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Animal Research Section

Role of Ascorbic Acid in Ameliorating Testicular Tissue Damage Induced by Testicular Torsion and Detorsion: An Animal Model Study

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ABSTRACT

Introduction: Testicular torsion-detorsion induced tissue damage, in neonates or adolescents may hamper fertility potential in their future life. Ascorbic acid being a water-soluble antioxidant has a number of antioxidant properties and is the most important antioxidant in human plasma.

Aim: To evaluate the potential role of ascorbic acid in ameliorating testicular tissue damage induced by testicular torsion and detorsion in wistar rats.

Materials and Methods: Forty male albino wistar rats were randomly divided into four groups. Group I served as normal control while Group II underwent Sham operation. Animals of Group III underwent testicular torsion and detorsion without any treatment while animals of Group IV were pretreated with ascorbic acid for 30 days, followed by 3 hours of testicular torsion and one hour detorsion. In the animals that underwent testicular torsion the testis appeared devitalised and a mild testicular oedema was observed. On detorsion, there was a slight further increase in oedema beside reactive hyperaemia. Other than this no complications were seen in the rats during the observation period. All the animals were sacrificed 1 hour after the experimental procedure, and testicular tissue sample was collected and evaluated for Seminiferous Tubular Diameter (STD), Seminiferous Epithelial Height (SEH), tubular necrosis, tissue lipid peroxidation, tissue glutathione and superoxide dismutase levels. Statistical analysis was done using SPSS package version II. The analysis of multiple group variation was done by ANOVA. Intergroup comparison was done by post-hoc (LSD) test.

Results: Animals of Group III showed a decrease in STD and SHE compared to Groups I and II. A four-fold increase in lipid peroxidation was observed in these animals (Group III). Superoxide dismutase and tissue glutathione levels were considerably reduced in these animals. Whereas, animals of Group IV showed merely 25% of seminiferous tubular necrosis with no significant decrease in their STD and SEH. A significant reduction in lipid peroxidation was also observed in these animals with antioxidants showing near to normal value compared to their untreated controls.

Conclusion: Results of the present study display that pretreatment with ascorbic acid offers salvaging effect on the testicular torsion-detorsion induced injury in rats.

Keywords: Spermatic cord torsion, Testis, Vitamin C

INTRODUCTION

Testicular torsion, one of urological emergency cases, seen in neonatal or adolescent males requires early diagnosis and treatment to preserve future fertility [1]. Though the molecular mechanism of the testicular injury caused due to its torsion is yet being explored, the oxygen free radicals generated during the reperfusion period is said to cause further damage to the tissue than the ischaemic period [2]. Studies have also revealed that severity of the injury is associated to the extent and the degree of torsion, with spermatogonia and spermatocyte being the primary targets to be affected [3-5]. The association between the levels of testicular damage to different duration of ischemia has been studied in the past, in rats [6,7]. Since then, a growing number of animal studies showing the therapeutic role of various antioxidants have been documented [8,9].

Ascorbic acid, a water-soluble antioxidant, has a number of antioxidant properties and has been claimed to be the most important antioxidant in human plasma [10]. This antioxidant property of ascorbic acid is mainly related to its ability to react with many Reactive Oxygen Species (ROS) and to the fact that the resulting semidehydroascorbate is being converted back to ascorbate at the expense of Nicotinamide Adenine Dinucleotide (NADH) or reduced glutathione [11]. Ascorbic acid also contributes to the redox mechanism by salvaging other antioxidants such as vitamin E, urate and β-carotene from its oxidised form [12]. It also quenches the free radicals present in the lipid membranes, preventing lipid peroxidation when combined with the tocopherols (vitamin E) [13]. The present study was aimed to investigate the effect of ascorbic acid "an exogeneous antioxidant" on rat testicular torsion-detorsion induced injury.

MATERIALS AND METHODS

Forty adult male albino rats of wistar strain, weighing 200-250 gm was divided into four groups (n=10) in the present study. Animals were housed in the animal house of Kasturba Medical College, Mangaluru, Karnataka, India. This study was conducted as a part of PhD thesis from 2004-2007. The duration of the experimental work was about 16 months. Seven days of acclimatisation period was given to the animals before the commencement of experiment.

The protocol carried out in this animal experiment was reviewed and approved by the Local Committee on Medical Ethics for the use of laboratory animals of Kasturba Medical College, Karnataka, India.

The animals of Group I served as normal control while Group II animals underwent Sham operation. Group III animals were subjected to manual rotation of the testes (720° clockwise) for three hours followed by counter rotation (detorsion) for one hour without any treatment. Group IV animals were pretreated with ascorbic acid dissolved in distilled water (40 mg/kg.bw, orally) for a period of 30 days before undergoing three hours testicular torsion followed by one-hour detorsion. At each time point, animals were anaesthetised by pentobarbitone sodium (40 mg/kg.bw

intraperitoneal) under strict aseptic conditions. Scrotectomy, was performed through mid scrotal vertical incision for inducing torsion and detorsion. The left testis was manually rotated 720° clockwise to induce ischaemia. A silk suture was thereafter passed through its tunica albuginea to fix the testis to the scrotum to retain the same position. Counter rotation of the testis to its original alignment for one hour was performed to allow reperfusion, which was confirmed by observing the development of reactive hyperaemia. Animals that failed to develop reactive hyperaemia were excluded from the study. The testis was covered with gauze soaked in normal saline (0.9% sodium chloride) to keep it moist. After each surgical procedure, the incision was closed and the animals were allowed to recover from anaesthesia.

They were left free in their cages and were fed with standard laboratory pellet diet (Gold Mohur, Lipton India Ltd.,) and water ad libitum. The animals were sacrificed at the end of one hour of detorsion using a lethal dose of sodium pentobarbitone (100 mg/kg.bw). Sequential biopsies were immediately performed where testes were removed for morphological and biochemical evaluation.

Histopathological analysis of the testis: The testicular tissue removed for histopathological evaluation was processed and paraffin blocks were prepared as per standard protocols [14]. Sections of 5µ thickness were obtained, stained with haematoxylin and eosin for light microscopic analysis.

A-Quantitative Analysis of Testicular Damage

Measurement of Standard Tubular Diameter (STD): Five transversely cut sections from various fields of each testis was evaluated using a stage micrometer that was calibrated with ocular micrometer. Short and long diameter of the tubule, one perpendicular to the other was measured and the average of these two diameters was taken for each animal [15].

Measurement of Standard Epithelial Height (SEH): From each transversely cut sections of the testis, five seminiferous tubules per slide were randomly picked and measured for their epithelial height [16].

B-Qualitative Evaluation

Testicular tissue damage was graded conferring the presence of coagulation type necrosis detected in the sections of seminiferous tubules [17].

Grade 0: Absence of coagulation and necrosis.

Grade 1: Mild coagulation, with less than 25% of the tubules exhibiting necrosis varying from lack of spermatogenesis, disruption in the germinal layers and necrosis in individual cells.

Grade 2: Moderate coagulation, with 25% to 75% of the tubules presenting variable grades of necrosis ranging from lack of spermatogenesis with disruption and damage of maturation layers to necrosis in individual cells, considering common features of Grade 1 and Grade 3 evaluation.

Grade 3: Severe coagulation, with 75% or more of the tubules showing total necrosis.

Biochemical Analysis

Estimation of testicular lipid peroxidation: This assay was performed based upon the reaction of Thiobarbituric Acid (TBA) with Malondialdehyde (MDA) that is one of the aldehyde products of lipid peroxidation [18]. Spectronic D-20 spectrophotometer was used to measure the formation of a pink colour at 535 nm. TBA was expressed in terms of nanomoles of MDA/g of wet tissue.

Estimation of tissue Glutathione (GSH): Tissue GSH form the homogenate {10% w/v in 10 mM Phosphate-Buffered Saline (PBS; pH. 7.4)} was estimated [19]. Optical density was measured at 412 nm using a Spectronic D-20 spectrometer and was expressed as GSH in nmol/mg of tissue protein.

Superoxide Dismutase (SOD) assay: SOD was estimated as per the method of Kakkar P et al., [20]. The reduction of Nitro Blue Tetrazolium (NBT) by O_2 was measured at 560 nm using a Spectron D-20 spectrophotometer and the values were expressed in unit/mg protein of tissue homogenate.

Chemicals: Riboflavin, TBA, NBT, Reduced GSH, and L-Methionine were procured from Loba Chem Pvt. Ltd., Mumbai, India. Trichloroacetic acid and Folins reagent were obtained from Qualigens Fine Chemicals, India. Ethylenediaminetetraacetic acid was purchased from SDFCL Ltd., Mumbai, India. Dithiobis-2 Nitro benzoic acid and Ascorbic acid were bought from SISCO Research Laboratories Ltd., Mumbai, India. All chemicals used in this study were of analytical grade.

STATISTICAL ANALYSIS

All values in the text and figures are presented as Mean±Standard Deviation (SD). Statistical analysis was calculated using SPSS package version II. The analysis of multiple group variation was done by ANOVA. The post-hoc (LSD) test was done for inter-group comparison. Statistical significance was considered as $p<0.05$.

RESULTS

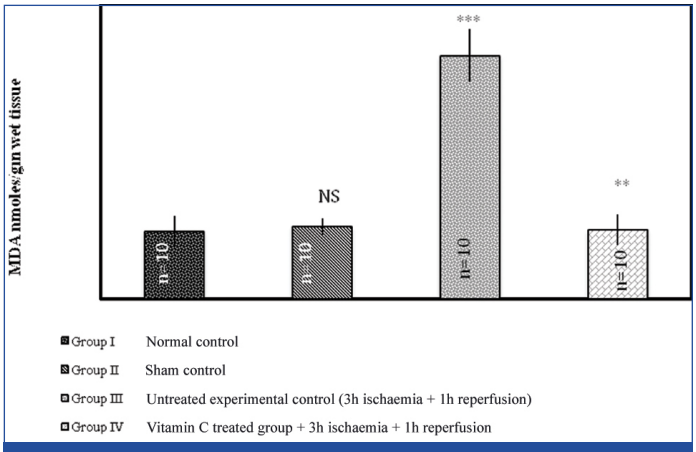
Biochemical Assay

Tissue lipid peroxidation (MDA assay): A significant increase in the level of tissue lipid peroxidation was observed in the untreated experimental group animals (Group III) and the animals pre-treated with ascorbic acid (Group IV) on the other hand showed a significant reduction in the level of lipid peroxidation compared to the untreated experimental control group [Table/Fig-1].

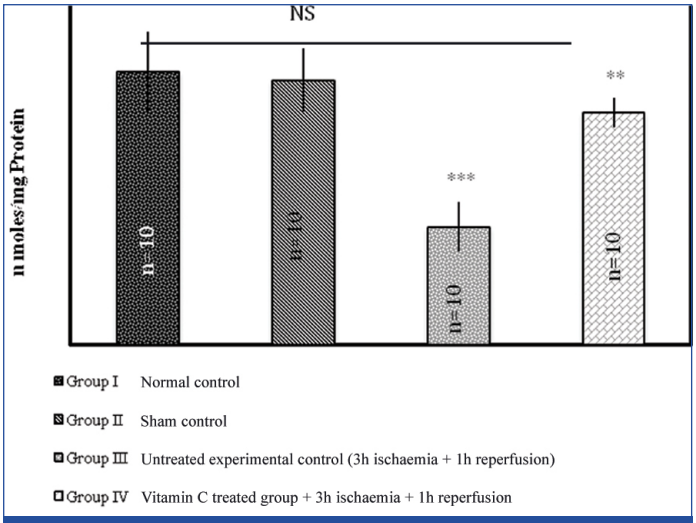
Tissue glutathione (GSH) and tissue superoxide dismutase (SOD): A significant decrease in the level of tissue GSH and SOD was observed in the animals of Group III when compared to normal control and sham control after 3 hour of torsion and 1hour of reperfusion ($p<0.0001$) as depicted in [Table/Fig-2,3]. However, the animals that were pre-treated with ascorbic acid (Group IV) showed near normal values to that of their control groups.

Histopathological Evaluation

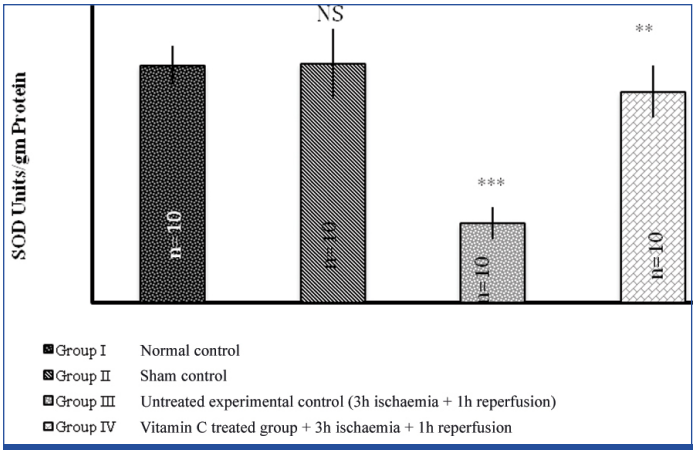
Quantitative analysis of testicular damage: The changes in STD and SEH, of the testis following 3 hour of ischaemia followed by reperfusion for 1 hour are summarised in [Table/Fig-4]. The animals in Group III showed a significant reduction in the STD and SEH compared to that of control groups (Group I and Group II). However, the animals of the group that was pre-treated with ascorbic acid (Group IV) prior to induction of 3 hour ischaemia followed by 1 hour reperfusion showed a near normal value in their STD and SEH [Table/Fig-4].



[Table/Fig-1]: Effect of 3 hour of ischaemia followed by reperfusion for 1 hour on the levels of tissue MDA.
The values are expressed as mean±SD, n=number of animals used in the study
*** $p<0.0001$ versus Gr II and Gr I. ** $p<0.0001$ versus Gr III. NS=Not significant versus Gr I.



[Table/Fig-2]: Effect of 3 hour of ischaemia followed by reperfusion for 1 hour on the levels of tissue GSH.
The values are expressed as mean±SD, n=number of animals used in the study.
*** p<0.0001 versus Gr II and Gr I. ** p<0.0001 versus Gr III. NS=Not significant versus Gr I.



[Table/Fig-3]: Effect of 3 hour of ischaemia followed by 1 hour of reperfusion on the levels of tissue SOD.
The values are expressed as mean±SD, n=number of animals used in the study.
*** p<0.0001 versus Gr II and Gr I. ** p<0.0001 versus Gr III and Gr I. NS=Not significant versus Gr I.

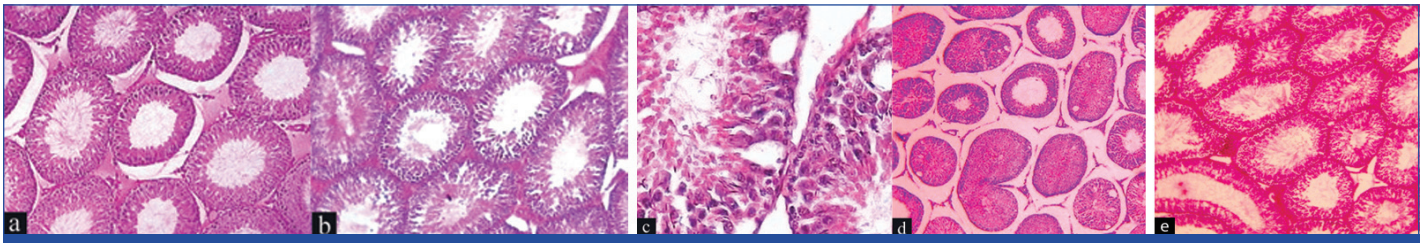
Groups	STD (μm)	SEH (μm)
I (Normal control)	597.51±28.4 (n=10)	75.14±9.38 (n=10)
II (Sham control)	561.76±38.5 NS (n=8)	76.35±4.60 NS (n=8)
III (Untreated experimental control)	450.42±66.2*** (n=8)	34.54±7.83*** (n=8)
IV (Pre-treated with ascorbic acid)	595.09±64.72** (n=8)	63.63±7.68** (n=8)

[Table/Fig-4]: Effect of 3 hour of ischaemia followed by reperfusion for 1 hour on STD and SHE.
The values are expressed as mean±SD; n=sample size.
STD: ***=p<0.0001 versus Gr. I and Gr. II, **=p<0.0001 versus Gr. III. NS=Not significant versus Gr. I.
SEH: ***=p<0.0001 versus Gr. I and Gr. II,**=p<0.0001 versus Gr. III. NS=Not significant versus Gr. I.

Qualitative evaluation: Animals of Group I and Group II showed normal testicular architecture with an orderly arrangement of germinal cells with absence of coagulation and necrosis in the STD [Table/Fig-5] respectively. In Group III, coagulative necrosis of germinal cells was observed within the STD with 75% or more of the tubules showing complete necrosis (Grade 3). On the other hand, the animals of the Group IV showed disordered, sloughed germinal cells within the STD and 25% or more of the tubules showed variable degrees of necrosis (Grade 2).

DISCUSSION

In the present study, the tissue levels of MDA were significantly increased in the testes of the untreated experimental control group rats (p<0.0001) when compared to that of normal and sham control group confirming oxidative injury that occurred in the testicular tissue. It has been documented that reduction in blood flow leads to hypoxia of the organs resulting in high levels of tissue lipid peroxide products [21]. Reperfusion of the ischaemic tissue will further lead to the formation of ROS and can additionally cause damage and atrophy of the testicular tissue [22]. MDA, a final product of lipidperoxidation, is used in assessing the formation of ROS after restoring the blood supply to the tissues [23,24]. This is agreeable in the present study as the animals that underwent testicular torsion and detorsion showed a fourfold increase in the levels of lipid peroxidation [Table/Fig-1] which simply proves that testes promotes severe cell membrane peroxidation [24]. Usually in the initial phases



[Table/Fig-5]: Photomicrograph of rat testis in different groups: a) Normal control group (group I) showing normal histological features (H&E, 10X); b) Sham control (group II) showing normal histological features in (H&E, 10X); c) Experimental control group rats that underwent 3 hours of testicular torsion and 1-hour counter-rotation (group III) showing cytoplasmic vacuoles in the seminiferous tubules of (H&E, 40X); d) Experimental control group rats that underwent 3 hours of testicular torsion and 1-hour counter-rotation (group III) showing necrosis in more than 75 percent of the seminiferous tubules (H&E, 10X); e) Pretreated experimental group of rats that were pre-treated with ascorbic acid prior to the induction of 3 hours of testicular torsion followed by counter-rotation for 1 hour (group IV) showing 25 percent or more necrosis of variable degrees in the seminiferous tubules (H&E, 10X).

the endogeneous antioxidants are said to counterbalance the injury. However, upon production of ROS beyond the capacity of defense mechanism the tissue injury occurs [25].

When the animals were pretreated with ascorbic acid prior to torsion and detorsion, a significant reduction in the level of tissue MDA indicates the prevention of lipid peroxidation. Reports show that rise in levels of oxidants or reduction in the tissue antioxidant levels is proved to be an indicator of oxidative stress [26]. Reduced concentration of GSH and SOD in the animals of untreated group in the present study ratifies the above statement [Table/Fig-2,3].

The duration of torsion chosen in the present study was based on the reports of Abasiyanik A and Dagdonderen L [27]. According to them one hour of torsion lead to loss of germ cells while two hours and above lead to loss of sertoli cells in adult rats. The histopathological evaluation in the present study showed decreased STD and SEH in the testis of animals of the untreated experimental group (Group III; [Table/Fig-4]), agreeing with the suggestion of above-mentioned authors. Besides, the histological sections of the seminiferous tubules in this group also showed interstitial oedema, vacuolisation, maturation arrest, haemorrhage and coagulative necrosis of germinal cells with 75% or more of the seminiferous tubules showing complete necrosis. Numerous intercellular areas were also observed inbetween the germinal cells lining the seminiferous tubules [Table/Fig-5] which is said to be caused due to the loosening of inter cellular connections that finally leads to shrinkage of both germ and sertoli cells [28].

On the other hand, there was no significant reduction/damage in the STD and SHE of the tubules in the animals of the pretreated group

(Group IV; [Table/Fig-4]). This suggests that pretreatment with ascorbic acid probably helped in maintaining the normal architecture of the testis and the seminiferous tubules without affecting the spermatogenesis.

Various researches have examined the effects of moderate and sustained reduction in testicular blood and its effects on the testis [7,29]. They observed that reductions in blood flow might play a major role in pathogenesis of male infertility. Aktas BK et al., demonstrated that administration of N-acetylcysteine to animals, prior to torsion and detorsion significantly increased the STD and SHE, in their experimental study [30]. Similar results of significantly less tissue injury to the seminiferous tubules were witnessed by Kemahli E et al., and his coworkers when the animals were pretreated with pyrrolidine dithiocarbamate15 minutes prior to detorsion [31]. In the present study, those animals that were pretreated with ascorbic acid and then underwent torsion-detorsion showed significantly less tissue damage. Testicular torsion and detorsion in animals exemplifies a practical model to that of humans and the production of ROS is the main cause of tissue damage. Growing evidence show that testicular torsion related injuries in newborns and young adolescents are being reversed when diagnosed and treated early [32]. Since exogenous antioxidants like vitamin C and E have proven beneficial in treating male infertility [33], the administration of ascorbic acid while restoring the blood flow after torsion in newborns and young adolescents, may potentially play a vital role in scavenging the free radicals produced during the tissue injury.

LIMITATION

A long-term follow-up particularly after the postoperative procedures to further check the testicular viability in the animals could not be performed. Since the contralateral testis was not investigated, a parallel investigation of both testes might provide further insight to this study. Moreover, we could not afford the cost to include any ROS mediated immunohistochemistry stains as this project was not funded.

CONCLUSION

Present study result deliberates that pretreatment with ascorbic acid significantly reduced testicular tissue damage induced by elevated level of ROS during testicular torsion-detorsion. Therefore, administration of ascorbic acid in the patients with testicular torsion from the time of diagnosis until the surgery and during recovery period might minimise the damage and salvage the testicular function.

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