0022-5347/03/1695-1870/0
THE JOURNAL OF UROLOGY®

Vol. 169, 1870–1873, May 2003
Printed in U.S.A.
DOI: 10.1097/01.ju.0000049228.37887.4d

THE EFFECT OF POLY (ADENOSINE DIPHOSPHATE-RIBOSE) POLYMERASE INHIBITORS ON BIOCHEMICAL CHANGES IN TESTICULAR ISCHEMIA-REPERFUSION INJURY

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ABSTRACT

Purpose: Poly (adenosine diphosphate [ADP]-ribose) polymerase inhibitors have been used successfully to decrease ischemia-reperfusion injury in several organ systems. We evaluated the efficacy of poly (ADP-ribose) polymerase inhibitors on biochemical changes in testicular ischemia-reperfusion injury.

Materials and Methods: Adult male Wistar rats were divided into 9 groups of 6 each. One group served to determine baseline values of biochemical parameters, 1 that underwent sham operation served as a control, 1 underwent 2 hours of testicular torsion and 4 hours of detorsion, 2 received pretreatment with vehicle (saline or dimethyl sulfoxide) before detorsion and 4 received pretreatment with the poly (ADP-ribose) polymerase inhibitor nicotinamide, 3-aminobenzamide, 1,5-dihydroxyisoquinoline or 4-amino-1,8-naphthalimide before detorsion. Lipid peroxidation products, nitric oxide content and myeloperoxidase activity, an indicator of neutrophil accumulation, were assessed in testicular and renal tissues.

Results: Testicular torsion-detorsion caused a significant increase in lipid peroxidation products, nitric oxide content and myeloperoxidase activity in ipsilateral testes ($p < 0.01$) but not in the contralateral testes or kidneys. Animals treated with poly (ADP-ribose) polymerase inhibitors had a significant decrease in these biochemical parameters compared with vehicle treated animals ($p < 0.01$).

Conclusions: These data emphasize that poly (ADP-ribose) polymerase may have a role in testicular damage caused by ischemia-reperfusion and the inhibition of poly (ADP-ribose) polymerase may be a novel approach to therapy for ischemia-reperfusion injury of the testis.

KEY WORDS: testis; reperfusion injury; poly(ADP-ribose) polymerases; spermatic cord torsion; rats, Wistar

Testicular torsion is a urological emergency that causes testicular injury and subfertility.[1] It appears that the main pathophysiology of testicular torsion is ischemia-reperfusion injury to the testis caused by the twisted spermatic cord and its release.[2] Although reperfusion is essential for the survival of ischemic tissue, there is good evidence that reperfusion causes additional cell injury, which has been attributed to neutrophil infiltration and generation of reactive oxygen species, such as the superoxide anion, hydrogen peroxide and the hydroxyl radical.[3] These generation of reactive oxygen species can damage various cellular components, for example by peroxidation of cell membrane lipids, protein denaturation and DNA damage.

It has been demonstrated that reactive oxygen species produce strand breaks in DNA, which triggers energy consuming DNA repair mechanisms and activates the nuclear enzyme poly (adenosine diphosphate [ADP]-ribose) polymerase, also called poly (ADP-ribose) synthetase.[4] The activation of poly (ADP-ribose) polymerase results in the depletion of intracellular nicotinamide adenine dinucleotide, which can only be replenished via a reaction that consumes adenosine 5′-triphosphate (ATP). Ischemia-reperfusion injury resulting in substantial DNA degradation requires that cells must consume large amounts of ATP to support poly (ADP-ribosylation). Moderate poly (ADP-ribose) polymerase activity protects cellular genome integrity, although excessive activation can lead to cellular dysfunction and eventual cell death secondary to ATP depletion.[4]

In recent years an increased proportion of basic science research has been directed toward evaluating mechanisms and treatments involving cell injury and the poly (ADP-ribose) polymerase pathway is involved in the pathogenesis of various forms of ischemia-reperfusion injury.[4,5] Administering poly (ADP-ribose) polymerase inhibitors led to a decrease in ischemia-reperfusion injury to the heart and skeletal muscle in rabbits,[6] protection against oxidative stress to the kidney,[7] a decrease in brain infarct volume in a model of focal cerebral ischemia[8] and an amelioration of ischemia-reperfusion damage to the retina in rats.[9] To our knowledge the role of poly (ADP-ribose) polymerase inhibitors in testicular ischemia-reperfusion injury is undefined. In the current study we examined whether there is a protective effect of poly (ADP-ribose) polymerase inhibitors on biochemical changes associated with testicular ischemia-reperfusion injury in the anesthetized rat.

MATERIALS AND METHODS

Study groups consisted of 54 adult male Wistar rats weighing 240 to 280 gm. All animal experiments followed a protocol approved by the ethics committee on animal research at our institution.

Chemicals. We used the poly (ADP-ribose) polymerase inhibitors nicotinamide, benzamide analogues such as 3-aminobenzamide, recently discovered and more potent isoquinoline derivatives such as 1,5-dihydroxyisoquinoline and

Accepted for publication November 1, 2002.
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4-amino-1,8-naphthalimide (Alexis Co., Lausen, Switzerland). Except for nicotinamide, which was dissolved in saline, all poly (ADP-ribose) polymerase inhibitors were dissolved in 10% dimethyl sulfoxide (DMSO). The administration modes and concentrations of the 4 poly (ADP-ribose) polymerase inhibitors used in this study correspond to those in previous experimental studies.[4, 6–9]

Animal preparation and surgical procedure. The rats were divided into 9 groups of 6 each. Surgery was done with the subject under intraperitoneal 1 shot ketamine (50 mg./kg.) anesthesia. All surgical procedures were performed through a standard ilioinguinal incision. Torsion was created by rotating the left testis 720 degrees clockwise and maintained by fixing the testis to the scrotum with a 4-zero silk suture through the tunica albuginea. During sham operations the testis was rotated 720 degrees clockwise and then immediately relieved with a 4-zero silk suture was placed through the tunica albuginea. After each surgical intervention the incision was closed. At the end of the experiments bilateral orchiectomy and right nephrectomy were performed.

Groups. In group 1 (baseline values) the described organs were harvested after anesthesia. In group 2 (torsion-detorsion) the organs were harvested after torsion for 2 hours and detorsion for 4. In group 3 (saline vehicle before detorsion) the same surgical procedure was done as in the detorsion group but saline was injected intraperitoneally for 30 minutes before detorsion and an additional dose injected into the left femoral vein immediately before detorsion. In group 4 (DMSO vehicle before detorsion) the same surgical procedure was done as in the detorsion group but DMSO was injected intraperitoneally for 30 minutes before detorsion and an additional dose was injected into the left femoral vein immediately before detorsion. In group 5 (nicotinamide before detorsion) the same surgical procedure was done as in the detorsion group but nicotinamide (10 mg./kg.) was injected intraperitoneally for 30 minutes before detorsion and an additional dose (5 mg./kg.) was injected into the left femoral vein immediately before detorsion. In group 6 (3-aminobenzamide before detorsion) the same surgical procedure was done as in the detorsion group but 3-aminobenzamide (10 mg./kg.) was injected intraperitoneally for 30 minutes before detorsion and an additional dose (5 mg./kg.) was injected into the left femoral vein immediately before detorsion. In group 7 (1,5-dihydroxyisoquinoline before detorsion) the same surgical procedure was done as in the detorsion group but 1,5-dihydroxyisoquinoline (1 mg./kg.) was injected intraperitoneally for 30 minutes before detorsion and an additional dose (0.5 mg./kg.) was injected into the left femoral vein immediately before detorsion. In group 8 (4-amino-1,8-naphthalimide before detorsion) the same surgical procedure was done as in the detorsion group but 4-amino-1,8-naphthalimide (1 mg./kg.) was injected intraperitoneally for 30 minutes before detorsion and an additional dose (0.5 mg./kg.) was injected into the left femoral vein immediately before detorsion. In group 9 (sham operation group) the organs were harvested after sham operation, placed in glass bottles with rubber caps, labeled and stored in deep freeze. Lipid peroxides, nitric oxide (NO) and neutrophil content of the tissues were determined.

Determination of lipid peroxides. Tissues were homogenized in 150 mM. ice-cold potassium chloride to make a 10% homogenate using a glass polytetrafluoroethylene homogenizer. The homogenate centrifuged at 4000 $\times$ gravity for 10 minutes at 4C and maintained cold until measurement. Lipid peroxides in tissues were determined by the method of Ohkawa et al.[10] Subsequently 0.2 ml. 8.1% sodium dodecyl sulfate, 1.5 ml. 20% acetic acid and 1.5 ml. 0.8% thiobarbituric acid solutions were added to 0.1 ml. 10% tissue homogenate pipetted into a tube. The mixture was heated in a 95C water bath for 30 minutes. After cooling color was extracted into 5 ml. n-butanol-pyridine at a ratio of 15:1 and absorbance was measured at 532 nm. using a Cary 50 Bio UV Visible Spectrophotometer (Pty 1 + 2, Varian, Australia). The amount of lipid peroxides was calculated as thiobarbituric acid reactive products of lipid peroxidation and reported as nmol. malondialdehyde per 100 mg. wet tissue.

Determination of NO. Tissues were homogenized in 50 mM. ice-cold potassium phosphate buffer, pH 7.4, to make a 10% homogenate using a glass polytetrafluoroethylene homogenizer. The homogenate was centrifuged at 4000 $\times$ gravity for 10 minutes at 4C and maintained cold until measurement. Since tissue nitrite (NO_2^-) and nitrate (NO_3^-) levels can be used to estimate NO production, we measured the concentrations of these stable NO oxidative metabolites. Quantification of NO_2^- and NO_3^- was based on the Griess reaction, in which a chromophore with strong absorbance at 540 nm. is formed by the reaction of NO_2^- with a mixture of N-(1-naphthyl)-ethylenediamine and sulfanilamide, that is the No. 2,746,081 nitrite/nitrate colorimetric method (Roche Diagnostics GmBH, Mannheim, Germany). Standard curves were established with a set of serial dilutions of sodium nitrite and potassium nitrate (5 to 0.05 mg. nitrite or nitrate per l.). Linear regression was done using peak areas from the NO_2^- and NO_3^- standard curves. The resulting equation was then used to calculate the unknown sample concentration. Results are expressed as nmol. NO_2^- or NO_3^-/100 mg. wet tissue.

Determination of neutrophil content. Neutrophil content of the tissues was determined by myeloperoxidase assay. Tissues were homogenized in 2 ml. ice-cold 20 mM. potassium phosphate buffer, pH 7.4, and centrifuged at 17,000 $\times$ gravity at 4C for 30 minutes. The pellets were resuspended in ice-cold potassium phosphate buffer, pH 7.4. Suspensions were then centrifuged twice more and 0.5% (weight per volume) hexacyltrimethylammonium bromide, 10 mM. ethylenediaminetetraacetic acid in 50 mM. potassium phosphate buffer, pH 6.0, was added to remaining pellet. Suspensions were re-homogenized, incubated at 4C for 20 minutes and centrifuged at 17,000 $\times$ gravity for 15 minutes at 4C. The protein concentration in the resulting supernatant was determined using the Cobas Integra 700 (Roche Diagnostics) pyrogallol-red molybdate complex colorimetric method. Sample supernatant was incubated with 0.2 mg./ml. o-dianisidine and 158 μM. H_2O_2 in 50 mM. potassium phosphate buffer, pH 6.0, at a ratio of 4:1. Changes in absorbance were detected at 450 nm. using a Reader 230 S (Organon Technica, Anthos Labtec Instruments, Austria) microtiter photometric plate reader. Myeloperoxidase activity is expressed in absorbance per minute per gm. protein.

Statistical analysis. All data are expressed as the mean $\pm$ SD. ANOVA 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

Tables 1 to 3 show intratesticular and intrarenal malondialdehyde, NO and myeloperoxidase values. The values of these 3 parameters in the testes and kidneys of sham operated animals did not differ significantly from baseline ($p > 0.05$, tables 1 to 3). Compared with baseline malondialdehyde, NO and myeloperoxidase in the torsion-detorsion group were significantly higher in the ipsilateral testes ($p < 0.01$) but not significantly different in the contralateral testes or kidneys ($p > 0.05$, tables 1 to 3). Ipsilateral testicular malondialdehyde, NO and myeloperoxidase values in the poly (ADP-ribose) polymerase inhibitor pretreatment groups were significantly lower than in the torsion-detorsion group ($p < 0.01$) (table 1). These values in the ipsilateral testes of the vehicle treated (saline or DMSO) groups were not statistically significantly different than in the torsion-detorsion

TABLE 1. *Malondialdehyde, NO and myeloperoxidase in the ipsilateral testes*

Groups	Mean Malondialdehyde ± SD (nmol./100 mg. wet tissue)	Mean NO ± SD (nmol./ 100 mg. wet tissue)	Mean Myeloperoxidase ± SD (absorbance/min./gm. protein)
Baseline	14.37 ± 6.80	19.53 ± 6.58	17.11 ± 6.39
Torsion-detorsion*	47.46 ± 8.29	36.06 ± 4.80	43.18 ± 7.16
Saline*	45.49 ± 9.38	36.82 ± 4.45	42.36 ± 6.82
DMSO*	44.93 ± 7.49	35.80 ± 4.22	40.09 ± 6.97
Nicotinamide†	26.14 ± 5.19	26.41 ± 5.93	28.44 ± 4.87
3-Aminobenzamide†	27.40 ± 5.80	25.72 ± 5.70	26.04 ± 4.82
1,5-Dihydroxyisoquinoline†	25.53 ± 6.04	25.22 ± 5.86	29.61 ± 5.08
4-Amino-1,8-naphthalimide†	26.36 ± 5.17	24.62 ± 6.05	29.09 ± 5.02
Sham operation	14.96 ± 6.85	18.53 ± 6.78	16.18 ± 4.92

* Versus baseline $p < 0.01$.
† Versus torsion-detorsion $p < 0.01$.

TABLE 2. *Malondialdehyde, NO and myeloperoxidase in the contralateral testes*

Groups	Mean Malondialdehyde ± SD (nmol./100 mg. wet tissue)	Mean NO ± SD (nmol./ 100 mg. wet tissue)	Mean Myeloperoxidase ± SD (absorbance/min./gm. protein)
Baseline	14.19 ± 7.71	19.25 ± 5.46	17.54 ± 4.65
Torsion-detorsion	16.20 ± 7.63	18.95 ± 6.22	18.23 ± 6.58
Saline	16.32 ± 8.97	18.46 ± 4.66	17.64 ± 6.63
DMSO	15.22 ± 6.19	19.05 ± 5.85	17.70 ± 6.27
Nicotinamide	15.33 ± 8.09	19.66 ± 6.14	17.92 ± 3.64
3-Aminobenzamide	14.35 ± 8.64	17.98 ± 5.25	17.46 ± 3.76
1,5-Dihydroxyisoquinoline	14.09 ± 8.91	19.45 ± 4.82	17.03 ± 3.69
4-Amino-1,8-naphthalimide	15.67 ± 7.95	19.36 ± 5.47	16.51 ± 4.13
Sham operation	15.65 ± 6.82	18.92 ± 7.12	16.84 ± 5.15

No statistically significant differences among the groups ($p > 0.05$).

TABLE 3. *Malondialdehyde, NO and myeloperoxidase in the kidneys*

Groups	Mean Malondialdehyde ± SD (nmol./100 mg. wet tissue)	Mean NO ± SD (nmol./ 100 mg. wet tissue)	Mean Myeloperoxidase ± SD (absorbance/min./gm. protein)
Baseline	19.48 ± 5.36	47.15 ± 6.69	97.68 ± 11.85
Torsion-detorsion	17.23 ± 5.56	45.95 ± 6.27	101.81 ± 12.65
Saline	22.19 ± 4.92	50.64 ± 8.13	98.82 ± 9.32
DMSO	20.96 ± 8.77	40.64 ± 4.17	98.78 ± 8.37
Nicotinamide	17.91 ± 4.98	44.54 ± 6.34	95.85 ± 11.74
3-Aminobenzamide	17.44 ± 5.43	52.67 ± 3.11	102.17 ± 10.11
1,5-Dihydroxyisoquinoline	21.23 ± 5.43	46.75 ± 7.12	100.66 ± 9.55
4-Amino-1,8-naphthalimide	18.68 ± 3.73	51.64 ± 8.63	94.74 ± 9.78
Sham operation	19.88 ± 4.76	48.15 ± 7.09	98.12 ± 9.35

No statistically significant differences among the groups ($p > 0.05$).

group ($p > 0.05$, table 1). The values of these 3 parameters in the contralateral testes and kidneys were not significantly different among these groups ($p > 0.05$, tables 2 and 3).

DISCUSSION

Testicular torsion is one of the few emergencies that requires immediate intervention to untwist the affected testis. In animal models Akgur et al noted clear reperfusion injury to the testis after untwisting the spermatic cord.[2] Testicular injury after torsion had an ischemic and a reperfusion component. Although reperfusion is essential for the survival of ischemic tissue, there is good evidence that reperfusion causes pathophysiological cascades, including ATP depletion, intracellular calcium accumulation, phospholipase activation, membrane lipid alterations, cytoskeletal dysfunction, neutrophil infiltration and reactive oxygen species generation.[3] The activation of poly (ADP-ribose) polymerase is currently described as a final common effector in various types of tissue injury, including ischemia-reperfusion.[4] Recently poly (ADP-ribose) polymerase inhibitors were successfully used to decrease ischemia-reperfusion injury in multiple organ systems, including the heart, skeletal muscle,[6] kidney,[7] brain[8] and retina.[9] The successful results of poly (ADP-ribose) polymerase inhibitors in different organ systems led us to use this treatment in model of testicular torsion. However, to our knowledge the effect of pharmacological poly (ADP-ribose) polymerase inhibition on testicular ischemia-reperfusion injury has not been reported previously.

In the current study we examined 3 biochemical changes associated with reperfusion injury, namely lipid peroxidation products, NO concentration and neutrophil content in testicular tissue. Lipid peroxidation products of the testis have previously been shown to increase after torsion repair.[2] In this report 2 hours of ischemia followed by 4 hours of reperfusion caused a significant increase in malondialdehyde levels in the testis. Akgür[11] and Lysiak[12] et al reported that allopurinol, and catalase and superoxide dismutase plus catalase treatments caused a decrease in lipid peroxidation products and these treatment modalities protected the testis against reperfusion injury. The effect of poly (ADP-ribose) polymerase inhibitors on ischemia-reperfusion induced lipid peroxidation was investigated by Halmosi et al, who reported that the inhibitors prevented ischemia-reperfusion induced lipid peroxidation in cases of Langendorff perfused heart.[13] Under our experimental conditions administering poly (ADP-ribose) polymerase inhibitors before reperfusion caused a significant decrease in the level of testicular malondialdehyde compared with that in the torsion-detorsion group. Our data indicate that treatment with poly (ADP-ribose) polymerase inhibitors prevents a further increase in lipid peroxidation caused by ischemia-reperfusion. Thus, these compounds may protect the testis against reactive oxygen species related oxidative damage.

In addition to reactive oxygen species, including hydrogen peroxide, hydroxyl radicals and superoxide anions, the formation of NO is enhanced during reperfusion of the ischemic testis.[14] Nitric oxide, a gaseous molecule with diverse biological functions, is synthesized from L-arginine by a family of

isoenzymes termed NO synthases (NOSs).[3] Results in previous studies suggest that NO has an important role in damaging the testis via ischemia-reperfusion.[14,15] Similarly the current study showed that reperfusion for 4 hours after 2 hours of ischemia elevated NO production in a model of testicular torsion in the rat. It has been investigated in several organs that after ischemia superoxide is produced during the reperfusion phase, which rapidly reacts with NO and forms peroxynitrite.[3] Peroxynitrite initiates toxic oxidative reactions, directly inhibits mitochondrial respiratory enzymes, decreases cellular oxygen consumption and inhibits membrane sodium transport. Oxidative injury in response to oxygen radicals and peroxynitrite is related to DNA single strand breakage and the consequent activation of poly (ADP-ribose) polymerase.[4] Therefore, poly (ADP-ribose) polymerase activation leads to cell death through energy depletion. Inhibiting poly (ADP-ribose) polymerase protects against cellular oxidant injury triggered by peroxynitrite, a cytotoxic oxidant produced by the reaction of superoxide and NO.[4,5]

On the other hand, our study also demonstrated that treatment with poly (ADP-ribose) polymerase inhibitors before reperfusion significantly decreased the concentration of testicular NO. Poly (ADP-ribose) polymerase inhibition decreases NO production by inhibiting inducible NOS mRNA expression.[5] There may be a specific region of the inducible NOS promoter that is regulated by such inhibition. Suppressing inducible NOS expression may be an additional mechanism by which the inhibition of poly (ADP-ribose) polymerase suppresses the inflammatory response.[4] In light of these data one may anticipate that administering poly (ADP-ribose) polymerase inhibitors before reperfusion would protect the testis against NO related injury in ischemia-reperfusion.

Turner et al documented leukocyte margination and diapedesis 4 hours after torsion repair.[16] As previously stated, reperfusing leukocytes are potent generators of reactive oxygen species and the recruitment of neutrophils to the testis after torsion is essential for the observed pathological condition. Extravasated neutrophils become activated after they are at the inflammatory sites, secreting various substances such as growth factors, chemokines, cytokines, complement components, proteases, NO, reactive oxygen metabolites and peroxynitrite, which are important mediators of tissue injury.[3] We also found that testicular myeloperoxidase values, an indicator of neutrophil accumulation, determined 4 hours after torsion repair were significantly increased in the torsion-detorsion group compared with myeloperoxidase in the baseline group. Recent investigations indicate that poly (ADP-ribose) polymerase activation has an important role in the process of neutrophil recruitment and in various forms of inflammation.[4,5] Inhibiting poly (ADP-ribose) polymerase suppresses the inflammatory response and attenuates neutrophil recruitment. Based on the data in this study we propose that decreased myeloperoxidase activity in testicular ischemia-reperfusion represents an important additional protective effect provided by poly (ADP-ribose) polymerase inhibitors.

The effect of unilateral torsion on the contralateral testis is controversial. It has been demonstrated that ipsilateral torsion does not result in contralateral testicular damage in rats.[17] Similarly in our study, while 2 hours of torsion followed by 4 hours of detorsion resulted in significant changes in biochemical analysis of the ipsilateral testes, our results did not reveal any change in the contralateral testes.

CONCLUSIONS

Although several enzymes and drugs have been used for decreasing ischemia-reperfusion injury to date,[11,12,18,19] there has been relatively little research into the role of poly (ADP-ribose) polymerase within the testis.[20] To our knowledge our results provide the first evidence of the role of poly (ADP-ribose) polymerase in the pathogenesis of testicular ischemia-reperfusion injury. We propose that inhibiting poly (ADP-ribose) polymerase may be a novel approach to therapy for ischemia-reperfusion injury of the testis. However, further studies are required to elucidate the mechanism of ischemia-reperfusion injury of the testis that involves poly (ADP-ribose) polymerase activation.

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