

# Evaluation of Oxidant and Anti-Oxidant Balance in Experimentally Induced Testicular Injury by Ischemia Reperfusion in Rats

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## ABSTRACT

**Aim:** The aim of the study is to evaluate the oxidant and antioxidant balance in experimentally induced testicular injury by ischemia reperfusion in rats with different time intervals of torsion/detorsion of the spermatic cord.

**Method:** Male rats were divided into thirteen groups and each group containing six rats. All rats were subjected to right spermatic cord torsion followed by detorsion for different time intervals except the sham control group.

**Result:** Malondialdehyde, super oxide dismutase, catalase levels were estimated in testicular tissue of each rat. Spermatic cord torsion/detorsion induced a significant increase in testicular malondialdehyde contents and significant decrease in SOD, catalase levels when compared to sham control group in a time dependent manner.

**Conclusion:** Our findings clearly represents that torsion for 4 hours followed by detorsion for 4 hours induces the highest oxidative stress with a great extent of lipid peroxidation and drastic reduction of SOD and catalase levels of the testis and is, therefore, a valid model for studying the oxidative antioxidant effects of the ischemia/ reperfusion injury in rat testis.

**Key words:** Oxidative stress, testicular torsion, detorsion, reperfusion Injury, antioxidant

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## **Ratlarda İskemi-Reperfüzyon Metodu ile Deneysel Olarak Oluşturulmuş Testiküler Hasarda Oksidan ve Antioksidan Dengenin Değerlendirilmesi**

**Amaç:** Çalışmanın amacı ratlarda değişik zamanlarda torsiyon/detorsiyon yapılarak oluşturulan iskemi-reperfüzyon deneysel modelinde meydana getirilen testiküler hasarda oksidan ve antioksidan dengenin değerlendirilmesidir.

**Metod:** Erkek ratlar 13 farklı gruba ayrılmıştır ve her grupta 6 rat bulunmaktadır. Kontrol grubu hariç tüm ratların sağ spermatic kordu belirli zaman aralıklarında torsiyone/detorsiyone edilmiştir.

**Bulgular:** Her ratın testiküler dokusunda malondialdehit ve superoksit dizmutaz seviyeleri ölçülmüştür. Torsiyone/detorsiyone edilen testislerde malondialdehit artmış ve superoksit dizmutaz ve katalaz seviyeleri kontrol grubuna göre azalmıştır.

**Sonuç:** Sonuçlarımız açıkça göstermektedir ki 4 saatlik torsiyon/detorsiyon sonrası testislerde oksidatif stress ve lipid peroksidasyonu en üst düzeye çıkmış, Sod ve katalaz seviyelerinde belirgin bir azalma olmuştur. Bu nedenle bu model iskemi/reperfüzyon hasarında oksidan ve antioksidatif etkiyi göstermede geçerli bir modeldir.

**Anahtar kelimeler:** Oksidatif stress, testiküler torsiyon, detorsiyon, reperfüzyon hasarı, antioksidan

### **INTRODUCTION**

Testicular torsion is a surgical emergency and affects newborns, children and adolescent boys and caused mainly by a twist in the spermatic cord. The torsion must be treated promptly to avoid loss of function of ipsilateral and contralateral testis. The surgery of torsion followed by detorsion may reperfuse the tissue which is essential at that point of time for the survival. Reperfusion of a previously ischemic testicular tissue results in some new cellular damage that blunts the beneficial effects of reperfusion itself. Such damage is called reperfusion injury (1).

Therefore reperfusion may be considered as double edged sword as it is essential for the survival of the tissue, may actually exacerbate rather than diminish cell injury. This syndrome often leads to infertility of the ipsilateral (torted) and contralateral (non torted) testis, due to various morphological and biochemical changes by both ischemia and reperfusion of the tissues.

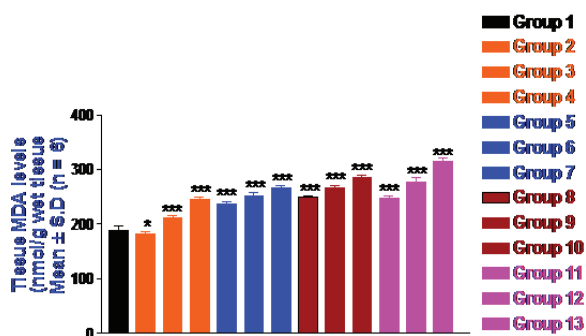
Ischemia Reperfusion injury is associated with over generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in testis just as in other organs such as brain, heart and kidneys. Testicular torsion is a serious problem in male children and, if not treated at the right time, can lead to subfertility and infertility (2).

It has been demonstrated that reperfusion of the ischemic tissue promotes the generation of ROS, which arise from activation of the xanthine oxidase system in parenchymal cells or from leukocytes penetrating into interstitial tissue (3, 4). Therefore, treatment by detorsion may further damage the testis. With the resumption of blood flow, oxygen needed for the conversion of hypoxanthine to uric acid is supplied to testis resulting in the production of

large amounts of free radicals. These free radicals react with lipids in the cell and mitochondrial membranes, changing their permeability and disrupting cell integrity (5). 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. As studies with experimental models of testis ischemia/reperfusion could bring new information for a better understanding of the local and distant repercussions of the oxidative stress induced by the torsion of the spermatic cord, many authors' evaluated changes in the testis of rats in different time schedules of torsion and detorsion. Testicular damage depends on duration of torsion and detorsion of spermatic cord. This paper is aimed to identify a better model by experimenting in different time intervals and to find levels of common parameters i.e. malondialdehyde, super oxide dismutase and catalase in the respective time intervals of ischemia and reperfusion injury. This identification can be utilized for the screening of various drugs for the treatment of testicular ischemia reperfusion injury.

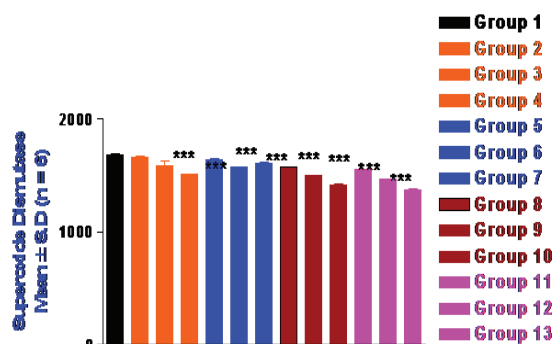
### **MATERIALS AND METHODS**

Prepubertal, male, Sprague Dawley rats weighing 180-200g were selected. Animals were maintained under standard laboratory conditions at 25±2°C relative humidity 50±15% and normal photoperiod (12 h dark/12 h light and were used for the experiment. Commercial pellet diet (Rayon's Biotechnology Pvt Ltd, India) and water were provided ad libitum. The experimental pro-



\* $p < 0.05$ , \*\*\* $p < 0.001$  vs sham control group by One way ANOVA/Tukey's test

Figure 1. Levels of MDA



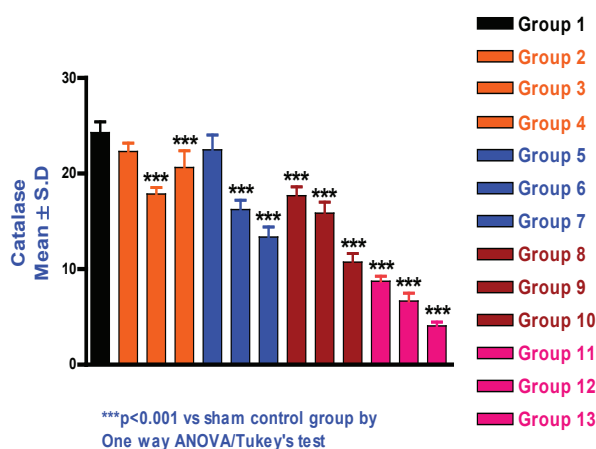
\*\*\* $p < 0.001$  vs sham control group by One way ANOVA/Tukey's test

Figure 2. Levels of SOD

protocol has been approved by the Institutional Animal Ethics Committee and by the Animal Regulatory Body of the Government (Regd. No. 516/01/A/CPCSEA). On the day of the experiment, animals were anesthetized with Thiopentone sodium (30 mg/kg, i.p). Later surgical procedure as described below was performed.

#### Chemicals

Thiopentone sodium was purchased from Neon-labs, Mumbai, India. Unless otherwise specified all the chemicals and reagents used were of analytical grade.



\*\*\* $p < 0.001$  vs sham control group by One way ANOVA/Tukey's test

Figure 3. Levels of catalase

#### Surgery

All the animals were divided into thirteen groups and each group contains six rats. All the groups underwent procedure as described below. Initially anesthesia is induced by using thiopentone sodium and a right scrotal incision was made.

The tunica vaginalis was opened, and the right testis was delivered to the surgical field. The testicle was rotated 720° in a clockwise direction and maintained in this torsion position by fixing the testicle to the scrotum with a 5-0 silk suture. At the end of testicular ischemia period, the testicle was released and restored to normal position to perform reperfusion. At the end of the experiment, the testes were removed and divided longitudinally into two halves for biochemical measurements (protein, MDA, SOD, catalase). Subsequently all the animals were sacrificed by cervical dislocation (6).

#### Experimental Design

Animals were divided into thirteen groups each containing six rats. Group 1 rats, Sham control group (animals have not undergone any surgical procedure). Groups 2, 3 and 4 underwent 1 hour of Torsion followed by 1, 2 and 4 hours of detorsion respectively. Groups 5, 6 and 7 have undergone torsion for 2 hours followed by detorsion for 1, 2 and 4 hours respectively. Groups 8, 9 and 10 have undergone torsion for 3 hours followed by detorsion for 1, 2, 4 hours respectively. Groups 11, 12 and 13 have undergone torsion for 4 hours followed by detorsion for 1, 2 and 4 hours respectively.

## Biochemical analyses

### Determination of MDA levels in testicular tissue

MDA levels in the testicular tissue were measured by the method developed by Ohkawa et al. Briefly, the testicular tissue was homogenized with 1.15% KCl (10% w/v). The assay mixture consists of 0.1 ml of tissue homogenate, 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid (adjusted to pH 3.5 with NaOH) and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid (TBA) was heated for 60 min at 95 ° C. Thereafter, the mixture was cooled and extracted with 5 ml of mixture of n-butanol and pyridine (15:1, v/v). After centrifugation at 4000 rpm for 10 min, the organic phase was assayed spectrophotometrically at 532 nm. Tetraethoxypropane (in amounts of 2, 4, 6 and 8 nmol) served as an external standard. MDA levels in testicular tissue were expressed as nmol /g of tissue (7, 8).

### Determination of superoxide dismutase (SOD) in testicular tissue

Superoxide dismutase (SOD) activity was determined by the method developed by Beauchamp, Fridovich. A testis was homogenized with a polytron homogenizer. To 0.5 ml of homogenate 1 ml of Sodium carbonate, 0.4 ml of nitroblue tetrazolium chloride, 0.2 ml of EDTA were added. The reaction was initiated by adding 0.4 ml of Hydroxyl amine hydro chloride. Zero time absorbance was taken at 560nm using spectrophotometer, followed by recording absorbance after 5 min at 2600C. The control was simultaneously run without homogenate. Units of SOD were expressed as amount of enzyme required for inhibiting the reduction of NBT by 50%. The specific activity was expressed in terms of units for mg of protein. (9)

### Determination of catalase (CAT) in testicular tissue

Catalase activity was measured by the method of Aebi. Testes were homogenized with a polytron homogenizer in ice-cold Tris-buffer to produce a 10 % w/v homogenate. The homogenate was centrifuged at 10,000 rpm at 4 ° C for 15 min. supernatant 0.1 ml was added to cuvette containing 1.9 ml of 50 mM phosphate buffer. To this mixture, 1.0 ml of freshly prepared 30 mM H<sub>2</sub>O<sub>2</sub> was added and changes in absorbance for 3 min at 240 nm at an interval of 30 sec. A control was prepared using 0.1 ml of distilled water devoid of 0.1 ml of homogenate. One unit of the enzyme activity is defined as enzyme concentration required inhibiting the change in

the absorbance by 50% in one min in the control sample. Activity of Catalase was expressed as units per mg protein.

### Estimation of protein

Protein was estimated by the method of Lowry et al(11). The level of protein was expressed as mg protein/g tissue for SOD and Catalase.

### Statistical analysis

The results are expressed as mean  $\pm$  S.D. Differences in tissue lipid peroxide levels, SOD and catalase were determined by factorial one-way analysis of variance. Individual groups were compared using Tukey's test. Differences with  $P < 0.05$  were considered statistically significant.

## RESULTS

In Sham control group, MDA, SOD, Catalase levels were found to be 187.9 $\pm$ 8.71 nmol /g of tissue, 1681.21 $\pm$ 8.06 Units/mg protein, and 24.30 $\pm$ 1.12 units /mg protein respectively. All the other groups were compared with sham control group. By increasing the time period of torsion and detorsion, there was significant and gradual rise in MDA levels and fall in SOD, catalase levels when compared to sham control group animals. Difference observed was statistically significant. Animals with 4 hours of torsion followed by 4 hours of detorsion shown maximum rise in MDA levels and a reduction in antioxidant reserves like SOD and catalase levels in a greater extent when compared to all other groups with different time intervals. It indicates testicular torsion for 4 hours and reperfusion for 4 hours will be a suitable animal model to perform screening procedure to identify a potential drug to treat testicular torsion induced reperfusion injury.

## DISCUSSION

There is increasing evidence that testicular torsion and detorsion induces biochemical changes caused by both ischemia and reperfusion of the tissues. Ischemia Reperfusion injury is caused by oxygen free radicals. Experimental testicular torsion and detorsion in the rats produces a variable acute response depending on the duration of torsion and detorsion, just as in clinical cases of testicular torsion seen in humans. Oxidative stress

was quantified at various time intervals of torsion and detorsion. Studies with time pattern of 1 hour torsion and 4 hours detorsion (12) produced higher MDA levels and decreased SOD and catalase levels when compared to Sham control. This indicates the oxidative stress produced by reperfusion injury. Group of rats in our experimentation also showed the similar results. Two separate studies conducted on rats with 2 hours of testicular torsion followed by 2 hours of testicular detorsion induces reperfusion injury and there was significant rise in MDA levels in testicular tissue. These results were similar to our experimental results with 2 hours of torsion followed by 2 hours detorsion (13,14). In another study, reperfusion injury was created by inducing torsion/ischemia for 2 hours and detorsion for 4 hours (15). There was rise in the MDA levels, similar to our results. Studies in the same lines by inducing ischemia for 2 hours and reperfusion for 4 hours (16) also had shown the identical results. There was increase in the MDA and fall in the levels of SOD and catalase after reperfusion injury. Our results (2 hours Torsion and 4 hours detorsion) also shown the similar values. Experiments with higher intervals of Testicular Torsion and Detorsion i.e. ischemia for 4 hours and reperfusion for 4 hours (17) produced maximum MDA value and maximum downfall in SOD and Catalase when compared to all other groups with different time intervals. Rats exposed to the 4 hours torsion and 4 hours detorsion in the present experiment also reveals the similar results when compared to all other groups.

It was determined that torsion up to 720 degrees for 4 hours and 4 hours detorsion produced a totally infarcted testis with elevated MDA levels and reduced anti oxidant enzymes like SOD and catalase. This result allows us to identify experimental protocol that produces maximum reperfusion injury, so with this model it is possible to study the ability of drugs to protect testicles with testicular torsion induced reperfusion injury.

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