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Research Article

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Effect of immobilization stress on organ indices, sperm quality, and testosterone level in rats: role of N-acetyl cysteine

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ABSTRACT

Reproductive function in male is suppressed by psychogenic or somatic stress. Epidemiological evidences emphasize that oxidative stress plays an important role in the etiology of male infertility. Antioxidants may act at different levels in the oxidative process by scavenging initiation of free radicals and protect against oxidative stress and prevent damage of cells. Group1: Normal Control animal received Distilled water, Group 2: Positive control (Only Stress), Group 3: Stress + Low dose NAC (50mg/kg/day), Group 4: Stress+ Intermediate dose NAC (100mg/kg/day), Group 5: High dose NAC (200mg/kg/day). All the drugs were given through orogastric tube before half an hour subjected to stress. Rats were exposed to Immobilization (IM) stress daily between 10.00am to 12.00pm for 15days. Effect of IM stress on organ indices, sperm quality and testosterone level were studied. IM stress significantly decreased the whole body weight, extreme significant reduction in sperm count, motility and viability in the stress group when compared with the corresponding control group of animals. The NAC very significantly improved the body weight, sperm count, motility and viability at low dose, Intermediate dose, however at high dose decreased bodyweight but it was insignificant. Our results suggest that NAC supplementations at low and intermediate doses have improved the stress induced reproductive changes. However the high dose of NAC has not shown any improvement in comparison with intermediate dose, rather high dose have shown deleterious effects on sperm quality.

Key words: Oxidative stress, Male fertility, N-Acetyl Cysteine, Immobilization stress.

INTRODUCTION

Recently oxidative stress has become the focus of interest as a potential cause of male infertility [1]. Epidemiological evidences emphasize that oxidative stress plays an important role in the etiology of male infertility [2]. Stress is an imbalance between production of reactive oxygen species and antioxidant defense [3]. Stress is defined as a general body response to initially threatening external or internal demands.[4, 5]. Normally, equilibrium exists between reactive oxygen species (ROS) production and antioxidant scavenging activities in the male reproductive organs [6]. Various forms of physical and psychological stress are believed to reduce sexual functions [7]. Reproductive function in male primates or animals is suppressed by psychogentic or somatic stress [8]. Antioxidants play an important role in sexual behavior in male rats [9].

The high levels of polyunsaturated fatty acids (PUFA) in the testicular membranes are susceptible to peroxidation injury [10]. In accordance, antioxidant enzyme activity has been shown to decrease in stress conditions, resulting in

increased lipid peroxidation (LPO) [11]. Spermatozoa produce small amounts of reactive oxygen species (ROS) under stress conditions. However excessive amount of ROS produced by immature spermatozoa can cause damage to the normal spermatozoa by inducing LPO. Drugs with multiple mechanisms of protective action, including antioxidant properties, may minimize tissue injury [12].

To counter oxidative stress, cells constitutively produce endogenous antioxidant enzymes that detoxify the ROS. These mainly consists of antioxidant enzymes (Superoxide dismutase, catalase) and small molecule antioxidant (vitamin E, Vit C, N-acetyl cysteine) [3,13,14]. Antioxidants may act at different levels in the oxidative process by scavenging initiation of free radicals, binding metal ions, scavenging peroxyl radicals and removing oxidatively damaged bio-chemicals [15].

Immobilization induced stress: Immobilization has been used extensively as a stressor for the study of stress-related biological, biochemical and physiological responses in animals. Immobilization as a model of stress, it produces an inescapable physical and mental stress. Brain regions of rats are very sensitive to acute immobilization stress. [16]

N-Acetyl Cysteine (also known as NAC, N-Acetyl Cysteine) is the amino acid L-Cysteine plus an acetyl (-CO-CH_3) group attached to the amino (NH_2) group. Amino acids which contain a sulfhydryl group (–SH) group have antioxidant properties. NAC is the most widely investigated drug with antioxidant properties that has been used in both experimental and clinical settings for many diseases. It has been also used a chelator of heavy metals to protect against oxidative stress and prevent damage of cells, but the evidence is not consistent for stress induced infertility [17,18,19].

EXPERIMENTAL SECTION

Animal: For the present study adult male albino rats weighing 250 – 280g and aged 10-12 weeks old were used. The animals were kept under standard conditions with a 12 - h light-dark cycle at 25 ± 1° C and animals were provided with ad libitum tap water 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 IEC and it followed the CPCSEA rules for animal protection [20]
Drug preparation: N-Acetyl Cysteine (NAC): Pure form of NAC was obtained from Vijaya trades scientific chemical distributor (Loda Make manufacturer). NAC was dissolved in double distilled water [21] with light heating and obtained a clear, colorless solution and administered orally for 15 days half an hour before subjected to stress by orogastric tube as per body weight.

Low dose: 50mg/ Kg body weight, Intermediate dose: 100mg/Kg body weight, High dose: 200mg/kg body weight, were selected taking into account the previous studies showing effects in dose range minimum dose 50mg/kg, maximum as 200mg/kg. [21, 22, 23, 24]

Experimental Design: Experimental animal based study.

Sample size: N=6 in each group

Sampling method: The rats were randomly divided into five groups of 6 rats each.

Grouping: Group1: Normal Control animal received Distilled water, **Group 2**: Positive control (Only Stress), **Group 3**: Stress + Low dose NAC (50mg/kg/day), **Group 4**: Stress+ Intermediate dose NAC (100mg/kg/day), **Group 5**: Stress+ High dose NAC (200mg/kg/day) for 15days per orally.

Stress Procedure: **Immobilization Stress:** [4,5, 16, 25, 26]

Protocol: Body weight of each rat was taken at 9:00am, oral administration of drugs at 9:30am and rats were exposed to immobilization stress daily at 10.00AM to 12.00PM for 15days [27]. Animal was kept immobilized in a semi cylindrical acrylic tube (4.5cm diameter and 12 cm long) with proper holes in it for air to pass.

The following parameters were studied:

Copulatory Index (CI) [28]**:** On 12^{th} day (to maintain three days abstain from sexual activity before sperm analysis) male rats were housed with 2 female rats for 24hrs on the end of 24hrs vaginal smear was taken for microscopic examination for spermatozoa. This indicated number of rats mated. The CI was calculated by dividing the number of female rats mated then multiplied by 100.

Body weight: At the beginning and end of the experimental period (Twenty four hours after the last treatment i.e., on 16^{th} day), the body weight of each individual rat was measured and the rats were sacrificed by cervical dislocation.

Reproductive organ indices
Reproductive organ weights: Reproductive organs; testes, prostate, seminiferous tubules and vas deferens were dissected out and were weighed with an electronic analytical and precision balance.

Testicular Index (Left) [29,30]: The testis index was calculated by dividing the left testis weight by the body weight and then multiplied by 100. **Right**: The right testicular index was calculated by dividing the right testis weight by the body weight and then multiplied by 100. **Total**: The total testicular index was calculated by dividing the average weight of right+left testis by the body weight and then multiplied by 100.

Collection and preparation of samples:
Blood samples: Blood samples were collected via cardiac puncture. Serum was obtained by centrifugation at 3000rpm for 20minutes. Serum was used for testosterone hormonal assay.

Semen samples: Cauda epididymis was dissected out and it was immersed in 1ml of Phosphate Buffer Solution (PBS) pH 7.2 and homogenized by using manual Homogenizer. The aliquot was used for semen analysis.

Sperm functions analysis:
Sperm count [31, 32]: The semen suspension was filtered through 80µ nylon mesh. According the standard method of Neubauer's chamber, the sperm counting was done. The sperm suspension (up to 0.5) was taken in WBC hemocytometer and diluted with PBS up to the mark 11 and the suspension was charged in to Neubauer's counting chamber. The total sperm count in 8 squares (except the central erythrocyte area) of 1 mm^2 each was determined and multiplied by $5x\ 10^4$ to express the number of spermatozoa/ epididymis.

Mass motility [33]: This was evaluated by an earlier method by Sonmez et al. [33]. A drop of aliquot semen solution was taken on the slide and percentage of motility was evaluated visually at a magnification of X400. The three different fields were examined for the motility in each sample. The final motility score was considered as mean value of three estimations

Live and dead sperms (Viability) [34]: A drop of the aliquot semen solution of each rat was mixed with an equal drop of 1% eosion stain prepared in accordance with Barth and Oko (1994). Thin films were made and examined under the microscope. Viable sperm remains colorless. The viability score (percent) was determined by examine one hundred sperm cells per rat.

Biochemical analysis:
Hormonal assay [17, 35]: Serum samples were used for testosterone was quantification by Testosterone CLIA kit (Chemilumience assay method kit details: Biomerieus India Pvt. Ltd, expiry date: 2015-10)

Statistical Analysis The data were expressed as Mean ± SD. The statistical analysis done by student's t test with the level of significance set at $P<0.05$.

RESULTS

Table.1: Effect of IM stress, NAC on the body weight, reproductive organ weight, testicular index and copulatory index

Parameter	Group I Control	Group II IM stress	Group III Stress+ NAC 50mg/ Kg	Group IV Stress+ NAC 100mg/ Kg	Group V Stress+ NAC 200mg/ Kg
Copulatory index (%)	91.67±20.4	83.3±25.8	75.0±27.4	91.7±20.4	66.7±25.8
Body weight difference between 1st & 15th day (g)	5.33±5.61	-8.00±1.41***	-4.67±1.37$^{**\$\$}$	-1.17±3.43$^{*\$\$\#}$	-4.17±1.47$^{**,\$\$\$}$
Testis L (g)	1.069±0.06	1.010±0.02	1.024±0.02	1.022±0.01	1.014±0.01
Seminiferous tubules (g)	0.727±0.15	0.612±0.01	0.617±0.02	0.625±0.02	0.618±0.02
Vas deferens (g)	0.109±0.005	0.099±0.004**	0.101±0.003**	0.103±0.003*	0.101±0.002**
Prostate (g)	0.125±0.01	0.109±0.004**	0.112±0.01*	0.112±0.01*	0.110±0.01*
Testicular index (L) (%)	0.387±0.02	0.397±0.01	0.397±0.002	0.405±0.006$^{\#}$	0.406±0.003$^{*,\#\#\#}$
Testicular index (R) (%)	0.349±0.13	0.358±0.006	0.359±0.13	0.362±0.10	0.372±0.18
Testicular index (T) (%)	0.370±0.15	0.378±0.005	0.376±0.001	0.384±0.007	0.383±0.007$^{\#}$

Testicular index (L=Left, R= Right, T= Total)

$^{*,\$,\#,¥}$ Significant (p<0.05) $^{**,\$\$,\#\#,¥¥}$ Very significant (p<0.001) $^{***,\$\$\$,\#\#\#,¥¥¥}$extremely significant (p<0.0001)

**comparison with Group I, $^{\$}$ comparison with Group II, $^{\#}$Comparison with Group III, $^{¥}$Comparison with Group IV*

Table 1 Shows that in comparison to control group the Immobilization stress significant decreased bodyweight. In comparison with Group II, Low dose 50mg/kg/day, intermediate dose 100mg/kg/day, high dose 200mg/kg/day of NAC significantly improved bodyweight. In comparison between low dose (group III) and intermediate dose (group IV) treated with NAC, the intermediate dose of NAC further significantly improved bodyweight. In comparison with intermediate dose (group IV) and high dose (group V) treated with NAC no significant improvement in body weight, rather than improvement it was decreased body weight. However; it was statistically insignificant.

In comparison to control group the Immobilization stress significant decreased Vas deferens and prostate weight. Supplementation of various doses of NAC doesn't improve the vasdeferens, and prostate weight.

In comparison with control group, Immobilization stress does not show any effect on testicular index (left, Right and total). Supplementation of low dose NAC do not show any effect. Intermediate dose (group IV) of NAC in comparison with low dose (group III) treated with NAC shown significant changes in left testicular index only. High dose of NAC in comparison with control group it was significant change in left testicular index, in comparison with low dose the left, total testicular index was significant changed, but in comparison with intermediate dose there was no significant change in testicular index.

Immobilization stress does not show any effect on copulatory index, testicular and seminiferous tubules weight. Supplementation of NAC doesn't show any effect on copulatory index, testicular weight and seminiferous tubules weight.

Table.2: Effect of stress, NAC on the sperm parameters, Testosterone level

Parameter	Group I Control	Group II IM stress	Group III Stress+ NAC 50mg/ Kg	Group IV Stress+ NAC 100mg/ Kg	Group V Stress+ NAC 200mg/ Kg
Sperm count ($10^{6/cumm}$)	778.3±21.3	602.7±10.6***	635.7±10.0$^{***\$\$\$}$	686.2±20.9$^{***\$\$\$\#\#\#}$	622±10.4$^{***\$\$\#¥¥¥}$
Sperm Motility (%)	80.33±3.14	68.2±1.83***	68.2±1.17***	69.0±1.1***	66±2.28$^{***¥}$
Sperm Viability (%)	80.00±3.41	69.3±2.16***	68.3±0.82***	69.2±1.17***	65.2±1.83$^{***\$\$\#\#¥¥}$
Sperm LDR (%)	4.10±0.89	2.27±0.24***	2.16±0.08***	2.24±0.12***	1.88±0.15$^{***\$\$\#\#¥¥¥}$
Testosterone (ng/ml)	2.55±0.48	0.99±0.04***	1.20±0.10$^{***\$\$\$}$	1.39±0.16$^{***\$\$\$\#}$	1.10±0.11$^{***¥¥}$

$^{*,\$,\#,¥}$ Significant (p<0.05) $^{**,\$\$,\#\#,¥¥}$ Very significant (p<0.001) $^{***,\$\$\$,\#\#\#,¥¥¥}$extremely significant (p<0.0001)

**comparison with Group I, $^{\$}$ comparison with Group II, $^{\#}$Comparison with Group III, $^{¥}$Comparison with Group IV*

Table 2 shows that in comparison to control group the immobilization stress extreme significantly decreased sperm count, motility, viability, LDR and testosterone. In comparison with group II, treatment with NAC (50mg/kg/day) extreme significantly increased the sperm count and testosterone levels, but in comparison with control group still showing significant decreased sperm count, motility, viability, LDR, Testosterone levels. In comparison with Group IV (NAC 100mg/ Kg) with Group III (NAC 50mg/ Kg), sperm count was extreme significant improved and testosterone was significant improved, but no effect on sperm motility, viability, LDR. Group IV with Group II significantly improved sperm count and testosterone but in comparison with control group still showing significant

decreased sperm count, motility, viability, LDR, Testosterone levels. In comparison with group V (NAC 200mg/ Kg) with IV extreme significantly decreased sperm count, LDR, very significantly decreased Viability, testosterone levels and significantly decreased sperm count, in comparison with Group III the high dose of NAC show significantly decreased sperm count, viability, LDR. Group II the high dose of NAC show significantly improved sperm count, but significantly decreased viability, LDR, in comparison with control group the sperm count, motility, viability, LDR and testosterone levels were extreme significantly decreased.

DISCUSSION

Stress, whether it is acute or chronic, may affect sexual function [7]. Previous studies concerning the stress and sexual behavior in male subjects have reported that stress significantly modifies sexual behavior. [7]. Oxidative stress due to free radicals precipitates a range of pathological changes that affect the reproductive functions in men.[36]

NAC reacts with highly oxidizing radicals such as $^{\bullet}OH$, $^{\bullet}NO_2$, $CO_3^{\bullet-}$, and also bind redox active metal ions. Thiosl can also afford radio protection through the donation of reducing equalents i.e.., the carbon centered radicals formed on DNA backbone or protein by $^{\bullet}OH$ attack can be restituted via hydrogen donation from RSH, and NAC plays an important role in the production of glutathione, which provides intracellular defense against oxidative stress. [14, 17]

Immobilization significantly decreased the whole body weight as compared to control group. This effect is accordance with other reports in chronic exercise [37]. Corticotrophin Releasing Hormone (CRH) is commonly released during the stress and might be a factor that suppressed food appetite in the stress; this may influence the body weight [31]. The NAC very significantly improved the body weight at low dose, Intermediate dose, however at high dose decreased bodyweight but it was insignificant. In comparison to low and intermediate dose of NAC showed very significant improvement in body weight.

In present study there is no effect on testicular weight. This is according to the Hiroshi et al **[8] &** Almedia et al.,[28] IM stress does not show any effect the testicular weight and seminiferous tubules**;** however it showed very significant effect on prostate**,** Vas deferens**.** The observed weight loss of the accessory sex organs may be due reduced bioavailability of sex hormones. Our results are in agreement with Nashwa etal[17]. NAC does not show any significant improvement in testicular, seminiferous tubules, prostate, vas deference weight.

Epididymal sperm count, motility and viability provide a direct measure of fertility in animals. (Nashwa) IM stress showed extreme significant reduction in sperm count, motility and viability in the stress group when compared with the corresponding control group [37]. The higher membrane lipids content of testes, sperm plasma membrane are presumed to make them vulnerable to oxidative stress [7,31]. The testicular germ cells might have been destroyed due to membrane damage by ROS leading to a significant decrease in sperm count increased the incidence of abnormal sperms [20, 31, 38]. Increased ROS levels have been correlated with decreased sperm motility, but underlying mechanism is not understood [39]. NAC at low and intermediate dose showed significant improvement on sperm count [18] but no affect on motility and sperm viability; however at high dose it shown significant decreased the sperm count, motility, viability and sperm LDR.

Testosterone is the main steroid sex-hormone, it modulates the sexual behavior in response to sexual stimulates in male albino rats. [7] For normal spermatogenesis, development, maintenance of sperm morphology critically required high level of testosterone in testis [40]. The significant decrease of testosterone level may be a result of direct damage of Leydig cells [8]; apart from the central dysfunction of the HPG axis alternately the most likely mechanism decreased testosterone biosynthesis. The ROS produced by the spermatogenesis, if unchecked by intracellular antioxidants, can also damage mitochondrial membranes and contribute to the inhibition of subsequent steroid production [35]. In the present study IM stress significantly decreased the testosterone levels. The similar hormonal changes observed in Hiroshi etal [8]. NAC significantly at low and intermediate dose extreme significantly improved but at high dose significantly decreased the testosterone.

Immobilization stress does not shown any effect on copulatory index. Supplementation of NAC does not show any effect on copulatory index.

CONCLUSION

Decreased testosterone level and sperm quality have shown that immobilized stress is an effective model for producing stress in albino rats. Our results suggest that NAC supplementations at low and intermediate doses were improved the stress induced reproductive changes, however the high dose has not shown any improvement in comparison with intermediate dose, rather high dose have shown deleterious effects on sperm quality.

Limitation of study: It is an animal study. The result needs to be confirmed in human subjects because of species variation.

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