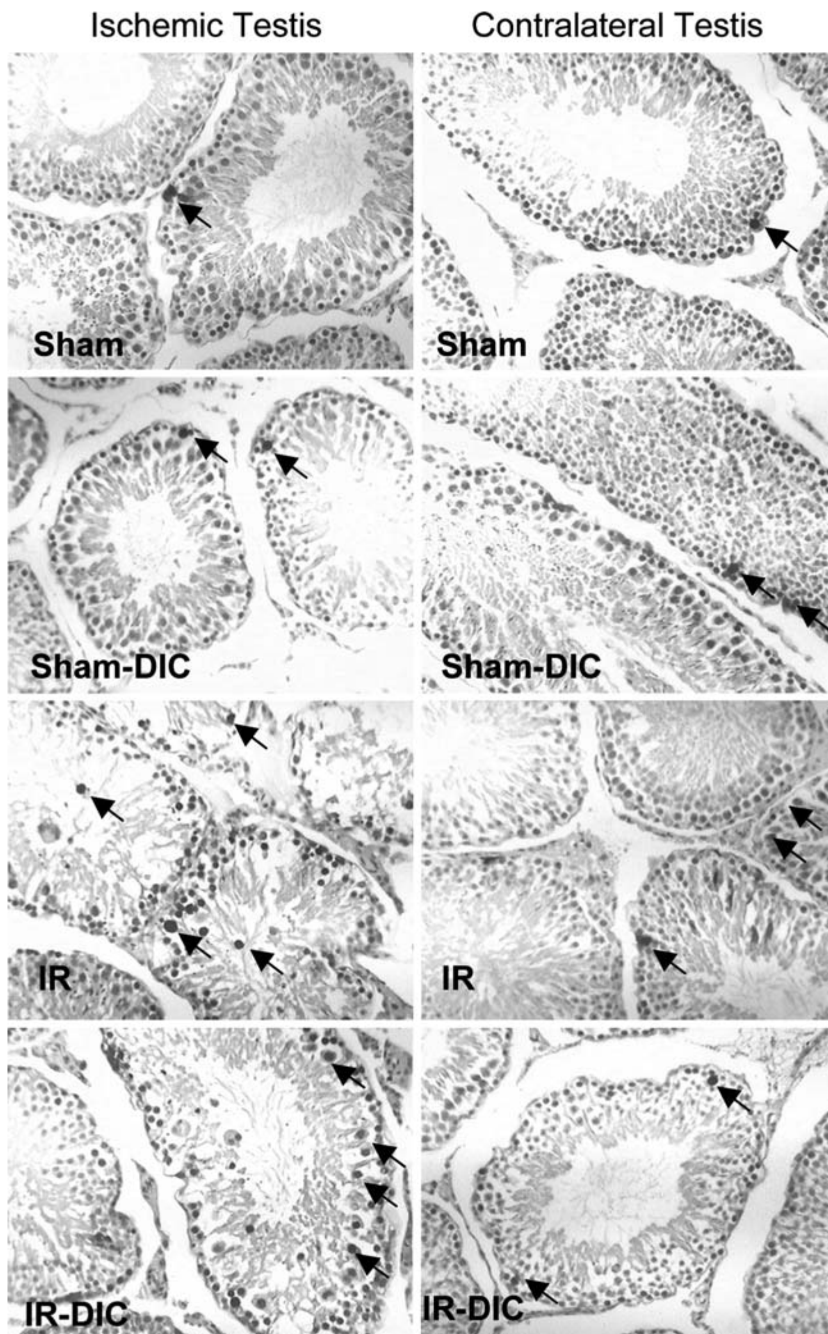

Fig. 1 Immunohistochemistry for apoptosis in the ipsilateral and in the contralateral testes in the four experimental groups. Single apoptotic cells (*arrows*) are present in sections from Sham and Sham-diclofenac treated rats. A marked tissue disorganization and multiple apoptotic cells are observed in ischemic testis. The number of apoptotic cells decreases in the ischemic testis following administration of DIC. Labeled cells are also frequently found in the contralateral testis of IR rats. The number of apoptotic cells decreases significantly in the contralateral testis following treatment with DIC

ischemia [19]. We concluded that increased germ cell apoptosis in the contralateral testis combined with ischemia-induced cell death in the ischemic testis, may lead to a significant decrease in the germ cell mass and may cause loss of fertility. In this context, identification of those agents or drugs that can decrease germ cell apoptosis may offer novel, scientifically relevant and practical approaches to prevent fertility loss following testicular IR.

Nonsteroidal anti-inflammatory drugs are the most widely used drugs for treating pain, arthritis, cardiovascular diseases, and more recently for colon cancer prevention [23]. However, NSAIDs produce gastrointestinal ulcers and delay ulcer healing. A major mechanism of the action of NSAIDs is generally thought to be inhibition of COX, which is a key enzyme which catalyzes the rate-limiting step of the prostanoid cascade. Arachidonic acid is converted by COX to prostaglandin H_2 (PGH_2); PGH_2 is then metabolized by different synthases into more biologically active products, such as other PGs (PGD_2, PGE_2, $PGF_{2\alpha}$, and PGI_2) and thromboxane (TXA_2). There are two distinct isoforms of COX: COX1 and COX2. COX1 is constitutively expressed in most tissues, while COX2

expression is barely detected at a constitutive level and is markedly inducible in specialized cell types [18]. COX1 is important in regulation of platelet aggregation and in parturition, while COX2 regulates specific physiological functions, such as the inflammatory process, ovulation, implantation, perinatal kidney development, ductus arteriosus remodeling, and ulcer healing [18]. COX1 and COX2 activities are differentially inhibited by NSAIDs. For example, aspirin and indomethacin (INDO) inhibit both enzymes, whereas NS-398 is a selective inhibitor for COX2. In addition to their anti-inflammatory properties, NSAIDs have immunosuppressive activities related to their capacity both to inhibit COXs and to act as peroxisome proliferator-activated receptor (PPAR) ligands.

NSAIDs have been shown to promote apoptosis in some cells, whereas they inhibit apoptosis in other cells depending on the drug amount, duration of treatment, and kind of target cells. An emerging body of literature has recently defined a reduced risk of cancer following the use of NSAIDs, which are generally able to exert a pro-apoptotic property and an inhibitory effect on the activity and/or expression of matrix metalloproteinases [15]. The chemopreventive efficacy of NSAIDs has been established for colorectal cancer and carcinoma of the esophagus, stomach, breast, lung, prostate, urinary bladder and ovary [10].

Because of their anti-inflammatory and analgesic activities, NSAIDs are frequently used in the treatment of many testicular disorders. The major concern about NSAIDs administration following testicular torsion is related to the ability of NSAIDs to promote cell apoptosis. Cheuk et al. [4] have shown that the COX-2 inhibitor NS-398 induced apoptosis of epididymal cells in a dose- and time-dependent manner. The second concern is an ability of NSAIDs to inhibit angiogenesis through alteration in vascular endothelial growth factor (VEGF), increased endothelial cell apoptosis, inhibition of endothelial cell migration, recruitment of inflammatory cells and platelets, and/or thromboxane A2 mediated effects [20].

The purpose of the present study was to evaluate whether treatment with DIC could affect germ cell apoptosis in both the ischemic and contralateral testes following testicular IR in a rat. Our data demonstrate that administration of DIC had a different effect on germ cell apoptosis and histologic criteria of spermatogenesis in normal (Sham) and IR rats. In Sham rats, DIC had a mild stimulating effect on germ cell apoptosis in both ipsilateral and contralateral testes; however, only changes in the ipsilateral testis achieved statistical significance. This increase in germ cell programmed death was associated with small changes in spermatogenesis. This is evident from a mild but significant decrease in the number of germ cell layers in both the ipsilateral and contralateral testis and in a decrease in Johnsen's score in the contralateral testis compared to Sham-nontreated animals. This pro-apoptotic effect of DIC was similar to that observed by Cheuk et al. [4] in epididymal cells following administration of another NSAID such as NS-398. Consistent with our previous study [19], testicular IR induced degeneration of germ cells and impaired spermatogenesis. A marked decrease in the average number of germinal epithelial cell layers and Johnsen's criteria in the ischemic testis support this conclusion. The measurement of germ cell apoptosis in the ischemic testis demonstrated an increase in cell programmed death following testicular IR. The present data are consistent with the results of other investigators who demonstrated that loss of germ cells after IR is from germ cell specific apoptosis [12, 22]. Treatment with DIC did not prevent damage caused by testicular IR in the ischemic testis and did not improve the recovery of testicular tissue from the ischemic injury, but resulted in a significant decrease in the germ cell apoptosis the in ischemic testis. Consistent with our previous study [19], in the present experiment testicular IR did not produce contralateral testicular histologic damage. IR rats showed normal histologic architecture of the seminiferous tubules in the contralateral testis. These data also fit in with reports from other investigators who have also noted no contralateral damage following ipsilateral experimental torsion [12]. We have previously reported increases in germ cell apoptosis in the contralateral testis 24 h following IR [19]. The present data indicate that 96 h after IR, germ cell apoptosis in the contralateral testis remains significantly increased. With the observed increase in germ cell apoptosis, the majority of seminiferous tubules maintain a normal architecture. It may be reasonable to presume that histological manifestation of impaired spermatogenesis will appear after a long period of enhanced programmed cell death. Our experiment demonstrates that treatment with DIC significantly decreased germ cell apoptosis in the contralateral testis. This is evident from the decrease in the number of apoptotic cells compared to the IR-nontreated animals. Further experiments are needed to clarify the mechanisms of the affect of DIC on germ cell apoptosis in both the ischemic and contralateral testes following testicular IR. Decreased production of prostanoids that are involved in signal transduction pathways activated by distinct ILs [24] as well as a decrease in production of nitric oxide [5] may be responsible for the anti-apoptotic effect of DIC on germ cell apoptosis after IR.

In conclusion, the NSAID DIC stimulates germ cell apoptosis in the normal testis, but produces a strong inhibitory effect on germ cell programmed death in the ischemic and contralateral testes following testicular IR. The use of NSAIDs may be beneficial in preserving germ cell mass and in preventing fertility loss after testicular IR.

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