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Original Article

Effect of chronic unpredictable stressors on reproductive parameters in male wistar rats: role of ascorbic acid

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ABSTRACT

AIM: Chronic unpredictable stress (CUS) may be more applicable as a model of post-traumatic stress disorder because of the lack of predictability, which could permit habituation.The present study, was aimed to investigate the effect of chronic unpredictable stressors on reproductive parameters and their possible relation with testicular ascorbic acid level.MATERIALS AND METHOD: Adult male Wistar rats were divided into two groups as non stressed group (n = 10) and stressed group (n = 10). The stressed groups were exposed to 10 days of CUS. The epididymal sperm count, sperm abnormalities, testicular weight, testicular lipid peroxidation and ascorbic acid level of the testis were estimated. All experimental procedures and animal maintenance confirmed to the strict guidelines of Institutional Ethics Committee. RESULTS:The results were analyzed statistically by using student't' test. $P < 0.05$ was considered as significant. Exposure to CUS showed a significant ($P < 0.001$) decrease in the weight of the reproductive organs, sperm count, and ascorbic acid level. Further, significant increase ($P < 0.001$) was observed in testicular lipid peroxidation level and incidence of sperm abnormality. CONCLUSION:The present data suggest that CUS has deleterious effect on spermatogenesis Further, stress induced oxidative damage might be mediated through its effect on reducing ascorbic acid level.

1. Introduction

In the recent years concern has aroused for the decrease in the fertility in man. One of the goals when evaluating an infertile man is to identify reversible conditions that are responsible for infertility. Chronic stress has been a potential risk factor for reproductive function [1,2]. In males, physical and psychological stressors might inhibit reproductive function mainly through the suppression of hypothalamus-pituitary-gonadal (HPG) axis and activation of hypothalamus-pituitary-adrenal (HPA) axis [3]. Several reports have suggested a stress related decline in semen quality, sperm concentration, morphology and percentage of motility [4,5]. This leads to a close correlation between the sperm concentration and fertility potential of males.

The Vitamin C is one of the most commonly available and accessible molecules and is a part of food commonly consumed by most [6]. The ascorbic acid is a known antioxidant present in the testis with the precise role of protecting the latter from the oxidative damage[7]. Deficiencies of vitamins C have been lead to a state of oxidative stress in the testes that disrupts both spermatogenesis and the production of testosterone[8,9]. In recent years, Supplementation with Vitamin C has also been shown to increase total sperm output and sperm concentration. Lipid peroxidation has been considered as a serious consequence of free radical toxicity leading to profound changes in the membrane structure and function that may cause even cell death [10, 11]. Malondialdehyde (MDA) is one of the end products of lipid peroxidation and extent of lipid peroxidation is measured by estimating MDA levels most frequently. Increased serum level of MDA has been reported in cardiovascular, neurological and other diseases [10, 11].Chronic unpredictable stress (CUS), one of the most clinically relevant stress paradigms in rodents, mimics a number of behavioral characteristics observed in patients with

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anxiety, depression and related mood disorders [12].Rats exposed to CUS show reduced body weight gain and increased adrenal gland weight, but not changes in basal or restraint stress stimulated plasma corticosterone levels [13].

In this study, a different model of chronic unpredictable stress was chosen in order to avoid habituation to the stressor and to maintain robust the stress response.The purpose of the present study was to determine whether exposure to chronic unpredictable stressors applied to adult male rats induces changes in reproductive parameters and its possible relation with testicular ascorbic acid level.

2. MATERIALS AND METHODS

Adult male rats of Wistar strain weighing between 140 -220 g was taken in this study. All the rats were given standard rat chow and tap water ad libitum and were housed at 25 ± 2°C on a 12-hour dark/light cycle. All experimental procedures and animal maintenance confirmed to the strict guidelines of Institutional Ethics Committee and that of Federal laws for the use of animals in the experiment. The animals were divided into two groups as non stressed group (n =10) and stressed group (n =10).

Experimental stress procedure [14]:Rats assigned to the chronic stress group were exposed to the following CUS protocol : Day one - 11:00 a.m. 50 min forced swimming stress (The rats were forced to swim in the plastic tub, circular in shape with a height of 60cm and a diameter of 40cm (water temperature; 28°C; water level kept at 30cms from the bottom), and 12:00 p.m. 60 min cage tilting (the rats are placed in a polycarbonate cage and tilted at an angel of 45°); Day two- 1:00 p.m. 4 h wet bedding (400 ml tap water in home cage), and 6:00 p.m. lights on overnight; Day three- 12:00 p.m. 3 h overcrowding stress, and 3:00 p.m. 60 min restraint stress (6 x 21.6 cm Plastic tube restrainer); Day four- 6:00 p.m. 50 min cage tilting, and food and water deprivation overnight (15 h); Day five - 3:00 p.m. 1 hour overcrowding stress (pooling the rats from 3 cages into 1 (size; 24x39x23 cms), and 4:00 p.m. isolation housing overnight (17 h); Day six - 11:00 a.m. 4 h wet bedding, and 3:00 p.m. 2 h forced swimming stress ; Day seven - 1:00 p.m. 30 min overcrowding stress, and 6:00 p.m. 1 h lights on; Day eight - 10:00 a.m. 20 min cage tilting, and 3:00 p.m. 60 min restraint stress; Day nine - 10:00 a.m. 4 h wet bedding, and 6:00 p.m. food and water deprivation; Day ten - 6:00 p.m. isolation housing and lights on overnight. Immediately after the last stressor, Animals were sacrificed. The laparotomy was performed and the reproductive organs were exposed. The reproductive organs (testis, epididymis, seminal vesicle and prostate) were weighed. The epididymis was carefully separated from the testis.

Sperm count: The epididymis was minced in 1ml of phosphate buffered saline (pH 7.2) to obtain a suspension [15]. The suspension was filtered through a nylon mesh. The sperm count was conducted in the filtrate as per the standard method in Neubauer'schamber . Briefly, an aliquot from the suspension (up to 0.5) was taken in leukocyte hemocytometer and diluted with phosphate buffered saline up to the mark 11. The suspension was well-mixed and charged into Neubauer's counting chamber. The total sperm count in 8 squares (except the central erythrocyte area) of 1mm2 each was determined and multiplied by 5×104 to express the number of spermatozoa/epididymis.

Sperm morphology test: For the evaluation of the sperm morphology the filtrate obtained was stained with 1% eosin Y or periodic acid-Schiff's reaction and morphological defects were analyzed as explained elsewhere [16]. Briefly, the sperms in the smears were visualized under oil immersion objectives and any abnormalities of either heads or tails were noted. The microcephaly, which was also a type of head abnormality, and cephalo-caudal junction defects (CC), which were a type of tail defects have been classified separately. Two hundred sperms were screened for each animal and total abnormality was expressed as incidence/200 sperms/animal.

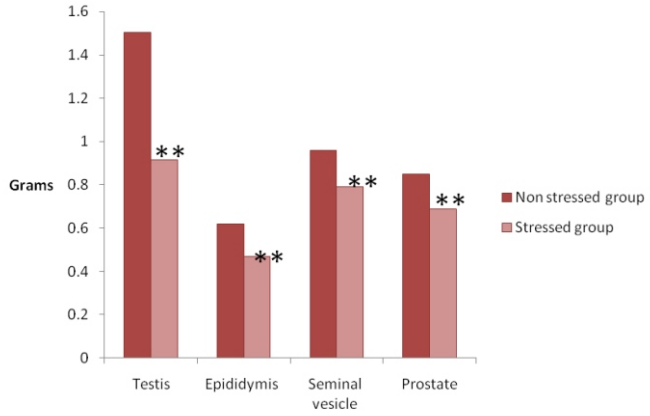
Ascorbic acid level in the testis: The right testis was removed and placed in phosphate buffered saline (pH 7.2) and the tunica albugenia was removed. Following this, the testis was homogenized in the same solution and the homogenate was used for the estimation of ascorbic acid level by 2,4-dinitrophenyl hydrazine method, calorimetrically[17]. Briefly, the ascorbic acid in the homogenate is oxidized by Cu2+ to form dihydro-ascorbic acid, which reacts with acidic 4-dinitrophenyl hydrazine to form a red hydrazone, which is measured at 520 nm.

Lipid Peroxidation: The testicular tissue (1g) was transferred to a homogenizer containing cold 10ml of 10mM cold potassium phosphate buffer (pH 7.4). The tissue was homogenized using a manual homogenizer. The unbroken cells and cell debris were removed by centrifugation at 3000 rpm for 10 minutes by using Remi C 24 refrigerated centrifuge(- 4^{0}C) . The obtained supernatant was used for the estimation of lipid peroxidation level. The lipid peroxidation was estimated according to the method of Kartha and Krishnamurthy[18]. This assay is based upon the reaction of TBA with malondialdehyde (MDA) , one of the aldehyde products of lipid peroxidation. Values were expressed as nanogram of MDA/gm tissue (taking molar extinction coefficient of MDA as 1.56 x 10^5

3. RESULTS:

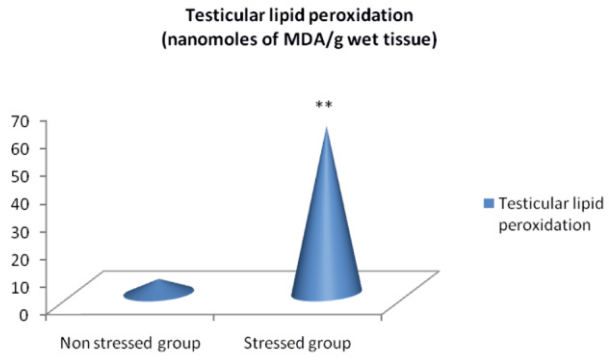
The data was expressed as Mean ± SD. The differences between groups were compared for statistical significance by student t test with the level of significance set at P< 0.05.Exposure to CUS showed a significant (P < 0.001) decrease in the weight of the reproductive organs, sperm count, and ascorbic acid level. Further, significant increase (P < 0.001) was observed in testicular lipid peroxidation level and incidence of sperm abnormality.

Figure I: Effect of chronic unpredictable stress induced alteration on reproductive organ weight (g) Mean ± SD, n=10 in each group.



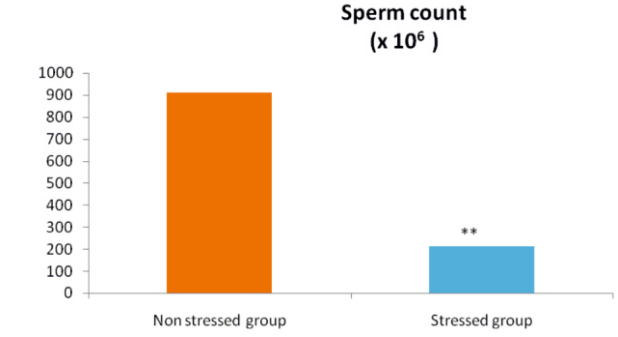
**** P< 0.001; NonStressed group versus Stressed group**

Figure 2: Effect of chronic unpredictable stress induced alteration on testicular lipid peroxidation (nanomoles of MDA/g wet tissue) in wistar male rats; Mean ± SD, n=10 in each group.



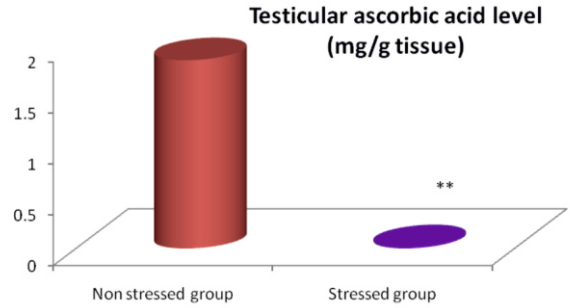
**** P<0.001 ; Non Stressed group versus stressed group**

Figure 3:Effect of chronic unpredictable stress induced alteration on testicular sperm count (x 10^6) in wistar male rats; Mean ± SD, n=10 in each group.



**** P< 0.001 Non Stressed group versus stressed group**

Figure 4: Effect of chronic unpredictable stress induced alteration on testicular ascorbic acid level in wistar male rats; Mean ± SD, n=10 in each group.



**** P<0.001 ; Non Stressed group versus stressed group**

Table I: Effect of chronic unpredictable stress induced alteration on sperm morphology in rats; Mean ± SD, n=10 in each group

Groups	Normal	HA	TA	MC	CC	Total abnormality
Non Stressed group	170.83 ± 2.24	12.52 ± 0.50	11.24 ± 0.25	0.60 ± 0.53	2.30 ± 0.22	22.25 ± 1.20
Stressed group	118.20 ± 1.12 **	30.07 ± 2.50 **	30.17 ± 1.20 **	2.00 ± 0.30 **	3.10 ± 0.72 **	72.10 ± 2.50 **

HA – Head abnormality ;TA – Tail abnormality;MC – Microcephaly CC – Cephalocaudal junction

**** P< 0.001 Non Stressed group versus stressed group**

4. DISCUSSION

In human life, prevalence of Stress has deleterious impacts on brain and body physiology[19, 20].Exposure to stress leads to the tremendous amount of free radicals[10].The present study indicates that chronic unpredictable stress significantly increased

the oxidative damage biomarker of lipid peroxidation in the testis.Stress induced increased in reactive oxygen species might have led to the significant decline in the sperm count and ultimately testicular weight loss.Reactive oxygen radicals are detrimental to the testicular functions and therefore, are regularly being scavenged by a variety of endogenous antioxidants and quenchers including vitamins, enzymes, tripeptidesand ascorbic acid could be one of them [21].Ascorbic acid functions as a most important free radical scavenger trapping free radicals protecting bio membrane from oxidative damage [22].Further, the role of ascorbic acid has long been established as an agent to play a crucial role in the differentiation process of the spermatogonial cells to sperm [23]. In the present context, ascorbic acid level in the CUS exposed rat testes have been declined significantly possibly indicating its role as a potential scavenger of reactive oxygen species. The insufficiency of the ascorbic acid incurred in the CUS exposed rats might have led to decrease transformation of sperm, thereby resulting in a significant decline in the sperm count. Further, increase in the percentage of sperm abnormalities in the stress induced rats coupled with increased lipid peroxidation level in the testicular tissue, emphasizes the possibility of gene alteration in germ cells induced by reactive oxygen species generated during chronic stress exposure.

CONCLUSION:

The present study suggest that at least one of the more possible mechanism in the stress induced toxic effect on the male reproductive system might be mediated through its effect on reducing ascorbic acid level and generating ROS. The assessment of the different types of stress and the different antioxidants during the exposure to chronic unpredictable stress will be an interesting step- forward to identify the mechanisms involved

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