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Raxofelast, a hydrophilic vitamin e-like antioxidant, reduces testicular ischemia-reperfusion injury

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Abstract Testis torsion is a surgical emergency that lead to permanent gonad damage. The damage has been ascribed to mechanisms of ischemia-reperfusion similar to other tissues. The mechanisms involved are different, but the lipid peroxidation of plasma membrane, caused by reactive oxygen species (ROS), generated particularly during reperfusion, is one of the most accredited. In the present study, we aimed to evaluate the effects of raxofelast, a vitamin E-like antioxidant with potent action and no systemic toxicity, on lipid peroxidation and histopathology in both testes after unilateral testicular torsion and detorsion. Adult male Wistar rats were subjected to total occlusion (3 h) of the left testis followed by 4 hours of reperfusion (TI/R). Sham testicular ischemia-reperfusion rats (SHAM TI/R) were used as controls. The animals were then randomized to receive either vehicle (1 ml/kg/i.p. of a dimetylsulphoxide/NaCl 0.9% 1:10 v/v solution, injected either 15 min before detorsion and 15 min after detorsion) or raxofelast (20 mg/kg i.p. 15 min before detorsion and 15 min after detorsion). Conjugated dienes (CD) levels, an index of lipid peroxidation, and testis histopathology were evaluated. Testicular ischemia reperfusion (TI/R) in untreated rats produced high testicular levels of CD (3.6 ± 0.3 ΔABS/g protein on the left side and 2.5 ± 0.2 ΔABS/g protein on the right side). Furthermore, histological examination revealed marked damage to the testis interstitium with severe haemorrhage and edema. The administration of raxofelast lowered CD levels (2.8 ± 0.2 ΔABS/g protein on the left side and 1.9 ± 0.1 ΔABS/g protein in the right side) and significantly reduced histological damage. These data suggest that the hydrophilic vitamin E-like antioxidants are good candidates for designing a novel therapeutic strategy to halt the oxidative stress that follows acute testis torsion.

Keywords Ischemia-reperfusion injury · Vitamin E · Antioxidants · Conjugated dienes

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Introduction

Unilateral testicular torsion is a common surgical emergency that can damage, with irreversible changes, the affected testis, as well as the unaffected contralateral testis [1].

Pathophysiological mechanisms that regulate such alteration have been ascribed to the direct damage caused by ischaemia during torsion, and to a secondary effect due to reperfusion during the untwisting of the spermatic cord [2]. In fact, if the restoration of testicular flow to the ischemic gonad is essential for tissue salvage, organ reperfusion may have detrimental effects.

The mechanism of tissue damage due to ischemia-reperfusion is common to other organs such as the brain, myocardium and kidneys. Neutrophil infiltration and the generation of reactive oxygen species (ROS) can cause tissue damage through cell membrane lipid peroxidation, protein denaturation and DNA damage [3].

Recently, the serum malondialdehyde (MDA) concentration in patients with testis torsion has been identified as a reliable marker of lipid peroxidation and tissue damage. For the above reasons different therapeutic strategies [4, 5] have been investigated with the aim of reducing short- and long-term testis reperfusion damage.

Endogenous vitamin E plays an important role in antioxidant defence mechanisms [6]. Unfortunately, the clinical use of vitamin E as a protective agent against ischemia-reperfusion injury has not been proved valuable. This has been attributed to the lipophilic nature of the compound that does not allow it to penetrate into the damaged tissue to play its antioxidant role. New synthetic analogues of vitamin E have been shown to have powerful antioxidant properties and a hydrophilic character. Particularly raxofelast (IRFI-016; 2,3-dihydro-5-hydroxy-4,6,7-trimethyl-2-benzofuranacetic acid) is a potent antioxidant with no systemic toxicity that reduces free radicals and interrupts lipid peroxidation of the plasma membrane. Different studies have demonstrated the efficacy of this hydrophilic vitamin E analogue in reducing the inflammatory cascade and protecting during hemorrhagic shock, septic conditions or ischemia-reperfusion of the myocardium [7, 8, 9].

The aim of the present study was to evaluate, in an experimental model of testis ischemia/reperfusion, the effects of raxofelast on lipid peroxidation and histopathology in both testes after unilateral testicular torsion and detorsion.

Materials and methods

Animals

All procedures complied with the standards for care and use of animals as stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, Md.). A total of 40 male Sprague-Dawley rats (250–300 g), fed on a standard diet, with tap water ad libitum, and with a 12 h light-dark cycle, were used. The animals were divided in four groups of ten animals each. The temperature of the animals was maintained at approximately 37°C using an overhead lamp.

Experimental protocol

Testis ischemia-reperfusion injury (TI/R) was induced by torsion of the left testis, with a 720° twisting of the spermatic cord so as to produce a total occlusion of the testis for 3 h. The same testis was then detorted. Following 4 h of reperfusion, the rats were killed with an overdose of sodium pentobarbital and bilateral orchidectomies were performed. Sham operated rats (SHAM TI/R) were subjected to the same surgical procedures as the TI/R rats except for the testis occlusion. The animals were randomized to receive either vehicle (1 ml/kg/i.p. of a dimetylsulphoxide/NaCl 0.9% 1:10 v/v solution, administered either 15 min before detorsion and 15 min after detorsion), or raxofelast (20 mg/kg i.p. 15 min before detorsion and 15 min after detorsion)

Conjugated dienes evaluation

Estimation of the content of conjugated dienes (CD) in the tissue was carried out to evaluate the extent of lipid peroxidation in damaged tissue. Samples of testis were frozen at −80°C until the assay. Next, they were collected in polyethylene tubes and the washed with 1 ml butylated hydroxytoluene (BHT) (1 mg/ml in phosphate buffer). After drying on absorbent paper, they were frozen at 4°C until the analysis. The biochemical assay of CD required previous lipid extraction from the tissue samples by chloroform/methanol (2:1). The lipid layer was dried under a nitrogen atmosphere and then dissolved in cyclohexane. CD contents of the testis were measured at 232 nm using a spectrophotometric technique. The amount of wound CD was expressed as ΔABS/mg.

Histology

Testes collected after reperfusion were divided longitudinally into two parts which were individually fixed in formaldehyde 7% in 0.1 mol/l phosphate buffer (pH 7.4) for 12 h, after which they were dehydrated in alcohol and embedded in paraffin. Serial sections of 4-μm thickness were cut, deparaffinized and stained with hematoxylin-eosin.

As part of the histological evaluation, all slides were examined by a pathologist without knowledge of the previous treatment, using masked slides under the microscope from 20 to 100× magnification.

Interstitial injury was graded on a scale from 0 to 3; 0 normal interstitium; 1 interstitial edema; 2 interstitial hemorrhage and 3 hemorrhagic infarct [10].

Statistical analysis

All data are expressed as the mean plus or minus the standard error of the mean (mean ± SEM). Between ipsilateral and contralateral sides, between groups, data were analyzed by ANOVA for a multiple comparison of the results. Duncan's multiple range test was used to compare the group means. In all cases, a probability error of less than 0.05 was selected as the criterion for statistical significance.

Drugs

Raxofelast, a lipid peroxidation inhibitor, was supplied by Biomedica Foscama Research Centre, Ferentino, Italy. The compound was administered intraperitoneally in dimetylsulphoxide/NaCl 0.9% (1:10 v/v) solution. All substances were prepared fresh daily and administered in a volume of 1 ml/kg.

Results

Testis tissue CD levels

The determination of testicular CD levels was performed to estimate free radical damage in the tissue. Very low CD levels were measured in testis obtained from SHAM TI/R animals treated either with vehicle (0.22 ± 0.03 ΔABS/g protein on the left side and $0.18 \pm 0.0.1$ ΔABS/g protein on the right side) or raxofelast (0.15 ± 0.012 ΔABS/g protein on the left side and 0.2 ± 0.01 ΔABS/g protein on the right side). There were no differences between the groups or between the ipsilateral and contralateral sides within the same group (Fig. 1). By contrast testicular ischemia-reperfusion injury (TI/R) caused a marked CD increase in both the left and right testis (3.6 ± 0.3 ΔABS/g protein on the left side and 2.5 ± 0.2 ΔABS/g protein on the right side). These values were significantly higher than in the SHAM TI/R groups ($P < 0.05$), suggesting the presence of a strong lipid peroxidation process. The administration of raxofelast significantly reduced ($P < 0.05$) the CD concentrations in treated rats in both the left and right testes (2. 8 ± 0.2 ΔABS/g protein on the left side and 1.9 ± 0.1 ΔABS/g protein on the right side). The CD level of the occluded left testis was significantly higher than that of the contralateral side in the treated group ($P < 0.05$).

Histology

Figure 2A and B show the normal histology of the left testis. Seminiferous tubules, germ cells, Sertoli and Leydig cells appear complete, without infiltrations and hemorrhagic signs. By contrast, light microscopy showed that the left testis torsion caused interstitial space dilatation and the presence of edema and hemorrhage (Table 1 and Fig. 2C). Raxofelast administration markedly reduced left testis lesions and alterations (Table 1 and Fig. 2D).

Discussion

Different pathogenic mechanisms have been proposed to explain the tissue damage that occurs during acute testicular torsion. In particular, recent reports have focused attention on the oxidative status that is generated during the reperfusion period [11, 12]. In fact, although reperfusion is essential to interrupt the progression of the cellular injury associated with decreased oxygen and nutrient delivery, it can also generate negative events such as leukocyte infiltration, subsequent production of inflammatory mediators and free-radical production that can be responsible for lipid peroxidation. This is associated with detrimental consequences to the reversibly injured cells and is related with the development of testis damage. Testicular torsion also seems to have a negative effect on the contralateral side as well. Different hypotheses have been put forward to try to explain this phenomenon. The most recent and accredited one proposes a reflex mechanism that can be responsible for reduced contralateral testicular blood flow, thus being responsible for tissue hypoxia [13].

Table 1 Histology of both left and right testes collected from SHAM TI/R animals or animals after ischemia-reperfusion (TI/R) treated either with raxofelast (20 mg/kg) administered intraperitoneally (15 min before detorsion and 15 min after detorsion), or its vehicle (1 ml/kg/i.p. of a dimetylsulphoxide/NaCl 0.9% 1:10 v/v solution). Interstitial injury was graded on a scale from 0–3: 0 normal interstitium; 1 interstitial edema; 2 interstitial hemorrhage, and 3 hemorrhagic infarct [10]. These data represent the mean ± SEM of ten testes

Groups	Histological grade	
	Left testis	Right testis
SHAM TI/R + vehicle	0	0
SHAM TI/R + raxofelast	0	0
TI/R + vehicle	2.1 ± 0.632	0.6 ± 0.210
TI/R + raxofelast	0.7 ± 0.206	0.1 ± 0.054

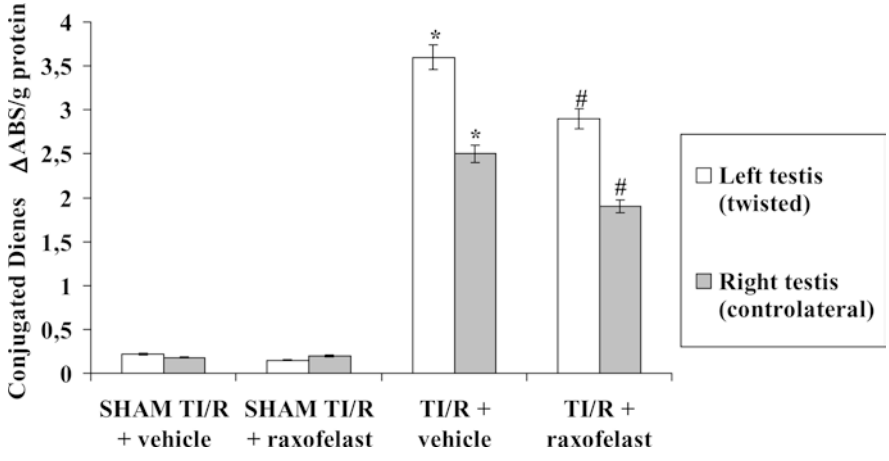

Fig. 1 Testes content of conjugated diene (CD) levels in SHAM TI/R operated animals or animals after ischemia-reperfusion (TI/R). Animals were randomly assigned to receive either raxofelast (20 mg/kg) in DMSO administered intraperitoneally (two doses given 15 min before detorsion and 15 min after detorsion) or its vehicle (1 ml/kg/i.p. of a dimetylsulphoxide/NaCl 0.9% 1:10 v/v solution) at the same time points. *Bars* represent the mean ± SD of ten animals. * $P < 0.05$ vs SHAM TI/R. # $P < 0.05$ vs TI/R + vehicle

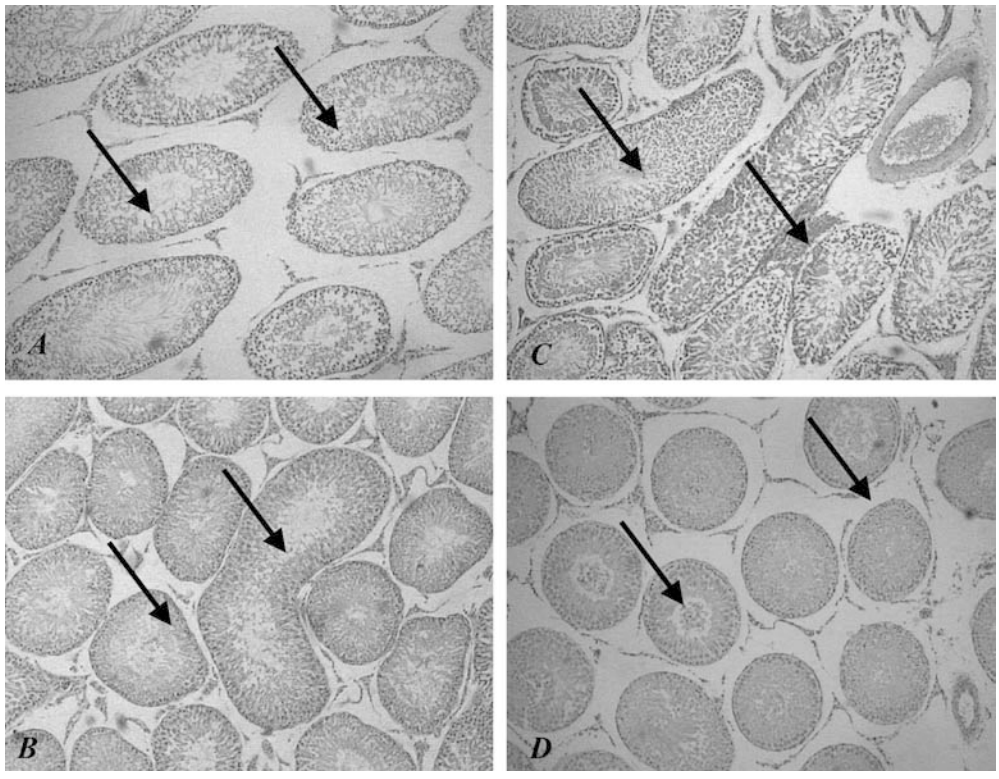

Fig. 2 A Histology of a representative testis collected from a SHAM TI/R rat. *Arrows* indicate normal architecture. Original magnification 100×. **B** Histology of a representative testis collected from a SHAM TI/R rat + raxofelast. *Arrows* indicate normal architecture. Seminiferous tubules, germ cells, Sertoli and Leydig cells appear complete, without infiltrations and hemorrhagic signs. Original magnification 100×. **C** Histology of a representative testis collected from a rat which had undergone testicular ischemia-reperfusion (TI/R). *Arrows* indicate the presence of interstitial edema and haemorrhage. Original magnification 100×. **D** Histology of a representative testis collected from a rat which had undergone testicular ischemia-reperfusion treated with raxofelast (20 mg/kg i.p.). *Arrows* indicate a reduction in both interstitial edema and haemorrhage. Original magnification 100×

Oxidative stress during post-ischemic reperfusion therefore plays a pivotal role in activating interconnected inflammatory cascades that have already been recognized to be involved in the development of reperfusion induced damage [14]. Antioxidants would theoretically have a dual effect in testis ischemia-reperfusion injury: they would limit the development of damage by decreasing free radicals generated by lipid peroxidation and counteracting ROS mediated activation of inflammatory reaction.

Under normal conditions, free radicals are produced and their effects are counterbalanced by the endogenous antioxidant system. When ROS generation exceeds the defence mechanism's capacity to control, oxidative stress is generated and contributes to reversible or irreversible cell injury. Recent reports indicate that superoxide dismutase, catalase and non-peptidyl superoxide dismutase mimic, enzymes that metabolize ROS, can partially prevent testis ischemia-reperfusion injury [12, 15].

The anti-oxidant vitamin E plays an important role in the endogenous defence mechanism, since its deficiency is responsible for increased tissue injury caused by oxidative stress [14]. Unfortunately, due to its marked lipophilicity, the incorporation of vitamin E into tissue is very slow, causing very low levels of this potent antioxidant in the ischemic/reperfused tissue. This peculiar chemical property has been responsible for the impracticability of the acute clinical use of vitamin E. As a consequence, vitamin E analogues with a less lipophilic character have been synthesised.

Raxofelast is an analogue of vitamin E with a hydrophilic character and powerful antioxidant properties. The drug is converted in vivo to the deacetylated active metabolite IRFI 005. In vivo, experimental and clinical studies have shown that plasma concentrations of raxofelast are very low after administration, whereas high levels of the active metabolite, IRFI 005, have been recorded [16].

IRFI 005 is a potent scavenger of superoxide anions, and in rat liver mitochondria and microsomes decreases the extent of lipid peroxidation [17]. This group of vitamin E analogues shows no systemic toxicity, even following high doses (up to 1 g/kg) [18]. In the present study, we demonstrated that raxofelast administered 15 min before detorsion and 15 min after detorsion effectively reduced oxidative stress and the morphological changes that follow ischemia-reperfusion of the testis. The source for free radical overproduction depends mainly on the reperfusion time. For this reason, we chose to administer raxofelast twice 15 min before detorsion and 15 min after detorsion so as to obtain, according to its pharmacokinetics, a constant concentration in the tissue during reperfusion. This experimental condition could also mimic the clinical situation in which pharmacological protection is possible only after the ischemic event but before reperfusion has been achieved by surgical intervention. CD levels were significantly increased in both testes after left testis torsion, confirming the effect of ischemia-reperfusion on the injured side as well as the contralateral side. Raxofelast was able to significantly reduce CD levels on both testes.

The histological changes observed under this experimental condition represent the microscopic features of an acute event caused by torsion and detorsion. Lesions appeared to be localized mainly on the extratubular compartment and restricted to the affected side without major changes on the contralateral side. The pharmacological protection significantly attenuated the interstitial edema and haemorrhage.

Raxofelast not only blunted, bilaterally, biochemical parameter alterations caused by oxidative stress but also reduced the histopathological changes. Therefore, our results demonstrate that the new antioxidant raxofelast strongly attenuates acute testis damage after torsion. This may provide a potential therapeutic approach to post ischemic testicular damage. Further studies will clarify the role of this antioxidant in preventing long-term sequelae responsible for subfertility.

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