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Antioxidative gene therapy using superoxide dismutase in ischemia-reperfusion injury of testes in rats

Research Article

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Abbreviations: Ischemia-reperfusion injury, (IRI); Luria-Bertani, (LB); malondialdehyde, (MDA); reactive oxygen species, (ROS); superoxide dismutase, (SOD); Thiobarbituric acid reacting substances, (TBARS)

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Summary

Ischemia-reperfusion injury (IRI) is associated with increased production of reactive oxygen species and thus with oxidative stress. Antioxidative pre-treatment prevents the free radical induced tissue impairment in experiment, as has been shown previously. However, clinical use requires long-term treatment with high doses of antioxidants. Gene therapy using safe vector constructs provides a useful tool for gene transfer *in vivo*. To evaluate the effects of superoxide dismutase (SOD) gene therapy on oxidative stress induced tissue impairment in an experimental model of IRI of testes. Male Wistar rats (n=18) were pre-treated either by single intratesticular injection of 500 μg of plasmid pcDNA3 containing the Mn-SOD cDNA or by adequate volume of saline two days prior to the IRI. Ischemia (30 minutes) was induced by torsion of a random testis in each rat. After 30 minutes of reperfusion (detorsion), both testes were removed. Malondialdehyde (MDA) as major product of lipoperoxidation were measured in testes homogenates. Samples were also analysed by electron microscopy. Although not reaching the level of statistical significance our results show that IRI increased the oxidative stress induced tissue impairment and SOD gene pre-treatment could partly compensate and reduce the MDA level. This decrease is even superior without IRI. This study does not provide any evidence, but indicates the possibilities of antioxidative gene therapy in IRI of testes. Further larger studies should prove the efficiency of this approach on other organs using wider palette of markers of tissue impairment.

I. Introduction

Damage of various tissues caused by ischemia-reperfusion injury is related to formation of many different oxygen and oxygen-derived free radicals called reactive oxygen species (ROS). These toxic molecules are natural side-products of many metabolic pathways. Oxidative stress occurs if the level of newly generated ROS is higher than the antioxidative status of the cells. It is one of the main factors contributing to reperfusion injury of the ischemic tissues such as myocardium (Chen et al, 1998; Li et al, 1998; Zhu et al, 2000) or testis (Koksal et al, 1999, 2003; Visser and Heyns, 2003). Reasons of oxidative stress are relatively well studied and contain the formation of free radicals after increased oxygenation during reperfusion of ischemic tissue that has adapted to an

environment of low oxygen concentration during hypoxia/ischemia (Bertuglia and Giusti, 2003).

Free radicals and ROS involved in damaging processes of oxidative stress are mostly superoxide anion (O2-), hydroxyl radical (OH) and singlet oxygen ($^1O_2$). Inability to degrade these ROS is the primary cause of irreversible ischemia-reperfusion injury (Zhu et al, 2000). To scavenge free radicals, except low molecular weight scavengers like ascorbic acid, vitamin E etc. (Llesuy et al, 1995), which are mostly of exogenous origin, there are also endogenous enzymatic antioxidant systems including superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, which are acting on different level in various tissues (Chen et al, 1998) and endogenous non-enzymatic antioxidants like gluthatione and urate (Miller et al, 1993).

Superoxide dismutase (SOD) seems to be the best described and mostly studied antioxidant enzyme, that catalyses the dismutation of superoxide anion (mainly as a product of intracellular respiration) to oxygen and hydrogen peroxide, which is later degraded by catalase to form water and molecular oxygen. Superoxide anions can take part in a reaction that generates much more toxic hydroxyl radicals, what emphasizes the important role of SOD in ROS reduction. There are three biological forms of SOD bearing an important antioxidant activity: 1. mitochondrial tetrameric manganese-containing Mn-SOD, which is mainly present in mitochondria, but is synthesized in cytosol and coded by a nuclear gene; 2. cytosolic dimeric copper/zinc-containing Cu/Zn-SOD; 3. extracellular SOD - tetrameric glycoprotein containing Cu/Zn which occurs mostly in the extracellular and interstitial space. A major part of total intracellular amount of free radicals is generated in mitochondria, therefore we used cDNA coding mitochondrial Mn-SOD in our study.

Testicular disorders such as varicocele or torsion of testis are considered to be related with sperm dysfunction and male infertility (Koksal et al, 1999; Visser and Heyns, 2003). ROS-mediated oxidative stress is one of crucial reasons of infertility and decreased sperm viability (Koksal et al, 2003). Increased level of free radicals may cause degeneration of testicular tissue. Torsion of testis is a common clinical status occurring mainly in young male population (Greenfield et al, 2002) and caused by ischemia after twisting the testicular cord through several revolutions. Depending on the rate of twisting and duration of the torsion, various levels of damage and atrophy can be observed in the testicular tissue. This damage is caused by oxidative stress during ischemia-reperfusion injury. Reduced antioxidative status of spermatozoa, decreased motility and ineffective spermatozoon-oocyte fusion occur as a consequence of the rise of ROS production (Koksal et al, 2003). It is evident, that SOD plays an important role in scavenging of ROS in testes, because in comparison to rat liver, the activity of catalase and glutathione peroxidase is much lower in the testicular tissue (Peltola et al, 1992).

Besides other adverse effects, ROS also induce lipoperoxidation that changes membrane permeability, it leads to protein impairment, and to enzyme inactivation and at the end to DNA damage. Plasmatic membranes of spermatozoa contain high concentrations of polyunsaturated fatty acids and therefore are highly sensitive to oxidative stress. In this study we have examined the oxidative stress in testicular tissue and measured the level of malondialdehyde (MDA) that is a product of lipoperoxidation and can be considered as direct quantitative marker of ROS induced lipid impairment.

Gene therapy presents a great potential for ischemia-reperfusion damage protection in various tissues. We used an experimental rat model of ischemia-reperfusion (torsion and detorsion of testes) to prove the biological function of intratesticulary administered Mn-SOD gene. Considering the potential safety risk of viral vectors usage, we decided to utilize naked DNA (plasmid) vector.

II. Materials and methods

A. Vector

The vector molecule for introduction the Mn-SOD gene was obtained from Dr. Larry W. Oberley and Dr. Yuping Zhang from University of Iowa (**Figure 1**).

B. Vector isolation and purification

Plasmid pcDNA3 containing the Mn-SOD gene was transformed into competent cells of host strain *E.coli* DH1 (F^-, rec A, hsd R, sup E, end A, gyr A96) using $CaCl_2$ and heat-shock method. DH1 strain is suitable for production of large amounts of plasmid. Transformed cells and cultures were selected by cultivation on Petri dishes with Luria-Bertani (LB) cultivation medium containing ampicilin (100 μg/ml LB) as a selective marker. For consecutive isolation of plasmid we used the Birnboim and Doly (1979) protocol. The purification of DNA was done using phenol-chlorophorm extraction. The purity and concentration of extracted plasmid DNA was approved by photometric methods.

C. Animal experiment

Male Wistar rats (n=18) were involved in the experiment. The plasmid was introduced randomly into right or left testis by injection of 100 μl of plasmid DNA solution in sterile water. This represented 500 μg of pure DNA for each rat. Corresponding volume of saline solution was injected into the remaining testis of each rat. On the third day after plasmid administration (48 hours), torsion-T of testis (randomly right or left) was simulated by 5-fold clockwise rolling of testis.

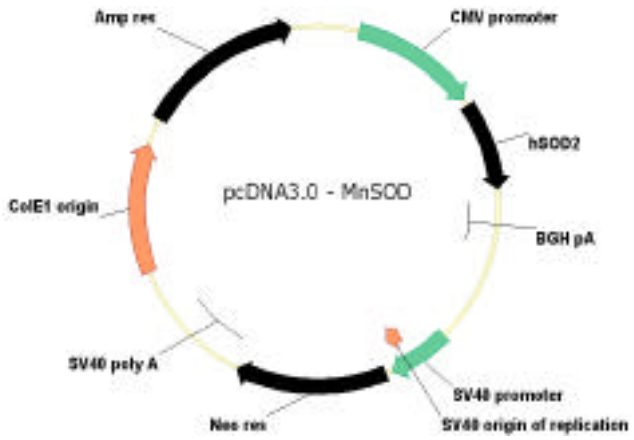

Figure 1. Map of the pcDNA3 vector containing the SOD cDNA used in this study.

After 30 minutes of ischemia, we restored the blood flow by 5-fold counterclockwise rotation (detorsion-DT, reperfusion phase). In this process, the increased oxygenation of ischemic tissue induced oxidative stress. After another 30 minutes of reperfusion, both the twisted and non-twisted testes were surgically removed, frozen until measurement of oxidative stress markers or fixed in 3% glutaraldehyde for ultrastructural examination. Acquired testicular tissues were divided into 4 groups: ctrl-testes with injected saline without T (n=9), SOD-testes with injected Mn-SOD plasmid without T (n=9), torsion-testes with injected saline with T-DT (n=9), SOD+torsion-testes with injected Mn-SOD plasmid with T-DT (n=9). One testis from the 2nd group (SOD) was atrophic and thus has not been involved in further examination.

D. MDA determination

Collected samples of testes were frozen (-20 °C) until measurement (2 days). MDA was measured in testes homogenates (10%) by spectrofluorometric method ($\lambda_{ex.}$=535nm, $\lambda_{em.}$=553nm) after derivatization with 0.6% thiobarbituric acid in acidic medium of acetic acid (100°C, 45 min.) After derivatization, the colored product was extracted to n-butanol, centrifuged (6000g, 10 min) and measured against a standard solution (1,1,3,3-tetrametoxypropan). MDA concentration in tissues was expressed on the basis of the calibration curve in $\mu mol.g^{-1}$.

E. Statistical analysis

Data was analyzed using One way ANOVA (MDA level as a parameter, groups as factor) with Bonferroni modified posthoc t-test with $\alpha = 0.05$. The results are presented as mean + standard error of the mean. The computations were done with SPSS 11.0 for Windows and Microsoft Excel 2000®.

III. Results and discussion

Many previous studies have examined the therapeutic effect after in vivo intravascular or local application of recombinant antioxidant enzymes (SOD, catalase). The disadvantage of the direct protein application is their low stability in circulation, inability to enter the cells and the induction of antibodies production after long-term administration. Gene therapy provides stable and endogenous source of therapeutic protein by introducing a gene, which codes such enzyme. Many protocols use replication of deficient adenoviruses as vectors for transferring cDNA of catalase (Zhu et al, 2000), or SOD (Li et al, 1998) into myocardium or intravenously. These studies have confirmed the protective effect of antioxidant-coding introduced genes on ischemia-reperfusion damage of rabbit hearts with highest level of antioxidant expression on second and third day after gene transfer. Another study demonstrated an important role of Cu/Zn-SOD in reducing the oxidative stress in mouse hearts by disrupting SOD I gene (coding Cu/Zn-SOD) (Yoshida et al, 2000). There was no activity of Cu/Zn-SOD in hearts of knock-out mice (SOD I $^{-/-}$) and high MDA level was detected. Increased activity of SOD was noticed in transgenic mice after ischemia and reperfusion (Chen et al, 1998).

Ischemia-reperfusion injury of various tissues is a typical experimental and clinical inducer of oxidative stress (Filho et al, 2004; Akgur et al, 1993; Gonzalez-Flecha et al, 1993). This does not affect only the myocardium, kidneys, organ transplants, but also testes. MDA as the end product of fatty acid breakdown is a widely used marker of lipoperoxidation (Celec et al, 2003). MDA belongs to the main components of thiobarbituric acid reacting substances. Although not significant, our results have shown indeed, that the ischemia-reperfusion injury in an experimental model of testicular torsion and detorsion increases concentrations of a lipoperoxidation marker like MDA (**Figure 2**). Swelling mitochondria from testicular tissue after ischemia-reperfusion injury without antioxidative treatment are shown on **Figure 3**. SOD gene therapy using naked plasmid DNA injected 48 hours before the onset of the reperfusion injury could partly compensate the increased ROS production and decrease the MDA level. The antioxidative effects of SOD are even more evident in the SOD group without torsion-detorsion (**Figures 2, 4**).

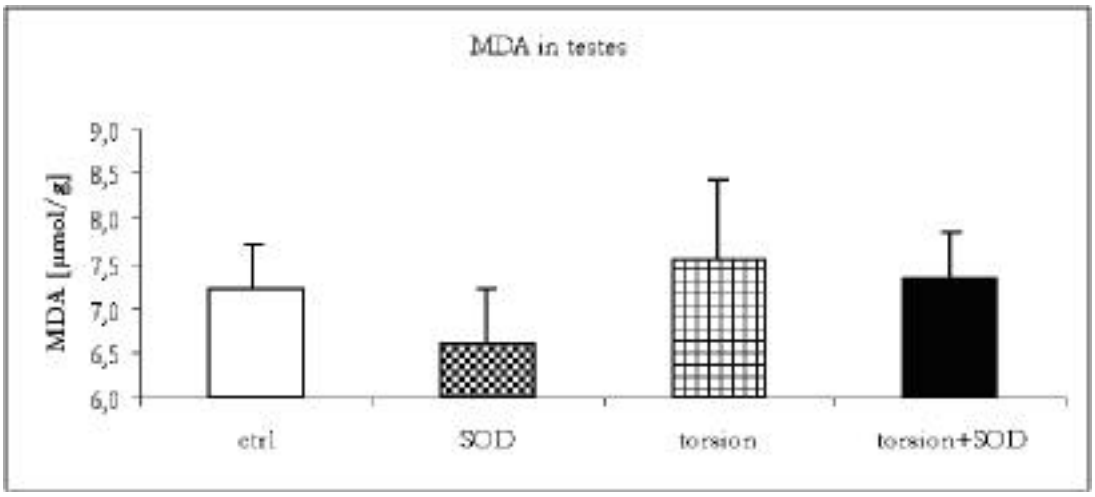

Figure 2. MDA concentrations measured in testes homogenates.

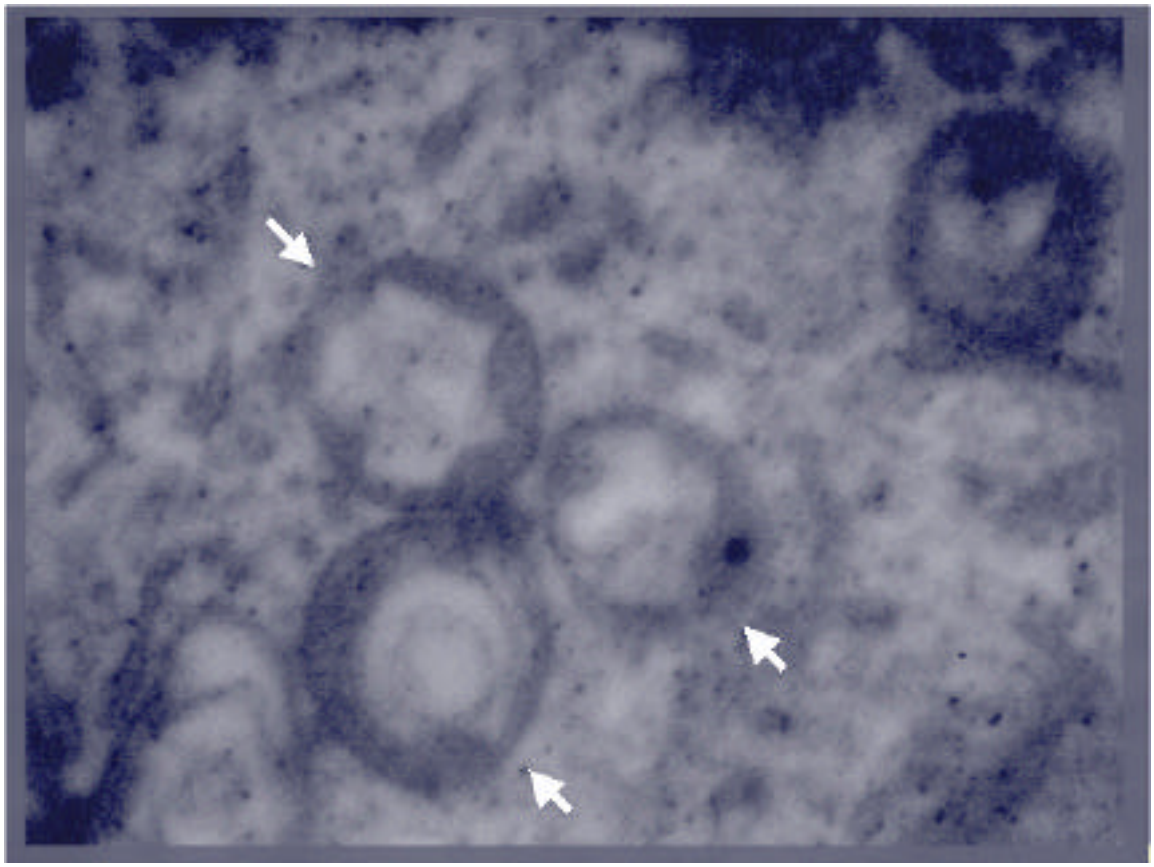

Figure 3. Arrows show swelling mitochondria from testicular tissue after ischemia-reperfusion injury without antioxidative treatment.

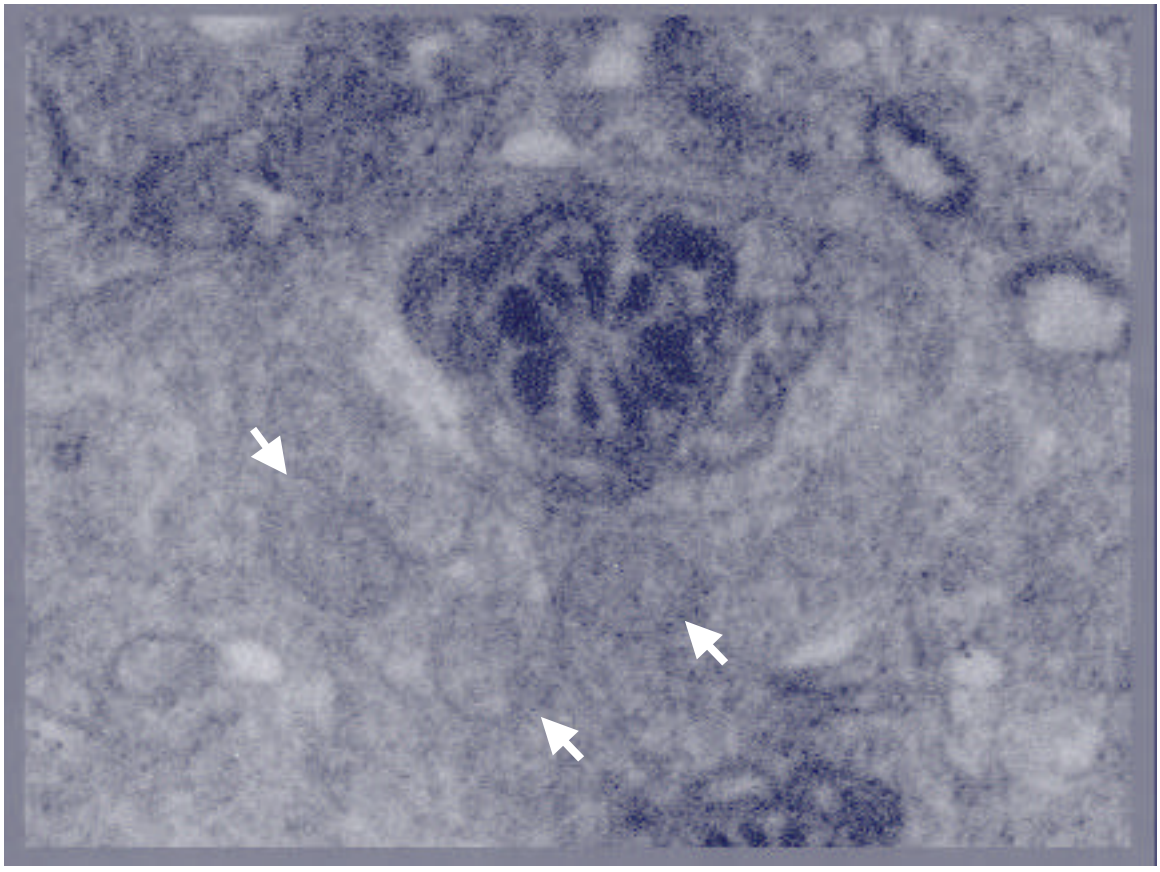

Figure 4. Arrows show mitochondria from testicular tissue after ischemia-reperfusion injury protected by antioxidative gene therapy using superoxide dismutase. The inner mitochondrial membrane is clearly visible, and no signs of swelling or other injuries are present.

The results of this study are similar to those obtained by other groups using SOD gene therapy in other organs. Nevertheless, data dealing with antioxidative gene therapy of testes are lacking. The main limitation of our study is the low number of animals and observed markers of tissue impairment. We are aware of the facts that other biochemical markers of oxidative stress like glutathione or hydroxynonenal might be measured. We have decided to concentrate on MDA because of the high susceptibility of the testicular tissue to lipoperoxidation. In addition, the relatively short reperfusion period might influence the results. Future research should concentrate on functional parameters like sperm count, viability and fertility. Further studies will also clarify the possibilities of using antioxidative gene therapy in clinical situations.

IV. Conclusion

According to our knowledge we are the first to describe the possibilities of SOD gene therapy in ischemia-reperfusion injury of testes. Our results should be proved in larger studies using other markers of tissue impairment.

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