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Original Article

Pharmacology Section

Effect of Vitamin C on Male Fertility in Rats Subjected to Forced Swimming Stress

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ABSTRACT

Introduction: Stress is defined as a general body response to initially threatening external or internal demands, involving the mobilization of physiological and psychological resources to deal with them. Recently, oxidative stress has become the focus of interest as a potential cause of male infertility. Normally, equilibrium exists between reactive oxygen species (ROS) production and antioxidant scavenging activities in the male reproductive organs. The ascorbic acid is a known antioxidant present in the testis with the precise role of protecting the latter from the oxidative damage. It also contributes to the support of spermatogensis at least in part through its capacity to maintain antioxidant in an active state.

Materials and Methods: Group1: Normal Control animal received Distilled water, Group 2: Positive control (Only Stress), Group 3: Normal rats received an intermediate dose of Vitamin C (20mg/kg/day), Group 4: Stress + Low dose Vitamin C (10mg/kg/day), Group 5: Stress+ Intermediate dose Vitamin C (20mg/kg/day), Group 6: High dose Vitamin C (30mg/kg/day). On 16th day effect of stress on body weight, Reproductive organ weight, sperm parameters, and hormonal assay was studied.

Results: In the present context, in stress group the sperm count, motility, testicular weight declined significantly. The intermediate dose and high dose of vitamin C showed significantly increased effect on the sperm count and motility.

Conclusion: Various physiological changes produced force swimming indicates that swimming is an effective model for producing stress in albino rats. The results suggest that Vitamin C supplementation improves the stress induced reproductive infertility due to both their testosterone increase effect and their antioxidant effect.

Keywords: Male fertility, Oxidative stress, Vitamin C

INTRODUCTION

Stress is an imbalance between production of reactive oxygen species and antioxidant defense. Stress is defined as “a general body response to initially threatening external or internal demands, involving the mobilization of physiological and psychological resources to deal with them” [1]. Various forms of physical and psychological stress are believed to reduce sexual functions. Several studies have examined the relationship between stress and sexual behaviour in male rats [2,3]. These reports show that chronic psychological and physical stress induce erectile dysfunction, which results from neurotransmission changes in various erectile response pathways, and a reduced blood flow in genital organs [4]. Oxidative stress has repeatedly been implicated as a leading cause of male factor infertility [3]. Based on these findings, we suspect that stress affects sexual function in both men and women.

Recently, oxidative stress has become the focus of interest as a potential cause of male infertility. Normally, equilibrium exists between reactive oxygen species (ROS) production and antioxidant scavenging activities in the male reproductive organs [4].

Stress situations can increase production of oxygen free radicals. Free radicals generate a cascade producing lipid peroxidation. Lipid peroxidation is one of the main events induced by oxidative stress [5].

Testicular membranes are rich in polyunsaturated fatty acids and thus susceptible to peroxidation injury [3]. In accordance, antioxidant enzyme activity has been shown to decrease in experimental cryptochidism, resulting in increased lipid peroxidation [5]. Increased lipid peroxidations in the testis contribute to the suggested vulnerability of this organ to oxidative stress [6,7].

Spermatogenesis is an extremely active replicative process capable of generating approximately 1,000 sperm a second. The high rates of cell division inherent in this process imply correspondingly high rates of mitochondrial oxygen consumption by the germinal epithelium [7].

Under stress conditions, spermatozoa produce small amounts of ROS, which are needed for the capacitation acrosome reaction and fertilization. However, excessive amount of ROS produced by leukocytes and immature spermatozoa can cause damage to the normal spermatozoa by inducing lipid peroxidation and DNA damage [8,9].

Swimming in small laboratory animals has been widely used for studying the physiological changes and the capacity of the organism in response to stress. Swimming has got a number of advantages over other types of exercise such as treadmill running. The amount of work done during the swimming exercise is far greater than that during the treadmill running of identical time duration [10].

Drugs with multiple mechanisms of protective action, including antioxidant properties, may be one way forward in minimising tissue injury in human disease. A number of plants and plant isolates including Ocimum sanctum, Piper, cubeba Linn., Allium sativum Linn., Terminalia bellerica, Camellia sinensis Linn., Zingiber officinale Roscoe and several Indian and Chinese plants have been reported to protect free radical-induced damage in various experimental models [11].

The ascorbic acid is a known antioxidant present in the testis with the precise role of protecting the latter from the oxidative damage [6]. It also contributes to the support of spermatogenesis at least in part through its capacity to maintain this antioxidant in an active state. Vitamin C is itself maintained in a reduced state by a GSH-

dependent dehydroascorbate reductase, which is abundant in the testes. Vitamin C has been shown to improve sperm motility and enhances semen quality and fertility of rats [12].

Deficiencies of vitamins C or E leads to a state of oxidative stress in the testes that disrupts both spermatogenesis and the production of testosterone. In recent years, vitamin C supplements have been widely used in rats diets and the levels for enhancing production and reproductive performance have been increased several fold. Supplementation with Vitamin C has also been shown to increase total sperm output and sperm concentration [6,12]. Apart from the protective effects of Vitamin C on membrane integrity that safeguarded cellular functions.

MATERIALS AND METHODS

Animal: Adult male albino rats weighing 250 – 300 g and aged 10-12 weeks old were used in present study. The animals were kept in wire bottomed cages in a room under standard condition of illumination with a 12 - h light-dark cycle at 25±1°C. They were provided with tap water ad libitum and balanced diet contains Ground wheat, toasted wheat, corn, flaked corn, whole oats, dehydrated alfalfa pellets, flaked field peas, flaked beans, soybean meal, straw pellets, oat middlings, and soybean oil (Protein 12.5%, fat 3.0%, Fiber 6.0%, Calcium 0.5% Phosphorus 0.3%) supplied by the Prasanth animal feed distributor, Pune. The study was approved by the Institutional Animal Ethics committee and followed the CPCSEA rules for animal protection.

Drug Preparation: Vitamin C procured from authentic distributor Vijaya traders Srirampur, Ahmednagar, manufactured by Sd Fine Chemical Ltd., Batch no K014-0201-2710-62 manufactured on July 2010. All reagents were of analytical grade.

Preparation of Vitamin C: 500mg of vitamin c disolved in 10ml in 1ml of double distilled water, given according the dose schedule by the oral route administration.

Experimental Design: Thirty six adult male rats of average body weight 250-300g were used for this study. The rats were randomly analysed into six groups of six rats each.

Grouping: Group1 Normal Control animal received Distilled water.
Group 2: Positive control (Only Stress).
Group 3: Normal rats received an intermediate dose of Vitamin C (20mg/kg/day).
Group 4: Stress + Low dose Vitamin (10mg/kg/day).
Group 5: Stress+ Intermediate dose Vitamin C (20mg/kg/day),
Group 6: High dose Vitamin C (30mg/kg/day).
Vitamin C administered daily, 30 min before expose to stress up to 15 days.

Stress Procedure: Rats were exposed to swimming stress daily between 09.00AM to 10.00AM until 15days. The swimming test developed by Porsolt et al., [13] has now become widely accepted model for physical stress in animals. It was modified according to previous researchers Nayanatara et al., [10], was used for this experiment.

Rats were swimming in plastic tanks (length 100cm, width 40cm, depth 60cm) containing tap water maintained at a temperature 36 ± 2°c. The water depth, 35cm was set so that the rats could not rest by supporting the tail on the bottom of the tank. The animals were assessed to be exhausted when they failed to rise to the surface of the water to breathe within 7sec. At this moment, the animals were removed from the tank [14].

Collection and preparation of samples

Twenty four hours after the last treatment (on 16th day), each animal was weighted and sacrificed by cervical dislocation.

- **Blood samples:** Blood samples were collected via cardiac puncture. Serum was obtained by centrifugation at 3000rpm for 20min. Serum was used for hormonal assay.
- **Semen samples:** Cauda epididymis was dissected out and it was immersed in 1ml of Phosphate Buffer Saline (PBS) and homogenized for 2min by using manual Homogenizer. The obtained aliquot and used for semen analysis [15].

METHODS

- **Body weight:** At the beginning and end of the experimental period, the body weight of each individual rat was measured.
- **Reproductive organ weights:** Rats were scarified by cervical dislocation on the 16th day, reproductive organs; testes, prostate, seminiferous tubules and Vas deferens were dissected out. The accessory tissue was removed and weighted.
- **Testicular Index:** Both testis were dissected out and weighted. The testicular index was calculated by dividing the left testis weight by the body weight and then multiplied by 100 [16].
- **Sperm functions analysis:**

Sperm count: The epididymis was minced in 1ml of PBS (pH 7.2) to obtain a suspension. The suspension was filtered through nylon mesh (80µm) [17]. The sperm count was conducted in the filtrate as per standard method Neubauer's chamber. Briefly, an aliquot from the suspension (up to 0.5) was taken in leuckocyte hemocytometer and diluted with PBS up to the mark 11. The suspension was well-mixed and charged in to Neubauer's counting chamber. The total sperm count in eight squares (except the central erythrocyte area) of 1 mm^2 each was determined and multiplied by 5x 104 to express the number of spermatozoa/ epididymis [12].

Mass motility: This was evaluated by an earlier method by Sallu et al., [18]. The epididymal sperm content was obtained by maceration of the tail of the epididymis in 1ml of PBS. An aliquot of this solution was on the slide and percentage of motility was evaluated microscopically at a magnification of 400 x. Motility estimates were performed from the three different fields in each sample. The mean of the three estimations was used as the final motility score [18].

Live and dead sperms (Viability): A drop of the epididymal content of each rat was mixed with an equal drop of 1% eosion stain prepared in accordance Metwally et al., [19]. Thin films were made by spreading the stained content onto clean slides and quickly dried. Viable sperm remains colorless. One hundred sperm cells per rat were scored for determining the viability percent.

Copulatory Index: On 12th day male rats were housed with four female rats for 12hrs (dark cycle). At the end of 24h (on 13th day) vaginal smear taken for microscopic examination for spermatozoa. This indicated no rats mated. The copulatory index was calculated by dividing the no. of female rats mated by four and then multiplied by 100 [20].

BIOCHEMICAL ANALYSIS

Hormonal assay: Serum samples were used for determination of testosterone was performed by Auto analyser method (kit details: Biomerieus India Pvt. Ltd, Lot No.1001025470. Exp; 2013-02).

STATISTICAL ANALYSIS

Statistical analysis was done by using Graphad Prism version 6.0. In the present study data was compared each group with all other group.

RESULTS [Table/Fig-1,2]

DISCUSSION

Ascorbic acid is a hydrophilic and functions as a most important free radical scavenger trapping free radicals in the aqueous phase thus protecting biomembrane from oxidative damage [15]. In the present context of stress group the sperm count, motility, declined significantly. The ascorbic acid has long been established

Parameter	Group I Normal rats (n=6)	Group II Stress group without treatment (n=6)	Group III Normal rats received Vit C 20mg/kg/day (n=6)	Group IV Stress + Vit C 10mg/kg/day (n=6)	Group V Stress + Vit C 20mg/kg/day (n=6)	Group VI Stress + Vit C 30mg/kg/day (n=6)
Body weight difference between 1st & 15th day (g)	+5.33 ±2.65	-16.50± 2.39 ***	+8.33 ±1.45 ^^^	-16.67 ±2.32 ***$$$	-15.83 ±1.76 ***$$$	-11.50 ±1.06 ***$$$
Testis Lt (g)	1.07 ±0.02	1.005 ±0.00**	1.07 ±0.02^^	1.02 ±0.01$	1.06 ±0.015^^	1.08 ±0.02^^#
Testis Rt (g)	0.954 ±0.06	0.885 ±0.03	0.952 ±0.08	0.904 ±0.02	0.903 ±0.007	0.929 ±0.02
Seminiferous tubules (g)	0.726 0.05	0.596 ±0.04	0.701 ±0.02^	0.632 ±0.00$$	0.613 ±0.05	0.667 ±0.01##
Vas deferens (g)	0.109 ±0.007	0.09 6±0.003	0.108 ±0.004^	0.110 ±0.007	0.105 ±0.007	0.110 ±0.010
Prostate (g)	0.125 ±0.004	0.106 ±0.007	0.129 ±0.002	0.101 ±0.001 ***$$$	0.102 ±0.002 ***$$$	0.11 3±0.003 *$$##¥
Testicular index (%)	0.38 ±0.006	0.40 ±0.012	0.39 ±0.005	0.36 ±0.006 *^$$	0.37 ±0.009	0.39 ±0.016#
Copulatory index (%)	70. 8±4.17	50.0 ±6.45*	70.8 ±4.17^	62.5 ±5.59	62.5 ±5.59	66.67 ±5.27

[Table/Fig-1]: Effect of stress, vitamin C on the body weight, reproductive organ weight, testicular index and copulatory index©

Para-meter	Group I Normal rats (n=6)	Group II Stress group without treatment (n=6)	Group III Normal rats received Vit C 20mg/kg/day (n=6)	Group IV Stress + Vit C 10mg/kg/day (n=6)	Group V Stress + Vit C 20mg/kg/day (n=6)	Group VI Stress + Vit C 30mg/kg/day (n=6)
Sperm count (106/)	778.3 ±8.72	567.5 ±28.7***	789.1 ±8.80^^^	623.3 ±9.80 ***$$$	640.33 ±12.0 ***^$$$	682.0 ±8.02* **^^$$$###¥
Sperm Motility (%)	80.0 ±1.39	66.33 ±1.56***	79.83 ±1.19^^^	66.67 ±1.05 ***$$$	71.17 ±0.98 ***^$$$#	72.23 ±0.67 ***^^$$$##
Sperm Viability (%)	80.0 ±1.39	68.0 ±2.16***	80.83 ±0.75^^^	69.17 ±1.96 **$$$	71.17 ±0.98 ***$$$	71.33 ±0.67 ***$$$
Sperm LDR (%)	4.10 ±0.36	2.19 ±0.21**	4.18 ±0.37^^^	2.27 ±0.20 **$$	2.38 ±0.18 **$$	2.47 ±0.08**$$
Testo-sterone ng/dl)	2.55 ±0.24	1.32 ±0.00***	3.44 ±0.06^^^	1.21 ±0.06 ***$$$	1.42 ±0.08 **$$$	1.66 ±0.07 **^^^$$$ ###¥

[Table/Fig-2]: Effect of stress, vitamin C on the sperm parameters, testosterone level ©

Data presented as Mean + SE,
Note: *Group I Vs all groups: * p<0.05), **<0.005, **p<0.0005
^ Group II Vs Group III,IV,V,VI : ^p<0.05, ^^p<0.005, ^^^p<0.0005
$ Group III Vs group IV,V,VI : $p<0.05, $$p<0.005, $$$ p<0.0005
# Group IV Vs group V,VI : #p<0.05, ##p<0.005, ###p<0.0005
¥ Group V Vs group VI: ¥p<0.05, ¥¥ p<0.005, ¥¥¥p<0.0005

as an agent to play a crucial role in the differentiation process of the spermatogonial cells to sperm [18]. Consequent upon its use as an antioxidant to fight against ROS. The testicular germ cells might have been destroyed either due to membrane damage or macromolecular degradation incurred by ROS leading to a significant decline in sperm count, increased the incidence of abnormal sperms and ultimately testicular weight loss [9].

Effect on body weight [Table/Fig-1]: Swimming stress significantly decreased the whole body weight in group II, IV, V, VI as compared to control group. This effect is in accordance with Nayanatara et al., [10] reports. Normal rats feed with Vit C (20mg/kg/day) does not show any changes in body weight in comparison to control group. The different dose of the Vit C (10.20, 30mg/kg/day) does not show an improvement on stress induced changes in body weight.

The decreased weight could be due to decreased food intake in the rats under the influence of stress. Corticotrophin Releasing Hormone (CRH) is commonly released during the stress and might be a factor that suppressed food appetite in the repeated swimming [21,22].

Effect on organ weight [Table/Fig-1]: Testis: In comparison with the group I; group II (stress) has decreased the Rt testicular weight significantly (p<0.05). The observed weight loss may be due to reduced sex hormones [22,23]. In comparison with group II; the group V, VI very significantly (p<0.005) recovered testicular weight. Group III does not produce any changes on testicular weight. In comparison with Group IV: Group V showed significant (p<0.05) effect on recovery of testicular organ weight. On Lt testis there was no significant effect of stress and no statistically significant changes in comparison to any group.

Seminiferous tubles: In comparison with group I: Stress does not show any significant effect on semniferous tubules weight. Group II, III no significant effect in comparison with any other group. Compared with IV group: Group VI very significantly (p<0.005) improvement of seminiferous tubules weight.

Vas deferens: Stress, Vit C supplementation does not show any significant effect on vas deferens weight. In comparison with Group II: Group III significant (p<0.05) difference on vas deferens weight.

Prostate: In comparison with Group I: In group II, VI significantly (p<0.005) decreased prostate weight, and extreme significant (p<0.005) decreased effect on Group IV, V. In comparison with Group IV: Group VI very significantly (p<0.005) recovered the prostate weight. Comparison Group V with VI : Group VI significantly recovered weight.

Testicular index [Table/Fig-1]: Stress doesn't show any significant effect on testicular index. Supplementation of low does Vit C to stress group (Group IV) significantly improved testicular index. In comparison with low dose vitamin C supplementation, high dose of vitamin further significantly improved testicular index.

Copulatory index [Table/Fig-1]: Swimming stress significantly (p<0.05) effected on copulatory index in comparison with normal group. Supplementation of Vit C does not show any effect on copulatory index.

Sperm count [Table/Fig-2]: In comparison with the group I; stress produced an extreme significant reduction in sperm count in the all groups when compared with the corresponding control group of animals. In comparison with the stress group (group II), the Intermediate dose of Vit C significant improved & high dose of Vit C very significantly improved in sperm count [23].

On the other hand, the administration of Vit C low dose does not show any improvement, but intermediate dose showed significant effect and high dose showed very significant improvement in sperm count.

Sperm motility: Stress has produced an extreme significant decrease effect in group II, IV, V, VI on sperm motility when compared with group I [Table/Fig-2]. The intermediate dose of Vit C significant improved sperm motility, and high dose Vit C very significantly improved sperm motility in comparison with low dose of Vit C [23].

Sperm Life Death Ratio (LDR) [Table/Fig-2]: Stress very significantly increased sperm life ratio in group II, IV, V, VI, with comparison to group I. The supplementation of Vit C does not show any effect on the sperm life death ratio.

Hormone study: Stress has extremely significantly decreased testosterone levels in comparison with Group I. CRH also a negative regulator of LH action in the Leydig cells. These inhibitory effects subsequently attenuate testosterone production by the Leydig cells. Apart from the central dysfunction of the HPG axis alternately the most likely mechanism decreased testosterone biosynthesis are perhaps testicular micro trauma or temperature increase [3,21,23].

Supplementation of Vit C to normal rats (Group III) improved testosterone level, but not significantly improved. High doses of vitamin C increased the testosterone level extremely significantly in comparison with the stress group (group II). In comparison with a low dose of vitamin C supplementation, the high dose of Vit C extremely improved testosterone level. Comparison with intermediate dose of Vit C supplementation, the high dose of vitamin significantly improved testosterone level [Table/Fig-2]. This effect is linked to ability of vit C to raise the blood testosterone levels and not only to this antioxidant, has it affected the hypothalamus pituitary testicular axis, thus elevating blood testosterone levels [15].

LIMITATION OF THE STUDY

It is an animal study. The results needs to be confirmed in human subjects because species variation.

CONCLUSION

Various physiological changes produced by force swimming indicate that swimming is an effective model for producing stress in albino rats. The results suggest that Vitamin C supplementation improves the stress induced reproductive infertility possibly mediated through increase in testosterone and antioxidant effect.

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