Rho-kinase Levels in Testicular Ischemia-reperfusion Injury and Effects of Its Inhibitor, Y-27632, on Oxidative Stress, Spermatogenesis, and Apoptosis

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OBJECTIVE To investigate testicular Rho-kinase levels and the effects of its inhibitor, Y-27632, on oxidative stress, spermatogenesis, and apoptosis in testicular ischemia-reperfusion rat model.

METHODS The study included 29 adult Wistar-Albino male rats weighing 150-200 g. The rats were divided into 3 groups. Group 1 underwent sham operation (n = 10). In group 2, left testicular torsion-detorsion was performed (n = 9). In group 3, Rho-kinase inhibitor Y-27632 (5 mg/kg) was injected intraperitoneally 30 minutes before detorsion (n = 10). Two months later, bilateral orchiectomy was performed in all the groups. Rho-kinase levels by Western blotting, apoptosis with terminal deoxynucleotidyl transferase dUTP nick end labeling method, testicular damage and spermatogenesis with modified Johnsen score, testicular total antioxidative status, and total oxidative status were measured.

RESULTS In the torsion-detorsion (T/D) group, Rho-kinase level increased significantly, compared with the sham group ($P = .025$). In the Y-27632 treatment group, Johnsen scores were significantly higher, and apoptosis indexes were significantly lower, compared with the T/D group ($P = .001$). Significantly higher total antioxidative status levels and lower total oxidative status levels were observed in the Y-27632 treatment group, compared with the T/D group ($P = .001$ and $P = .002$, respectively).

CONCLUSION Testicular ischemia-reperfusion significantly increased Rho-kinase levels in rats, and administration of Rho-kinase inhibitor, Y-27632, before detorsion might prevent ischemia-reperfusion injury. UROLOGY 83: 675.e13–675.e18, 2014. © 2014 Elsevier Inc.

Testicular torsion is a common urologic emergency among newborns, children, and adolescents. 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] Although reperfusion is essential for the survival of ischemic tissue, there is good evidence that reperfusion itself causes the pathophysiological cascades, including an activation of neutrophils, inflammatory cytokines, and adhesion molecules with a massive intracellular Ca^{2+} release, the generation of oxygen-derived free radicals, and increased thrombogenicity.[2] Reactive oxygen species (ROS) could cause deoxyribonucleic acid (DNA) damage, endothelial injury, and germinal cell necrosis. These free radicals react with lipids in the cell and mitochondrial membranes, changing their permeability and disrupting cell integrity.[3] Under normal conditions, free radicals are produced and their effects are counterbalanced by the endogenous antioxidant system. When ROS generation exceeds the defense mechanisms capacity to control, oxidative stress is generated and contributes to reversible or irreversible cell injury. In particular, sperm are highly sensitive to oxidative stress and lipid peroxidation because of their high content of polyunsaturated fatty acids in the plasma membrane. The fatty acids are an essential requirement for the male germ cell to maintain sperm functions.[4]

Ischemia-reperfusion injury in tissues induced extravascular recruitment of leukocytes is a multistep process comprising leukocyte rolling, adhesion, and transmigration.[5] Although numerous cellular pathways contribute to the inflammation in reperfusion injury, underlying mechanisms have yet to be elucidated, and the number of pathways involved has been rising continuously. Accordingly, it has been reported that Rho-kinase (ROCK) is expressed

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in polymorphonuclear leukocytes and that the ROCK inhibitor, Y-27632, suppresses the motile functions of these cells.[6] In addition, it inhibits aggregation of activated polymorphonuclear leukocytes and the production of O_2^-anions.[7] Furthermore, Rho/Rho-kinase signaling might play a significant role in renal,[8] hepatic,[9] and cerebral ischemia-reperfusion injuries.[10] ROCK activation has been described to be involved in the pathogenesis of septic organ injury and vascular inflammation. However, there is no study to investigate any roles of this pathway in testicular ischemia-reperfusion injury. In urogenital tissues, the expression of ROCK protein and its substantial role in smooth muscle cell contraction have been demonstrated, including penis, ureter, vas deferens, and urinary bladder.[11-14]

The aims of this study were to investigate testicular Rho-kinase (ROCK-2) level and the effect of Rho-kinase inhibitor, Y-27632, on oxidative stress, spermatogenesis, and apoptosis in testicular ischemia-reperfusion rat model.

MATERIALS AND METHODS

Surgical Procedure

Twenty-nine adult male Wistar-Albino rats weighing 150-200 g were obtained from the Experimental Medicine Unit at the University of Mersin School of Medicine. They were caged separately under a 12-hour light/dark photoperiod and a constant temperature (23°C ± 1°C) and received standard mice chow ad libitum. This study was approved by the institutional review board at the University of Mersin School of Medicine and was performed in accordance with the Guide for the Care and Use of Laboratory Animals of Experimental Medicine Unit.

The rats were randomly separated into the following 3 groups:

Group 1 (n = 10): sham operated rats.

Group 2 (n = 9): left testicular torsion and detorsion was performed.

Group 3 (n =10): Y-27632 (5 mg/kg, intraperitoneally) was administered 30 minutes before detorsion.

Under ketamine anesthesia (50 mg/kg intraperitoneally), all surgical procedures were performed through standard ilioinguinal incision.[15] In the torsion-detorsion (T/D) group (group 2), 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. Two hours after the 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 administered Y-27632 (TOCRIS Cookson, Avonmouth, UK) before detorsion (group 3), the same surgical procedure was done as in the T/D group, but Y-27632 (5 mg/kg) was administered intraperitoneally 30 minutes before detorsion and closure. In the sham-operated group (group 1), the same procedure was done as in the T/D group, except rotating the testis 720° clockwise, it was immediately relieved, and a 4-0 silk suture was placed through the tunica albuginea. After 60 days, bilateral orchiectomy was performed, and the rats were killed by administering overdose pentobarbital (200 mg/kg) and bilateral thoracotomy. Thereafter, testis tissues were stored at −80°C until the experimental procedures.

Histologic Evaluation

Testis tissues for histologic preparations were fixed in Bouin's solution (7.5 mL saturated picric acid, 2.65 mL glacial acetic acid) and embedded in paraffin blocks. Five-micrometers sections were obtained and deparaffinized. The bilateral testicles were removed and placed in 10% formaldehyde solution and processed to paraffin wax. Standard sections were prepared for light microscopic examination and *terminal deoxynucleotidyl transferase dUTP nick end labeling* staining method.

The seminiferous tubules were graded according to the modified Johnsen score.[16]

Score 1: no seminiferous epithelium

Score 2: no germinal cells, Sertoli cells only

Score 3: spermatogonia only

Score 4: no spermatozoa or spermatids, few spermatocytes

Score 5: no spermatozoa or spermatids, many spermatocytes

Score 6: no spermatozoa, no late spermatids, and few early spermatids

Score 7: no spermatozoa, no late spermatids, and many early spermatids

Score 8: <5 spermatozoa per tubule, few late spermatids

Score 9: slightly impaired spermatogenesis, many late spermatids, and disorganized epithelium

Score 10: complete spermatogenesis with many spermatozoa presence.

Apoptosis was evaluated using the terminal deoxynucleotidyl transferase (TdT)-FragEl DNA fragmentation kit (Oncogene, Cambridge, MA). This in situ apoptosis kit allows recognition of apoptotic nuclei in paraffin-embedded tissue sections by fragment end labeling of DNA. In this assay, terminal deoxynucleotidyl transferase targets DNA exactly at the 30-OH ends generated in response to apoptotic signals and catalyzes the addition of biotin-labeled and unlabeled deoxynucleotides. Biotinylated nucleotides are detected using streptavidin—horseradish peroxidase conjugate. Diaminobenzidine reacts with the labeled sample to generate an insoluble brown-colored complex at the site of DNA fragmentation. Counter staining with methyl green aids in the morphologic evaluation and characterization of normal and apoptotic cells.

An apoptotic index was calculated.[17] All tubules on the sections of the slides were evaluated for apoptosis. The groups of the experiments were blinded to the pathologist who counted the cells (D.D.A.). The number of apoptotic cells was divided into the number of tubules. Therefore, the number of apoptotic cells as a percentage of the total number of cells was counted. Results are evaluated as 5 graded scales:

Grade 0: no cells stained

Grade 1: 1%-5% of the cells were stained

Grade 2: 5%-10% of the cells were stained

Grade 3: 10%-20% of the cells were stained

Grade 4: >20% of the cells were stained.

Western Blot Analysis for ROCK-2

Small pieces of the rat testis tissues were homogenized with a lysis buffer (composition in mM; Tris-HCl (pH = 7.4) 50 mM,

NaCl 400 mM, EGTA 2 mM, EDTA 1 mM, dithiothreitol 1 mM, phenylmethylsulfonyl fluoride 10 μM, leupeptin 10 μg/mL, pepstatin 1 μg/mL, benzamidine 1 mM). The homogenate was centrifuged at 13,000 ×*g* for 10 minutes at 4°C, and the supernatant was removed. It was then used for protein analysis (with Bradford method) and Western blot analysis.[12,13] Equal amounts of the protein (30 μg) were loaded in wells, electrophoresed on 8% polyacrylamide-sodium dodecyl sulfate gels and then transferred to a polyvinylidene fluoride membrane overnight. The membrane was blocked with the blocking agent of the enhanced chemiluminescence (ECL Advance) kit (Amersham Biosciences, Freiburg, Germany) in Tris buffer solution containing 0.05% Tween-20 for 1 hour. It was then probed with a primary antibody raised against ROCK-2 (ROKα, monoclonal IgG, BD Science, 1:2000 dilution) or β-actin (Santa Cruz Biotechnology, CA, 1:500 dilution) followed by a horseradish peroxidase-conjugated secondary antibody (donkey antimouse, 1:2000, Santa Cruz Biotechnology Inc., CA). Protein blots were then detected with the advanced chemiluminescence detection kit (Amersham Biosciences, Freiburg, Germany) and visualized on commercial x-ray film.[11,14]

Biochemical Evaluation

Tissues were then prepared for the metabolic assays as described previously. Protein was detected by Lowry method.[18] Total antioxidant status (TAS) and total oxidant status (TOS) were measured using the methods described in the following sections.

Total Antioxidant Status. Antioxidants in the sample reduce dark blue-green colored ABTS radical to colorless reduced ABTS form. The change of absorbance at 660 nm is related with total antioxidant level of the sample. The assay is calibrated with a stable antioxidant standard solution, which is traditionally named as Trolox Equivalent that is a vitamin E analog.[19] Results are expressed in terms of μmol Trolox Eq/mg protein.

Total Oxidant Status. The TOS of the plasma was measured using a novel automated colorimetric measurement method.[20] In this method, oxidants present in the sample oxidize the ferrous ion–chelator complex to ferric ion. The oxidation reaction is prolonged by enhancer molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with chromogen in an acidic medium. The color intensity, which can be measured spectrophotometrically at 530 nm, is related to the total amount of oxidant molecules present in the sample.[20] The assay is calibrated with hydrogen peroxide, and the results are expressed in terms of nanomolar hydrogen peroxide equivalent per liter (nmol H_2O_2 Eq/L).

Statistical Analysis

Biochemical parameters and histopathologic scores were represented as mean ± standard deviation. TAS and TOS parameters of the right and the left testis tissues in all the groups were compared with paired sample *t* test. Wilcoxon test was also used for the other parameters. Differences between the groups for the parameters of TAS and TOS have been tested with one-way analysis of variance, and binary comparisons of groups were tested with the Tukey HSD. Kruskal-Wallis test was used to test differences between groups in other parameters (apoptosis index and modified Johnsen scores). Statistical significance, $P < .05$ was used. Analyses were made using SPSS 11.5 and MedCalc v11.0.1 statistical software programs.

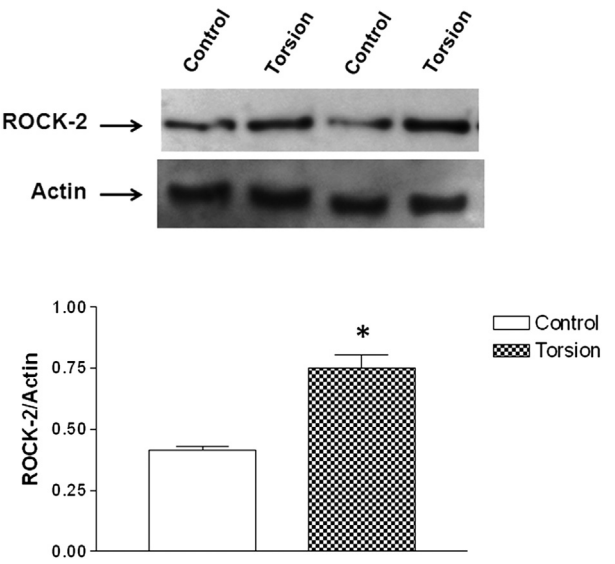

Figure 1. Demonstration of Rho-kinase (ROCK-2) level using Western blotting in the torsion and sham groups. The expression level of Rho-kinase (ROCK-2) increased significantly in the torsion-detorsion group, compared with the sham group ($P = .025$). The level of ROCK-2 was approximately 2-fold higher in ischemic group than in the control. * $P = .025$

For Western-blot analysis, all data represent mean ± standard errors of the mean of *n* observations. For statistical comparison, unpaired *t* test was used. A *P* value <.05 was considered significant. Graphs were drawn using a GraphPad Prism 3.0 program (GraphPad software, San Diego, CA).

RESULTS

Western blot analysis revealed that the expression level of Rho-kinase (ROCK-2) increased significantly in the T/D group, compared with the sham group ($P = .025$). As shown in Figure 1, the level of ROCK-2 was approximately 2-fold higher in ischemic group than in the control.

The mean values of modified Johnsen scores are shown in Figure 2. The mean modified Johnsen score was 9 ± 1.02 in the sham group, 3 ± 0.98 in the T/D group, and 6.5 ± 0.63 in the Y-27632 treatment group. The mean apoptosis index was 0 in the sham group, 2.3 ± 1.2 in the T/D group, and 0.7 ± 0.6 in the Y-27632 treatment group. Modified Johnsen scores increased ($P = .001$), and apoptosis indexes decreased significantly ($P = .001$) in the Rho-kinase inhibitor Y-27632 treatment group, compared with the T/D group. Figure 3 shows a microphotography of apoptotic cells in a rat testis of the T/D group.

As shown in Figure 4, the mean TAS values (μmol Trolox Eq/mg protein) were 18.02 ± 2.33, 6.06 ± 1.79, and 9.48 ± 2.79, respectively. The mean TOS values (nmol H_2O_2 Eq/L) were 6.85 ± 1.70, 7.63 ± 1.46, and 4.93 ± 1.33, respectively. The mean TAS values were higher ($P = .001$), and mean TOS values were lower

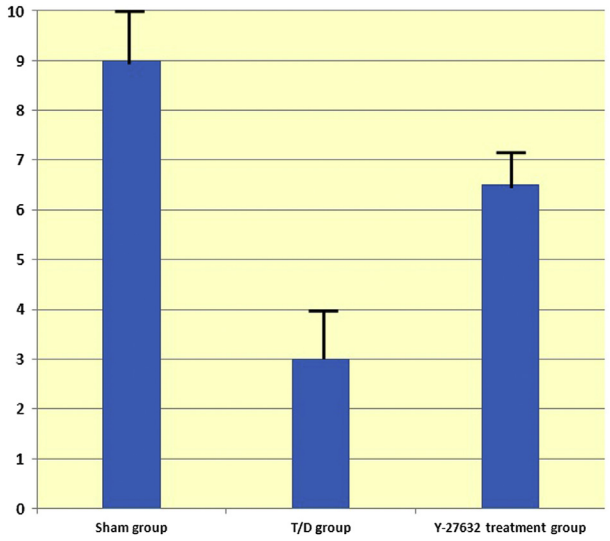

Figure 2. Modified Johnsen scores in all the groups. Modified Johnsen scores significantly increased in the Rho-kinase inhibitor Y-27632 treatment group, compared with the torsion-detorsion (T/D) group ($P = .001$).

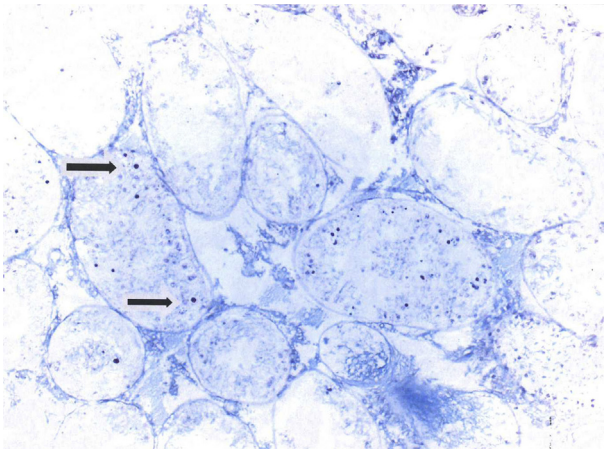

Figure 3. Apoptotic cells in a rat testis of the torsion-detorsion group. Arrows show apoptotic cells.

($P = .002$) in the Rho-kinase inhibitor Y-27632 treatment group, compared with the T/D group.

The histopathologic and biochemical parameters of the right testicles did not reveal any statistically significant differences among the groups ($P > .05$).

COMMENT

Testicular torsion is a surgical emergency. Late presentation or failure to diagnose and incorrectly manage this condition leads to testicular injury and subfertility. Although many attempts have been made to gain insight into the nature of ischemia-reperfusion injuries, definition of the underlying mechanisms is still unknown. Pathophysiological mechanisms in testicular ischemia-reperfusion damage are most likely multifactorial, involving hypoxia, inflammatory responses, and oxidative stress, which are characterized by an imbalance between ROS and the antioxidative defense system. Similarly, in our previous study, the testicular torsion caused a significant increase in testicular lipid peroxidation, nitric oxide (NO) production, and neutrophil accumulation with permanent loss of spermatogenesis.[21] In addition, the results of the present study have confirmed the involvement of ROS in testicular ischemia-reperfusion injury. However, further mechanisms in terms of cellular signaling are yet to be known. Such signaling has been recently reported to be involved in ischemia-reperfusion injuries, namely, Rho/Rho-kinase pathway.[10] Moreover, Rho/Rho-kinase signaling might play a significant role in renal, hepatic, and colonic ischemia-reperfusion injuries.[8,9,22] Furthermore, ROCK activation has been described to be involved in the pathogenesis of septic organ injury and vascular inflammation. However, there is no study to investigate any roles of this pathway in testicular ischemia-reperfusion injury. Therefore, we explored possible involvement of this signal pathway in testicular ischemia-reperfusion injury in rats.

Normally, it does not preferentially explore any effects of an enzyme inhibitor on the expression of the enzyme. However, the level of expression could reflect activation in that the more enzyme the more reaction in the case of enough substrate available. Instead, it is more sensible to explore effects of an enzyme inhibitor on the activation of the relevant enzyme. Because we did not investigate ROCK activation but merely protein expression level, we did not check any effects of Y-27632 on the expression of the enzyme it has already inhibited with. However, the functional effects of Y-27632 are much more important on testicular apoptosis and spermatogenesis that were already investigated in our study.

The present study demonstrates that unilateral testicular ischemia-reperfusion caused testicular damage in testes, as evidenced by biochemical and histologic changes. The pathophysiological mechanism in testicular damage owing to testicular torsion is an ischemic process for the testis. The ROS can oxidize cell membrane lipids, proteins, and DNA, which leads to cellular dysfunction and, sometimes, cell death. This cascade of events is known as reperfusion injury.[23,24] In addition, more neutrophils accumulated in the testis after testicular torsion-detorsion and generated excess ROS; this caused spermatogenic injury in the ipsilateral testis. The elimination of ROS has been shown to be beneficial in treating ischemia-reperfusion injury.[7] In this study, the reduction of TAS values in the torsion/detorsion group might be because of the consumption of antioxidants generated by enzymatic and nonenzymatic responses to damage. The administration of Y-27632 prevents antioxidant consumption compared with T/D group. The increment of TOS values in the T/D group might be because of increased oxidants after tissue damage. However, Y-27632 had protective effect against oxidant because it increased TAS and decreased TOS values in testicular tissues. During torsion, the testis showed significant cyanosis and edema. After detorsion, these findings were partially resolved. However, biochemical and

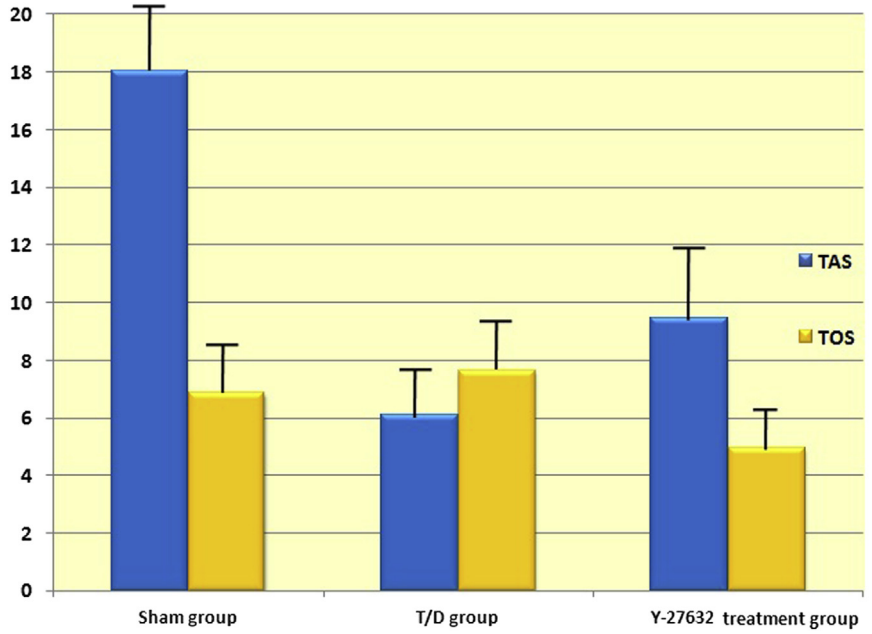

Figure 4. Total antioxidative status (TAS) and total oxidant status (TOS) values in all the groups. The mean total antioxidative status values were higher ($P = .001$), and mean total oxidant status values were lower ($P = .002$) in the Rho-kinase inhibitor Y-27632 treatment group, compared with the torsion-detorsion (T/D) group.

histologic parameters of the contralateral testis did not reveal any statistical differences.

Activated polymorphonuclear leukocytes play a major role in ischemia-reperfusion injury. Y-27632 inhibited the infiltration of polymorphonuclear leukocytes and inhibits the O_2^- production after reperfusion and consequently protected against hepatic injury.[9] Recent studies demonstrated that Y-27632 has antiapoptotic effect by regulation of Bcl-2 in myocardium. Neutrophils cause reperfusion injury by obstruction of capillary vessels, production of vasoactive substances, and release of cytotoxic agents. Proinflammatory cytokines promote further inflammatory cell adhesion and infiltration into myocardium and influence acute tissue injury. Treatment with Y-27632 resulted in a significant reduction in the accumulation of neutrophils in ischemic myocardium. The improvement of postischemic cardiac function by Y-27632 might also be mediated by inhibition cytokines release.[25] Rho-kinase blockade decreased the production of TNF-α, a well-known activator of endothelial cells in the reperfused colon.[22] In another study, renal myeloperoxidase activity in untreated acute renal failure rats increased significantly 2-6 hours after the reperfusion. This increase was suppressed by Y-27632 treatment, suggesting that the infiltration/migration of neutrophils in the postischemic kidney was attenuated by the Rho-kinase inhibition.[8] Taken together, the inhibition of Rho-kinase seems to be a promising target for the prevention and/or treatment of ischemic testis damage.

CONCLUSION

In conclusion, the ischemia-reperfusion injury in the rat testis impaired spermatogenesis. In addition, ischemia-reperfusion injury reveals apoptosis, oxidative stress, and upregulation of ROCK-2. Furthermore, the administration of Rho-kinase inhibitor, Y-27632, before detorsion, might prevent the histopathologic and biochemical deterioration induced by ischemia-reperfusion.

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