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Effect of Vitamin C, as an Antioxidant, on Immobilization-Induced Changes in Sexual Behavior and Sperm Count in Male Mice

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Abstract

Sexual behavior in males is vulnerable to stress and it has been suggested that alterations in sexual behavior during stress is concomitant with spermatogenesis dysfunction. In this study, we investigated the effects of immobilization on sexual behavior and whether or not these effects are accompanied by changes in spermatogenesis process. The effect of antioxidant treatment on the sexual behavior and sperm count is also evaluated. 21 male mice were divided into the following three groups: control, immobilization stress (3 hours daily), and immobilization stress (3 hours daily) plus administration of 500 mg/kg of body weight vitamin C. Sexual behavior and sperm count were assessed after 60 days of stress and vitamin C treatment. Mount latency increased significantly, and the number of mounts showed significant decreased in males exposed to immobilization but not in vitamin C treated mice. Moreover, sperm count decreased significantly in the stressed group but mice administrated vitamin C did not show a significant reduction in number of sperms as compared with control unstressed animals. These results indicate that stress has great effects on sexual behavior and sperm count. Interestingly, administration of vitamin C resulted in a reversal in stress-induced inhibition in sexual behavior parameters, and sperm counts.

Key words: Immobilization, Vitamin C, Sexual behavior, Sperm count

Introduction

Stress, as a threatening factor of homeostasis, may impact all systems of body including reproductive system (Eriksen and Ursin, 2002; Khandve *et al*., 2013). In response to stress, a series of behavioral and neurochemical changes occur (Anisman, 1999); if the stress is extreme, the homeostatic mechanisms of the organism become deficit (Rather, *et al*., 2013).

Immobilization is most commonly used by researcher as stress inducer (Bharihoke *et al*., 2000). Chronic immobilization in male animals results in suppression of testosterone secretion (Collu *et al*., 1984), sexual motivation, testicular maturation and spermatogenesis (Almeida et al., 2000a, 2000b). Studies have suggested that chronic stress reduces Hypothalamus-Pituitary-Gonadal axis activity (Monder *et al*., 1994). On the other hand, other researches have shown that acute immobilization decreases serum levels of luteinizing hormone (LH) and testosterone in macaques (Norman and Smith, 1992). Previous studies have shown the acute electrical shocks in rats facilitates sexual behavior (Goldfoot and Baum, 1972), while acute immobilization impairs sexual behavior (Menendez-Patterson *et al*., 1978). The impacts of chronic stress on sexual behavior have been barely studied.

Some reports confirm the effects of chronic immobilization on the spermatogenesis process. This stress delays testicular maturation, and decreases sperm density in rats (Almeida *et al*., 2000a). Chronic immobilization also degenerate the germinal epithelium of seminiferous tubes of testes and changes the spermatogenesis process (Cockett *et al.,* 1970, 1971). Other studies have shown that there is a relationship between stress and semen quality (Ameen, 2009; Abdul

Rahman *et al*., 2014) and stress may suppress sexual or reproductive function (Woo *et al.,* 2011).

Since quality of masculine sexual behavior depends on testosterone level (Meisel and Sachs, 1994), the hormone secretion impairments caused by stress might affect male sexual behavior (Retana-Marquez *et al*., 2003). Many studies have suggested there is a negative correlation between stress level and ejaculate volume, sperm production, viability, motility and concentration of spermatozoa, morphologically normal spermatozoa, serum testosterone concentration, and fertilizing capacity of spermatozoa in animal models (Nirupama and Yajurvedi, 2013).

Previous studies have shown the chronic immobilization may induce oxidative stress by excessive production of reactive oxygen species/ROS (Hu *et al*. 2000; Juliet, 2004). There are many reports on oxidative stress induced reproductive damage. Since there is high amount of polyunsaturated fatty acids in testicular membranes and there is a correlation between ROS production and testicular steroidogenesis, testis is sensitive to ROS (Ghosh *et al*., 2002; Manna *et al*., 2003). Spermatozoa show more susceptibility to oxidative damage due to presence of great amount of polyunsaturated fatty acids in their composition, thus, generation of ROS in semen leads to loss of membrane integrity, DNA damage and apoptosis of spermatozoa (Nirupama and Yajurvedi, 2013). The studies have shown immobilization stress may decrease amount of antioxidants in the testis, which in turn leads to decrease in testicular spermatogenesis, viability, motility and fertility rate of spermatozoa (Nirupama and Yajurvedi, 2013).

Other studies have suggested the potential for supplementary antioxidants to decrease free radical-induced oxidative stress. For instance, the studies have shown vitamin E and vitamin C are powerful antioxidants that prevent the propagation of free radical reaction and inhibit lipid peroxidation and oxidative damage (Hsu *et al*., 2002; Muthuvel *et al*., 2006). Thus, supplementation of vitamin C can be necessary for normal metabolic functioning of the body (Gonzalaz *et al*., 2005), and it serves important enzymatic, antioxidant, and regulatory functions (Padayatty *et al*., 2003).

Therefore, the aim of this study was to determine the role of oxidative stress in mediating the stress-induced changes in sexual behavior and sperm count. The present study also analyzed whether the protective effect of vitamin C, as an antioxidants, can block the effects of stress on sexual behavior and sperm quality in males mice exposed to chronic immobilization stress.

Materials and Methods

Animals and experimental design

Twenty one adult male BALB/c mice (eight weeks old, weighing 25-30 g) were used in this study. All of the animals were allowed to acclimatize for 2 weeks prior to the experiment. The mice were housed in cages specially designed to minimize field perturbation. The mice had free access to Laboratory chow and tap water *ad libitum.* Animals were maintained under standard laboratory conditions on a 12 h light/dark cycle in a temperature-controlled room at 23± 3 ºC. All applicable institutional guidelines for the care and use of animals were followed.

Stress was attained by immobilization of the animals in restrainer, for 3 hours a day between 9:30 am and 12:30 pm, over a period of 60 days. Control animals were left undisturbed in their cages.

The mice were randomly divided into three groups; each group containing seven mice. The first group was immobilized and was given water, named I. The mice in the second group were immobilized and take daily oral administration of 500 mg/kg of body weight vitamin C, and named IC. Only 0.5 mL water was administered to mice in the third group, and they served as control. Vitamin C and water were given by gavage daily for eight weeks.

Sexual behavior assessment

For analysis of the sexual behavior, control and stressed males (7 per group) with no previous sexual experience were placed in a Plexiglas

cage. Males were tested for masculine sexual behavior under dim red lights 2 hours after the onset of the dark phase of the light/dark cycle (Pattij *et al*., 2005). Masculine sexual behavior was assessed by placing the male 5 min before a female was presented. After the presentation of the female, tests lasted 30 min. Upon presentation of the female, the following parameters were recorded: latency to the first mount and number of mounts.

Sample collection

After the Sexual behavior assessment, all the mice were anesthetized and sacrificed. Then, they were dissected and the cauda epididymis was removed and was punctured with a needle; a mass of sperm were squeezed out into a Petri dish containing 1 ml of phosphate buffered saline (PBS, pH 7.2). The sperm suspension was poured to tube and the tubes were immediately placed into the incubator at 37ºC. After 30 minutes, the 0.5 ml of sperm suspension was diluted with 9.5 ml PBS (Narayana *et al*., 2002). The dilution was mixed thoroughly and charged into Neubauer's chamber and covered with a cover slip and viewed under a light microscope. The sperm count was conducted in eight randomly picked boxes from the counting chamber. The total count was then multiplied by correction factor.

Statistical analysis

All the data are expressed using mean ± standard error of mean (S.E.M). Statistical differences between the groups were analyzed using one-way ANOVA followed by Tukey's Post Hoc test using the SPSS version 21.0. The differences were considered statistically significant at $p<0.05$.

Results

Sexual behavior test

As shown in Table 1 and Fig. 1A, compared to control animals, stressed mice (group I) exhibited a significant increase latency for the first mount ($p<0.05$). Group I also showed a decreased frequency of mounting significant at, $p<0.05$ (Fig. 1B, Table 1).

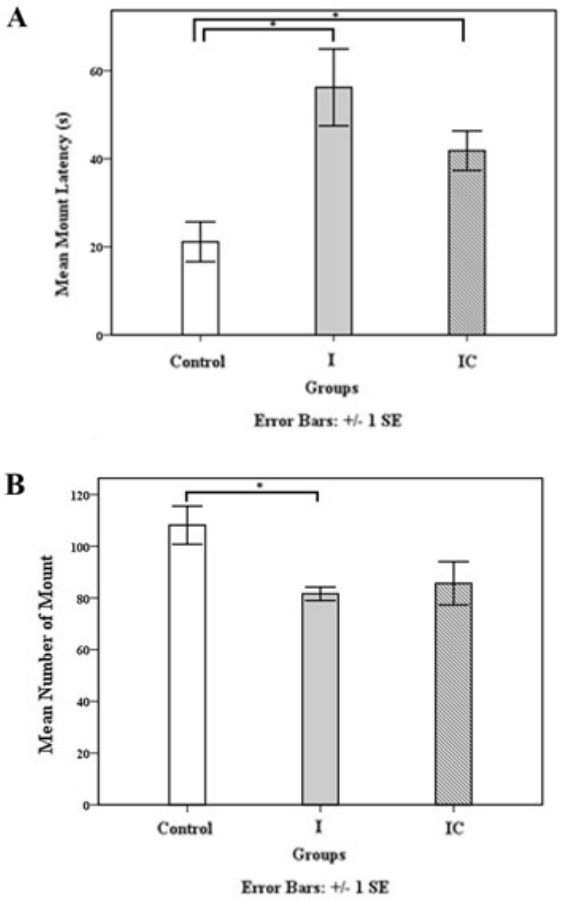

Fig. 1. Effect of immobilization and vitamin C on (A) mount latency and (B) number of mount: Mount latency increased and the number of mounts decreased significantly in males exposed to immobilization (I) but not in vitamin C treated mice (IC). Data are shown as Mean ± SEM. *p <0.05, in comparison to the control group.

Table 1. Number of mount and mount latency in control, immobilized (I) and immobilized and vitamin C supplemented (IC) mice (Mean ± SEM). *p <0.05, different from control group.

	Number of Mount	Mount Latency(s)
Group	Mean ± SE	Mean ± SE
Control	108.14±19.51	21.14±11.96
I	81.60± 5.81*	56.20±19.52*
IC	85.67±20.49	41.83±10.98*

Moreover, vitamin C decreased the mount latency in immobilized mice (group IC) compared to the mice only exposed immobilization stress (group I) (p>0.05). The mice supplemented with vitamin C also showed the increased number of mount compare to those exposed immobilization stress but not received vitamin C (I group) (p>0.05).
Results also indicated that group IC does not show significant increase in mount latency in comparison to control group. Indeed, the number of mounts did not decreased significantly in group IC compared to control group (p>0.05).

Sperm count

The effects of chronic immobilization and different antioxidants on total sperm count are presented in figure 2 and table 2. The results showed that the immobilization stress significantly decreased the sperm count in group I compared to the control group (p<0.05). As shown in table 2 the number of sperms in group IC have been increased compared to group I but not significantly (p>0.05). Results also showed that the number of sperms of group that received vitamin C (group IC) did not show significant reduction as compared with control group (p>0.05).

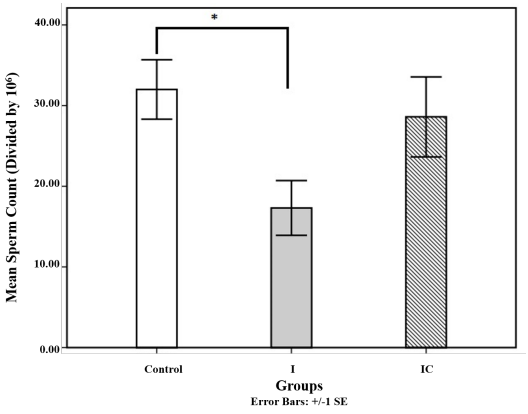

Fig. 2. Effect of immobilization and vitamin C on sperm count. Number of sperms decreased significantly in the immobilized mice (I) but group that received vitamin C or group IC did not show significant reduction in Sperm count as compared with control group. Data are shown as Mean ± SEM. *p <0.05, in comparison to the control group.

Table 2. Sperm count in control, I and IC mice (Mean ± SEM). *p <0.05 in comparison to the control group.

Sperm Count	
Group	**Mean ± SE**
Control	$32 \times 10^6 \pm 9.75 \times 10^6$
I	$17.31 \times 10^6 \pm 9.6 \times 10^6$ *
IC	$28.6 \times 10^6 \pm 11.09 \times 10^6$

Discussion

The results of this study showed that masculine sexual behavior in male mice may change by chronic immobilization stress. Similar results have been reported by other authors who exposed male rats to different stress conditions, and they observed that the time spent in sexual behaviors was reduced depending on the stress severity (Retana-Marquez *et al*., 2003).
In the present study, immobilization stress decreased the number of mounts and increased the latency of the first mounting. The decrease in the number of mounts induced by the stress suggests that motor copulatory events could consider as fragile parameters of male sexual behavior. This decrease in mount number indicates that stress can cause some sensorial dysfunction. The male sexual activity observed in this study are different from those reported by other authors, who have described that exposure of rats to immobilization causes a slightly increase in number of mount (Retana-Marquez *et al*., 2003). These differences can be attributed to the duration of exposure to immobilization in this study (60 days) and to the duration of the stress (3 hours per day). Those authors applied immobilization stress for shorter periods (20 days) and the duration of stress in each day was also shorter (2 hours). This implies that stress caused by our experimental conditions could be greater than in that study, causing a larger effect of the adrenal axis. Therefore, the intensity and duration of stress could be critical. On the other hand, the immobilized animals received vitamin C did not show the decreased mount frequency compared to control group (p>0.05), suggesting the improving effect of the antioxidant, vitamin C, on this sexual behavior parameter.

Compared to mice that exposure immobilization stress, stressed mice those received vitamin C exhibited a decreased latency for the first mount that might be suggestive of a higher sexual motivation (Melis and Argiolas 1995). These animals (group IC) presented also an increased number of mounting, indicative of enhanced sexual performance. Vitamin C serves as water-soluble chain-breaking antioxidants, and protects lipids, proteins, and membranes from oxidative damage. Vitamin C scavenges oxygen radicals in the aqueous phase (Moazzam *et al.*, 2012).

It has been proposed that stress play a role in semen quality changes and damage to reproductive organs (Al-Zahrani *et al.,* 2012). The production of ROS under stress causes disruption of the cell membrane integrity and cell damage (Abnosi *et al.,* 2015). Those are in accordance with our results which show that there is a significant reduction ($p<0.05$) in the sperm count of the animals that subjected to immobilization stress compared to the control group (Fig. 2). On the other hand, sperm count was found higher in mice given both supplementation vitamin C and immobilization stress as compared to those exposed only stress ($p>0.05$) (Fig. 2). In fact, this dose of vitamin C could not increase the number of sperm in our study significantly. Thus, it is supposed that a higher dose of vitamin C can improve the sperm parameters, effectively.

Since our results showed that sperm count in control group and IC group don't have significant difference, antioxidant consumption with stress can modify the harmful effects of immobilization on semen quality.

In the present study, a significant decrease in the mount latency coupled with a decrease in the number of mount, and also reduction in sperm count indicate that masculine behavior and number of sperms will change following chronic exposure to restraint. Our results confirm the role of vitamin C as an antioxidant on sexual behavior and sperm count because both of the number of sperms and mount frequency in stressed animals received vitamin C did not damage significantly in comparison with control group. In the other words, vitamin C may improve the destructive effects of immobilization stress.

Conclusion

The use of antioxidant supplementation is one of the means protecting the body from the harmful effects of stress. Intake of antioxidant vitamins can result in an increase in sexual activity and sperm count and in fertility rate of stressed persons.

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