ELSEVIER

INHIBITION OF POLY(ADENOSINE DIPHOSPHATE-RIBOSE) POLYMERASE DECREASES LONG-TERM HISTOLOGIC DAMAGE IN TESTICULAR ISCHEMIA-REPERFUSION INJURY

MURAT BOZLU, BANU COŞKUN, SELAHİTTİN ÇAYAN, DENİZ ACAR, SAVAŞ AKTAŞ, ERCÜMENT ULUSOY, AND ERDEM AKBAY

ABSTRACT

Objectives. To evaluate the effect of 3-aminobenzamide, an inhibitor of poly(adenosine diphosphate-ribose) polymerase (PARP), on the long-term histologic damage in testicular ischemia-reperfusion injury. PARP inhibitors have been used successfully to decrease ischemia-reperfusion injury in several organ systems.

Methods. Adult male Wistar rats were divided into four groups of 7 rats each. One group underwent 2 hours of testicular torsion; one received pretreatment with vehicle (dimethyl sulfoxide) before detorsion; one received pretreatment with 3-aminobenzamide, an inhibitor of PARP, before detorsion; and one group underwent a sham operation. All rats underwent bilateral orchiectomy 60 days after the experiment. The mean seminiferous tubular diameter, germinal epithelial cell thickness, and mean testicular biopsy score were determined by histologic examination of each testis.

Results. Testicular torsion-detorsion caused a significant decrease in the mean seminiferous tubular diameter, germinal epithelial cell thickness, and mean testicular biopsy score in the ipsilateral testes ($P < 0.001$), but not in the contralateral testes. The animals treated with 3-aminobenzamide had a statistically significant increase in these histologic parameters compared with the torsion-detorsion group ($P < 0.01$).

Conclusions. The results of this study show that PARP may have a role in the testicular damage caused by ischemia-reperfusion. Administering PARP inhibitors before reperfusion may have the potential to decrease the long-term histologic damage that occurs after testicular torsion. UROLOGY **63:** 791–795, 2004. © 2004 Elsevier Inc.

Testicular torsion is a surgical emergency. Late presentation or failure to diagnose and correctly manage this condition leads to testicular injury and subfertility.[1] Testicular injury due to torsion and detorsion is an ischemia-reperfusion injury attributed to neutrophil infiltration and generation of reactive oxygen species.[2–5] Reactive oxygen species, including superoxide anions, hydrogen peroxide or hydroxyl radicals, and nitric oxide or peroxynitrite, cause DNA damage. DNA damage by reactive oxygen species can lead to loss of cell viability by several mechanisms. It has been shown that reactive oxygen species produce strand breaks in DNA that trigger energy-consuming DNA repair mechanisms and activate the nuclear enzyme poly(adenosine diphosphate [ADP]-ribose) polymerase (PARP), also called poly(ADP-ribose) synthetase.[6]

Ischemia-reperfusion injury resulting in substantial DNA degradation requires that cells consume large amounts of adenosine 5′-triphosphate to support poly(ADP-ribosylation).[6] Massive ADP-ribosylation of nuclear proteins by PARP results in cellular energy depletion and injury. Although PARP is essential for DNA repair, its excessive activation during reperfusion of ischemic tissue may lead to cell death, and the activation of PARP is currently described as a final common effector in various types of tissue injury, including oxidative stress.[2,6–8] It has been demonstrated that activation of PARP is a consequence of ischemia-reperfusion

From the Departments of Urology and Histology/Embryology, University of Mersin School of Medicine, Mersin, Turkey

Reprint requests: Murat Bozlu, M.D., Department of Urology, University of Mersin School of Medicine, Zeytinlibahce Caddesi, Mersin 33079, Turkey

Submitted: September 2, 2003, accepted (with revisions): October 27, 2003

0090-4295/04/$30.00
doi:10.1016/j.urology.2003.10.062

injury in several tissues and that administration of PARP inhibitors leads to a decrease in ischemia-reperfusion injury to those organs.[9–12]

More recently, we demonstrated that administration of PARP inhibitors before detorsion had a protective role in the testicular biochemical changes associated with ischemia-reperfusion injury.[13] However, to our knowledge, the role of PARP inhibition on the histologic damage in testicular ischemia-reperfusion injury is undefined. In the present study, we examined whether 3-aminobenzamide (3-AB, Alexis, Lausen, Switzerland), an inhibitor of PARP, has a protective effect on histologic damage after testicular torsion followed by 60 days of recovery.

MATERIAL AND METHODS

Twenty-eight adult male Wistar rats weighing between 240 and 280 g were obtained and maintained on a 12-hour light/dark cycle. The ethical committee on animal research at our institution approved the protocol for all animal experiments. We provided appropriate care and use of the laboratory animals as recommended by the Board of Registry publication guidelines.

Chemicals

We used the benzamide analogue 3-AB, an inhibitor of PARP. 3-AB was dissolved in 10% dimethyl sulfoxide (DMSO). The administration mode and concentration of 3-AB in this study corresponded to those used in previous experimental studies.[6]

Animal Preparation and Surgical Procedure

The rats were divided into four groups of 7 rats each. One group underwent 2 hours of testicular torsion; the second received pretreatment with vehicle (DMSO) before detorsion; the third group received pretreatment with 3-AB before detorsion; and the fourth underwent a sham operation. Surgery was done with the subject under ketamine anesthesia (single intraperitoneal 50-mg/kg dose). All surgical procedures were performed through standard ilioinguinal incisions.

In the torsion-detorsion (T-D) group, the gubernaculum was divided, and the testis was freed from its longitudinal and distal pole attachment to the epididymis. Torsion was created by rotating the left testis 720° clockwise and maintained by fixing the testis to the scrotum with a 4-0 silk suture placed through the tunica albuginea. After 2 hours of torsion, the testis was counter-rotated back to the natural position and reinserted into the scrotum. The wound was closed using 3-0 catgut suture.

In the group receiving vehicle (DMSO) before detorsion (DMSO group), the same surgical procedure was done as in the T-D group, but DMSO was injected intraperitoneally for 30 minutes before detorsion and closure.

In the group receiving 3-AB before detorsion (3-AB group), the same surgical procedure was done as in the T-D group, but 3-AB (30 mg/kg) was injected intraperitoneally for 30 minutes before detorsion and closure.

In the control group (sham group), a sham procedure was performed. This consisted of the same procedure as in the T-D group, except that after rotating the testis 720° clockwise, it was immediately relieved and a 4-0 silk suture was placed through the tunica albuginea.

Histologic Preparation

After 60 days, bilateral orchiectomy was performed, and the rats were killed by pentobarbital overdose (200 mg/kg) and bilateral thoracotomy. The testes were fixed in Bouin's solution (7.5 mL saturated picric acid, 2.65 mL glacial acetic acid, and 2.5 mL 7% formaldehyde), postfixed in 70% alcohol, and embedded in paraffin blocks. Sections (5 μm) were obtained, deparaffinized, and stained with hematoxylin-eosin.

Histologic Evaluation

The testicular tissue was evaluated in random order with standard light microscopy by an observer unaware of to which group the rat had belonged. Three slides, prepared from the upper, lower, and mid portions of the testes, were evaluated completely for each testis. The mean seminiferous tubular diameter (MSTD), germinal epithelial cell thickness (GECT), and mean testicular biopsy score (MTBS) were used to evaluate in 20 seminiferous tubules of each section. The MSTD was calculated using a microscope-adaptable micrometer. The MSTD of each testis was determined in microns. GECT was determined by counting the number of epithelial cells from the basement membrane to the lumen at 90°, 180°, 270°, and 360°, and averaged. The MTBS was graded using Johnsen's score.[14] A score of 0 to 10 was given to each tubule according to epithelial maturation.

Statistical Analysis

All data are expressed as the mean ± standard deviation. Analysis of variance was used for statistical analysis of the data among the groups. Multiple comparisons were made using Tukey's procedure, with $P < 0.05$ considered statistically significant.

RESULTS

The results of the histologic evaluation for each group are shown in Table I and Figure 1. Compared with the sham group, the MSTD, GECT, and MTBS in the T-D group were significantly lower in the ipsilateral testes ($P < 0.001$). These three histologic parameters in the ipsilateral testes of the 3-AB group were significantly greater than the values in the T-D group ($P < 0.01$). The ipsilateral testicular MSTD, GECT, and MTBS in the vehicle DMSO group were not significantly different statistically than those in the T-D group ($P > 0.05$). The histologic parameters of the contralateral testes did not reveal any statistically significant differences among these groups ($P > 0.05$).

COMMENT

Testicular torsion is a urologic emergency. The salvage rate is directly proportional to the duration of torsion, and early diagnosis followed by detorsion is the current management for the preservation of spermatogenesis and fertility.[1] The results of the current investigation indicate that testicular torsion-detorsion induces progressive histologic changes as a result of ischemia-reperfusion injury. Previous studies using a rat model of testicular torsion have demonstrated that a 1-hour, 720° rotation of the testis followed by reperfusion results in

TABLE I. *Mean values of seminiferous tubular diameter, germinal epithelial cell thickness, and testicular biopsy score in ipsilateral and contralateral testes*

	MSTD ± SD (μm)		GECT (mean cell layers ± SD)		MTBS ± SD	
Group	Ipsilateral	Contralateral	Ipsilateral	Contralateral	Ipsilateral	Contralateral
Torsion-detorsion*	182.29 ± 21.29	263.29 ± 23.28	1.51 ± 0.38	8.12 ± 0.32	4.42 ± 1.91	9.89 ± 0.28
DMSO*	159.58 ± 27.64	266.66 ± 13.85	2.02 ± 1.10	7.80 ± 0.50	4.05 ± 2.01	9.90 ± 0.15
3-AB†	233.28 ± 23.83	265.46 ± 17.74	4.70 ± 1.45	7.85 ± 0.37	7.10 ± 1.37	9.95 ± 0.18
Sham	277.41 ± 13.26	264.69 ± 11.51	8.07 ± 0.52	8.02 ± 0.57	9.97 ± 1.22	9.97 ± 0.34

KEY: MSTD = mean seminiferous tubular diameter; GECT = germinal epithelial cell thickness; MTBS = mean testicular biopsy score; DMSO = dimethyl sulfoxide; 3-AB = 3-aminobenzamide.

No statistically significant differences in contralateral testes among groups (P >0.05).

** Versus sham in the ipsilateral testes (P <0.001).*

† Versus torsion/detorsion in the ipsilateral testes (P <0.01).

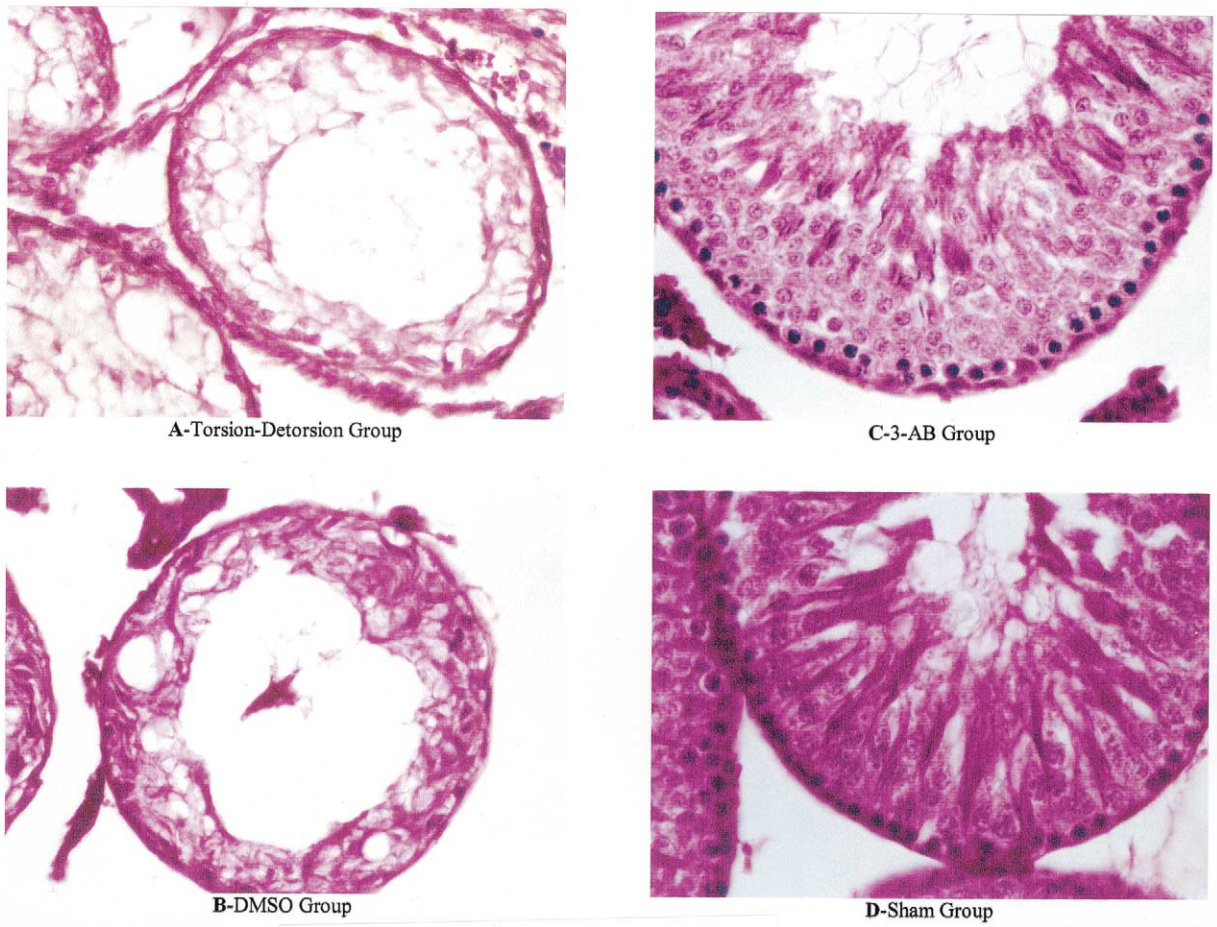

FIGURE 1. *Histologic findings of ipsilateral testes in (A) torsion-detorsion, (B) dimethyl sulfoxide, (C) 3-aminobenzamide, and (D) sham groups. Note, no recovery of spermatogenesis observed in testes of torsion-detorsion and dimethyl sulfoxide groups and preservation of spermatogenesis in 3-aminobenzamide group and normal spermatogenesis in sham group. Hematoxylin-eosin stain, reduced from ×400.*

the permanent loss of spermatogenesis.[15–17] The lesions in the current study were characterized by a decrease in seminiferous tubule diameter, germinal epithelial cell thickness, and degree of spermatozoal maturation. These observations are in agreement with those of previous studies.

Although reperfusion is essential for the survival of ischemic tissue, the main pathophysiology of testicular torsion is ischemia-reperfusion injury of the testis caused by the twisted spermatic cord and its release.[3] Ischemia-reperfusion injury initiates a pathophysiologic cascade including an inflammatory response with liberation of cytokines and reactive oxygen species generation.[2] Evidence has shown that reactive oxygen species induce cellular injury by inducing nicks in DNA. A recently dis-

covered mechanism of cell injury, the PARP pathway is involved in the pathogenesis of various forms of ischemia-reperfusion injury.[2,6–8] This pathway involves reactive oxygen species producing strand breaks in DNA, and consequent activation of the nuclear enzyme PARP. However, excessive DNA damage in the setting of ischemia-reperfusion injury may lead to excessive PARP activation that consumes large quantities of cellular nicotinamide adenine dinucleotide, resulting in adenosine 5′-triphosphate depletion. Moderate PARP activity protects cellular genome integrity, although excessive PARP activation leads to cell death secondary to massive adenosine 5′-triphosphate depletion. Overall, this process has been termed "the poly(ADP-ribose) polymerase suicide hypothesis."[18]

Use of PARP inhibitors after ischemia and reperfusion has been shown to improve the status of various organs after ischemia.[2,6–8] Administering PARP inhibitors led to a reduction of the ischemia-reperfusion injury to the heart and skeletal muscle in rabbits,[9] a reduction of brain infarct volume in a model of focal cerebral ischemia,[10] amelioration of the ischemia-reperfusion damage to the retina,[11] and protection against oxidative stress to the kidney in rats.[12] More recently, we showed that 2 hours of torsion with 720° of rotation of the testis followed by 4 hours of detorsion caused a statistically significant increase in the biochemical parameters, including malondialdehyde, nitric oxide content, and myeloperoxidase activity, an indicator of neutrophil accumulation, in testicular tissue related to ischemia-reperfusion injury.[13] In that study, we demonstrated that administration of four recognized PARP inhibitors (nicotinamide, 3-AB, 1,5-dihydroxyisoquinoline, or 4-amino-1,8-naphthalimide) before detorsion improved these biochemical parameters, and this treatment may protect the testis against ischemia-reperfusion injury.

Our successful results in the early period of testicular ischemia-reperfusion injury[13] led us to evaluate the effect of pharmacologic PARP inhibition on long-term histologic damage in the testicular torsion model of the rat. We used 3-AB, which was first recognized as a PARP inhibitor more than 20 years ago. It has been commonly used for this purpose in both in vivo and in vitro studies investigating its effects in many pathophysiologic conditions, including ischemia-reperfusion injury.[6–8] To our knowledge, we have demonstrated here, for the first time, that 3-AB has a beneficial effect on long-term histologic damage in testicular ischemia-reperfusion injury. When 3-AB was administered before detorsion, the preservation of the histologic parameters, including MSTD, GECT, and MTBS, were significantly maintained. The results of the present study support the hypothesis of improved seminiferous tubule diameter, survival of the germinal epithelium, and preservation of spermatogenesis with PARP inhibitor treatment before detorsion of the testis. All these histologic parameters may give an accurate measurement of the degree to which spermatozoal maturation is taking place within the seminiferous tubule and also the level of spermiogenesis that is related to fertility. In light of these findings, we propose that inhibition of PARP may be a novel approach for the therapy of ischemia-reperfusion injury of the testis.

It has been demonstrated that treatment with reactive oxygen species scavengers had a palliative effect on the histologic changes in testes that underwent 1 hour experimental torsion but no statistically significant rescue was seen after 2 hours of testicular ischemia.[19,20] In contrast, we found that inhibition of PARP provided a statistically significant decrease in the histologic damage after 2 hours of testicular torsion followed by detorsion. These observations could not be explained by a simple antioxidant effect of the PARP inhibitor. As previously stated, treatment with PARP inhibitors before detorsion prevented additional increases in lipid peroxidation of the testis and significantly reduced the concentration of testicular nitric oxide and myeloperoxidase activity, an indicator of neutrophil accumulation.[13] Recent studies have demonstrated that the loss of spermatogenesis after ischemia-reperfusion of the testis is due to germ cell-specific apoptosis, and an influx of neutrophils to the testis is essential for this pathologic finding.[4,5] Moreover, inhibition of PARP suppresses the inflammatory response and attenuates neutrophil recruitment.[13] One may anticipate that reduced leukocyte adhesion in testicular ischemia-reperfusion represents an important additional protective effect provided by PARP inhibitors. Additional studies are required to clarify the relationship between PARP and germ cell-specific apoptosis in relation to the recruitment of neutrophils.

Poly(ADP-ribosylation) is involved in the regulation of many cellular processes such as DNA repair, gene transcription, cell cycle progression, cell death, chromatin functions, and genomic stability.[2,6–8] In the present study, inhibition of PARP rescued the histologic changes observed after ischemia-reperfusion injury of the testis; however, we did not evaluate the DNA of the rescued germ cells. Because PARP is essential for DNA repair, it is possible that many of the rescued germ cells may have had defective DNA and thus were nonviable or perhaps carried mutated DNA. Our results may encourage other investigators to evaluate the DNA of the rescued germ cells in future studies.

The effect of unilateral torsion on the contralateral testis has been controversial. It has been demonstrated that ipsilateral torsion does not result in

contralateral testicular damage in rats.[21] Our previous study demonstrated no statistically significant effect of 2 hours of torsion followed by 4 hours of detorsion on the biochemical changes of the contralateral testis.[13] The histologic parameters of contralateral testes did not reveal any statistically significant differences, and the results of the present study support our earlier findings.

CONCLUSIONS

The results of the current study showed that inhibition of PARP before the reperfusion period of testicular torsion may result in prolonged testicular salvage. Administering PARP inhibitors before reperfusion may have the potential to decrease the long-term histologic damage that occurs after testicular torsion, with a subsequent improvement in fertility. We propose that PARP inhibition may be a novel approach for the therapy of ischemia-reperfusion injury of the testis. However, relatively little research has been done into the role of PARP within testicular torsion,[13] and additional studies are required to elucidate the mechanism of ischemia-reperfusion injury of the testis that involves PARP activation.

REFERENCES

1. Anderson JB, and Williamson RCN: The fate of human testes following unilateral torsion of the spermatic cord. Br J Urol **58:** 698–704, 1986.

2. Cuzzocrea S, Riley DP, Caputi AP, *et al:* Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. Pharmacol Rev **53:** 135–159, 2001.

3. Akgur FM, Kilinc K, Tanyel CF, *et al:* Ipsilateral and contralateral testicular biochemical acute changes after unilateral testicular torsion and detorsion. Urology **44:** 413–418, 1994.

4. Lysiak JJ, Nguyen QA, and Turner TT: Peptide and nonpeptide reactive oxygen scavengers provide partial rescue of the testis after torsion. J Androl **23:** 400–409, 2002.

5. Lysiak JJ, Nguyen QA, Kirby JL, *et al:* Ischemia-reperfusion of the murine testis stimulates the expression of proinflammatory cytokines and activation of c-jun *N*-terminal kinase in a pathway to E-selectin expression. Biol Reprod **69:** 202–210, 2003.

6. Szabo C, and Dawson VL: Role of poly (ADP-ribose) synthetase in inflammation and ischemia-reperfusion. Trends Pharmacol Sci **19:** 287–298, 1998.

7. Shall S, and de Murcia G: Poly (ADP-ribose) polymerase-1: what have we learned from the deficient mouse model? Mut Res **460:** 1–15, 2000.

8. Tentori L, Portarena I, and Graziani G: Potential clinical applications of poly (ADP-ribose) polymerase (PARP) inhibitors. Pharmacol Res **45:** 73–85, 2002.

9. Thiemermann C, Bowes J, Myint FP, *et al:* Inhibition of the activity of poly (ADP-ribose) synthetase reduces ischemia-reperfusion injury in the heart and skeletal muscle. Proc Natl Acad Sci USA **94:** 679–683, 1997.

10. Takahashi K, Greenberg JH, Jackson P, *et al:* Neuroprotective effects of inhibiting poly (ADP-ribose) synthetase on focal cerebral ischemia in rats. J Cereb Blood Flow Metab **17:** 1137–1142, 1997.

11. Lam TT: The effect of 3-aminobenzamide, an inhibitor of poly-ADP-ribose polymerase, on ischemia/reperfusion damage in rat retina. Res Commun Mol Pathol Pharmacol **95:** 241–252, 1997.

12. Chatterjee PK, Cuzzocrea S, and Thiemermann C: Inhibitors of poly (ADP-ribose) synthetase protect rat proximal tubular cells against oxidant stress. Kidney Int **56:** 973–984, 1999.

13. Bozlu M, Eskandari G, Cayan S, *et al:* The effect of poly (adenosine diphosphate-ribose) polymerase inhibitors on biochemical changes in testicular ischemia-reperfusion injury. J Urol **169:** 1870–1873, 2003.

14. Johnsen SG: Testicular biopsy score count—a method for registration of spermatogenesis in human testis: normal values and result in 352 hypogonadal males. Hormones **1:** 2–25, 1970.

15. Cosentino MJ, Nishida M, Rabinowitz R, *et al:* Histopathology of prepubertal rat testes subjected to various duration of spermatic cord torsion. J Androl **7:** 23–31, 1986.

16. Turner TT, and Brown KJ: Spermatic cord torsion: loss of spermatogenesis despite return of blood flow. Biol Reprod **49:** 401–407, 1993.

17. Becker EJ Jr, and Turner TT: Endocrine and exocrine effects of testicular torsion in the prepubertal and adult rat. J Androl **16:** 342–351, 1995.

18. Berger NA: Poly (ADP-ribose) in the cellular response to DNA damage. Radiat Res **101:** 4–15, 1985.

19. Prillaman HM, and Turner TT: Rescue of testicular function after acute experimental torsion. J Urol **157:** 340–345, 1997.

20. Greenstein A, Schreiber L, and Matzkin H: The effect of methylene blue on histological damage after spermatic torsion in a rat model. BJU Int **88:** 90–92, 2001.

21. Turner TT: On unilateral testicular and epididymal torsion: no effect on the contralateral testis. J Urol **138:** 1285–1290, 1987.