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Durational effects of chronic stress on the testicular damage and its reversibility in albino rat

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ABSTRACT

The study was to investigated whether stress induced testicular damage and its reversibility are duration of exposure dependent or not. Adult male rats were subjected to restraint for 1 hour and after a gap of 4 hours to forced swimming exercise for 15 minutes daily for 1, 4 and 6 months. There was a significant duration dependent decrease in testicular weight, number of germ cells in stage VII of spermatogenesis, concentrations of ascorbic acid and tocopherol; increase in counts of abnormal spermatozoa and degeneration of seminiferous tubules. In addition, in all the durations, a significant uniform decrease in the activities of testicular 3β-hydroxysteroid dehydrogenase and antioxidant enzymes, the serum testosterone levels and total sperm count and a significant increase in malondialdehyde levels were found. The rats exposed to stress for 1 month and allowed to recover for 4 months, showed restoration in the relative weight of testes, abnormal sperm counts, counts of A spermatogonia and spermatids and activities of superoxide dismutase, glutathione-s-transferase and the concentrations of malondialdehyde and tocopherol levels whereas other parameters showed significant increase over stressed rats, although they were lower than controls. All these parameters of rats exposed to stress for 4 or 6 months and allowed to recover for 4 months did not differ from respective stress groups. The results indicate that increase in duration of stress exposure leads to progressive deterioration in structure and functions of the testis and changes are irreversible following 4 months exposure in rats.

Key words: chronic stress, antioxidant enzymes, spermatogenesis, steroidogenesis, testis

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INTRODUCTION

Stress is a state of threatened homeostasis provoked by physical, psychological or environmental stressors [1]. It seems to be a potential risk factor for reproductive function. Reduced male fertility is one of the known consequences of psychological stress [2]. In males, physical and psychological stressors may inhibit reproductive function mainly through the suppression of hypothalamus-pituitary-gonadal (HPG) axis and activation of hypothalamus-pituitary-adrenal (HPA) axis [3]. Many studies have shown decrease in sperm production, sperm count and motility of spermatozoa, increase in percentage of morphologically abnormal spermatozoa, impaired spermatogenesis, decrease in levels of serum testosterone and LH levels, reduction in fertilizing capacity of spermatozoa, maturation arrest of germ cells, stage specific germ cell apoptosis and increase in cytoplasmic vacuolization in the Leydig cells due to stress in animal models [4-15]. Likewise stress is also known to affect human spermatogenesis. For instance, individuals exposed to psychological or occupational stressors showed a

negative correlation between stress level and ejaculate volume, decreased sperm production, reduction in viability, motility and concentration of spermatozoa, sperm abnormality, testicular shrinkage, suppression of spermatogenesis, increased sperm DNA damage and reduction in serum testosterone concentration [2, 16-22]. The oxidative damage, which is a central feature of many pathological conditions [23, 24], is known to be caused by stress like other factors in various organs. Spermatozoa are more susceptible to oxidative damage due to their unique structural composition i.e presence of polyunsaturated fatty acids [25]. Excessive generation of reactive oxygen species (ROS) in semen leads to loss of membrane integrity, DNA damage and apoptosis of spermatozoa [26, 27]. There are many reports on oxidative stress induced reproductive damage. For instance, a significant elevation in the free radical levels concomitant with a significant decrease in the activities of testicular antioxidant enzymes were observed following forced swimming exercise in rats [28]. Likewise, a long term exposure to psychological stress in men may enhanced the ROS [29] generation leading to decrease in viability, motility and fertility rate of spermatozoa [30]. In addition, semen antioxidant enzyme activities are significantly reduced due to psychological stress in human [21]. Our earlier study also showed decrease in testicular spermatogesis and steroidogenic activity coupled with decreased antioxidant status of the testis in rats following daily exposure to restraint and forced swimming for two months. Interestingly, in this study most of the stress effects were not reversible. Although effects of stress on testicular activity are well established, it is not known whether or not severity of damage and reversibility of stress effects are dependent on exposure period. Hence, the present study tests the hypothesis that severity of testicular damage as well as reversibility of the effects depend on the duration of stress exposure in rat.

MATERIALS AND METHODS

Animals:

Adult male Wistar rats weighing 180-200 g were obtained from the central animal facility, University of Mysore, Mysore, India, and housed two or three per cage. The rats were provided standard rat chow and water ad libitum and were kept in 27 ± 2^0 C, under 12 h : 12 h light: dark cycle (lights on 07:00-19:00 h). The experimental protocols were approved by the institutional animal ethics committee and the maintenance and treatment of animals were in accordance with the ethical guidelines of the committee.

Stressors:

Two kinds of stressors were used [31], r**estraint,** wherein rats were placed in an open-ended cylindrical restrainer (6.7 cm in diameter and 22.2 cm in length) for 1 h per episode followed by **forced swimming**, wherein rats were individually forced to swim for 15 minutes per episode in a glass jar (18 inches height×8.75 inches outer diameter) filled two thirds full of water at a temperature of $27\pm2^{\circ}$ C.

Experimental design:

Rats were randomly divided into control (n=20) and stress (n=30) groups and body weight (initial body weight) was recorded while commencing the experiment. The controls were kept without any disturbance whereas rats in stress group were restrained for 1 h and after a gap of 4 h forced to swim for 15 minutes every day for different durations viz. 1, 4 and 6 months. The sequence of stressors and timing of the exposure were randomly changed every day to minimize habituation. After each treatment period, five rats from stress as well as control group were killed and five rats in stress group were maintained without any treatment (recovery group) for 4 months and then killed. At autopsy, blood sample was drawn by heart puncture and the testes and adrenal glands were removed and weighed. The right testis of each rat was stored in −20ºC until biochemical analyses were conducted and the left testis was processed for histological studies. The epididymis was used for determining total sperm count, motility of spermatozoa and abnormal sperm counts.

Sperm parameters:

Motility of sperm:

The cauda epididymis of one side of each rat was minced in 1 ml phosphate buffer saline (PBS) (pH 7.2) to obtain suspension. The suspension was filtered through muslin cloth, an aliquot of this solution was placed on slide and numbers of motile and immotile spermatozoa were counted out of 200 spermatozoa in each of three different fields in each sample using the light microscope under 400X. The mean of the three estimations / sample was used to compute percentage of motility [32].

Total sperm count:
The filtered sperm suspension described above was mixed with a drop of 1 % aqueous eosin and kept for 30 min for the staining of the spermatozoa. An aliquot of stained filtrate was taken in a WBC pipette up to the 0.5 mark and diluted further up to the mark 11 with PBS, and mixed well and charged into Neubauer's counting chamber. The spermatozoa present in eight outer squares of 1 mm^2 each area except the central erythrocyte counting area was counted. The aggregate of counts of eight squares was multiplied by 5×10^4 factor to obtain the total sperm count/ epididymis [33].

Abnormal sperm count:
A drop of stained spermatozoa preparation was put on a clean glass slide and a uniform smear was obtained. One thousand spermatozoa per epididymis were observed under higher magnification (400X) in randomly selected areas of smear and number of spermatozoa showing head shape and tail abnormalities viz. amorphous head, pin head, bent mid-piece, curved tail, hook less head, double head was counted. The sum of counts of different abnormalities was expressed as total abnormal sperm count/1000 spermatozoa/epididymis [33].

Spermatogenesis:
The left testis was fixed in Bouin's fixative for 18 hours and dehydrated with 70 % ethanol, embedded in paraffin wax, and 5 μm sections were cut, mounted on slides, and were stained with hematoxylin and eosin. The number of each category of germ cells in stage VII of seminiferous epithelium cycle, i.e., type A spermatogonia, preleptotene spermatocytes, midpachytene spermatocytes, round spermatids and elongated spermatids was counted in ten round tubular cross sections of each rat testis. All the counts of the germ cells were converted to true counts by the formula, true counts = (crude count × section thickness) / (section thickness + nuclear diameter of germ cells) [34, 35].

Biochemical analyses
Activities of antioxidant enzymes i.e. superoxide dismutase (SOD) [36], glutathione peroxidase (GPx) [37], catalase (CAT) [38], glutathione reductase (GR) [39] and glutathione S transferase (GST) [40] were determined in the right testis of each rat. The testicular concentrations of non enzymatic antioxidants i.e. ascorbic acid [41] and tocopherol [42] and a product of lipid peroxidation, malondialdehyde (MDA) [43] were determined. Further, activity of the key steroidogenic enzyme, 3β-hydroxysteroid dehydrogenase (3β HSDH) [44] was determined in the adrenal gland and the testis.

Serum testosterone concentration:
Serum testosterone concentration was estimated by conducting enzyme linked immuno assay using the kit, manufactured by DRG, Germany following procedure of the manufacturer and expressed as ng/ml.

Statistical analysis
The one way analysis of variance (ANOVA) followed by Duncan's multiple range test were used to determine the significant difference among mean values of each parameter of different groups, fixing the minimum level of significance at $P \leq 0.05$.

RESULTS

Body and organ weights:
There was duration of exposure dependent significant reduction in the relative weight of the testes and percent gain in body weight in 1, 4 and 6 months stressed rats compared to control rats. The body weight of recovery group of rats after 1 month stress exposure did not differ from controls whereas that of recovery group rats after 4 or 6 months stress exposure was significantly lower than controls **(**Table 1). The relative weight of the testes in recovery group rats after 1 month exposure did not differ from controls whereas that of recovery group rats after 4 or 6 months stress exposure was significantly lower than controls and did not differ from respective stress group **(**Table 1). Relative weight of the adrenal gland in 1, 4 and 6 months stressed rats was significantly higher than respective controls. The adrenal weight of recovery group rats after 1, 4 or 6 months stress exposure did not significantly differ from controls (Table 1).

Table 1: Effects of chronic stress exposure for different periods on percent gain in body weight, relative weight of the adrenal gland and the testis of rat

Groups	% gain in body weight(g)	Relative weight of the adrenal gland(g)	Relative weight of the testes (g)
1month control	8.19±0.79^{d}	0.0186±0.0016^{ab}	1.266±0.043^{a}
1month stress exposure	-0.8±0.20^{ef}	0.0254±0.0027^{cde}	1.105±0.033^{b}
Recovery after 1month exposure	8.87±0.75^{d}	0.0164±0.0018^{a}	1.163±0.015^{ab}
4 months control	38.11±3.59^{a}	0.018±0.001^{ab}	1.258±0.043^{a}
4 months stress exposure	-2.14±1.47^{f}	0.0296±0.003^{e}	0.884±0.0204^{bc}
Recovery after 4months exposure	28.65±0.91^{bc}	0.0196±0.003^{abc}	0.940±0.033^{bc}
6months control	31.16±2.06^{ab}	0.0171±0.001^{ab}	1.137±0.016^{ab}
6months stress exposure	6.19±5.05^{de}	0.0282±0.002^{de}	0.661±0.028^{c}
Recovery after 6 months exposure	22.48±5.6^{c}	0.0232±0.014^{bcd}	0.913±0.046^{bc}
10 months control	30.23±2.46^{ab}	0.0178±0.002^{ab}	1.184±0.046^{ab}
ANOVA **F-value** **df = 40, 9**	 **25.692** **P≤0.05**	 **5.999** **P≤0.05**	 **3.456** **P≤0.05**

All the values are mean ± SEM,

Mean values with same superscript letters in the given column are not significantly different, whereas those with different superscript letters are significantly (P≤0.05) different as judged by Duncan's multiple test.

Steroidogenic activity in the adrenal gland and testis:

There was a significant increase in the adrenal 3β HSDH activity in 1 and 4 months stress group rats and it was further significantly elevated after 6 months stress exposure compared to controls (Table 2). The adrenal 3β HSDH activity of recovery group rats after 1, 4 or 6 months stress exposure did not significantly differ from controls. There was a significant decrease in the testicular 3β HSDH activity in 1, 4 and 6 months stressed rats compared to respective controls. The testicular 3β HSDH activity of recovery group rats after one month exposure was significantly higher than 1 month stressed rats but lower than controls and that of recovery group rats after 4 and 6 months exposure was significantly lower than controls whereas it did not significantly differ from respective stress group rats (Table 2).

Serum testosterone concentration:

There was a significant decrease in serum testosterone concentration in 1, 4 and 6 months stressed rats compared to respective controls. Though recovery group of rats after 1 month stress exposure showed significant increase in serum testosterone levels compared to stress group, it was lower than controls whereas that of recovery group rats after 4 or 6 months stress exposure did not differ from respective stress groups (Table 2).

Table 2: Effects of chronic stress exposure for different periods on the adrenal and testicular 3β HSDH activities and serum testosterone levels in rat

Groups	3β HSDH activity (nmol/mg//min)		Serum Testosterone concentration (ng/ml)
	Adrenal gland	testis	
1month control	0.14±0.02^{a}	0.61±0.05^{a}	1.60±0.11^{a}
1month stress exposure	0.27±0.02^{c}	0.26±0.02^{cd}	0.63±0.09^{de}
Recovery after 1 month exposure	0.13±0.02^{a}	0.51±0.08^{b}	1.21±0.01^{b}
4 months control	0.15±0.03^{a}	0.64±0.02^{a}	1.63±0.13^{a}
4 months stress exposure	0.25±0.02^{bc}	0.26±0.02^{cd}	0.47±0.12^{e}
Recovery after 4 months exposure	0.19±0.02^{a}	0.31±0.03^{c}	0.87±0.13^{cd}
6 months control	0.18±0.01^{a}	0.62±0.02^{a}	1.61±0.10^{a}
6 months stress exposure	0.34±0.03^{d}	0.22±0.03^{cd}	0.53±0.09^{e}
Recovery after 6 months exposure	0.21±0.01^{ab}	0.19±0.02^{d}	0.43±0.12^{e}
10 months control	0.18±0.01^{a}	0.55±0.03^{ab}	1.23±0.07^{b}
ANOVA **F-value** **df = 40, 9**	 **12.348** **P≤0.05**	 **28.84** **P≤0.05**	 **21.043** **P≤0.05**

All the values are mean ± SEM

Mean values with same superscript letters in the given column are not significantly different, whereas those with different superscript letters are significantly (P≤0.05) different as judged by Duncan's multiple test.

Sperm parameters:

There was a significant decrease in the total sperm count and percentage of motile spermatozoa and a significant increase in immotile spermatozoa in 1, 4 and 6 months stressed rats compared to respective controls. The total sperm

count and percentage of motile spermatozoa in recovery group rats after 1 month stress exposure showed a significant increase over stressed rats but were significantly lower than controls whereas those of recovery group rats after 4 months exposure did not significantly differ from 4 months stressed rats and those of recovery group rats after 6 months exposure showed further significant decrease compared to 6 months stressed rats (Table 3).

The counts of abnormal spermatozoa showed significant duration of exposure dependent increase after 1, 4 and 6 months exposure. The abnormal sperm counts of recovery group rats of 1 month exposure did not significantly differ from controls whereas that of recovery group rats after 4 months exposure though significantly decreased than stress group, it was higher than controls and that of recovery group rats after 6 months exposure did not differ from stressed rats (Table 3).

Table 3. Effects of chronic stress exposure for different periods on total and abnormal counts and motility of epididymal spermatozoa in rat

Groups	Total Sperm count/epididymis (millions)	% of motile of spermatozoa	% of immotile spermatozoa	Number of abnormal spermatozoa / 1000 spermatozoa
1month control	157.56±4.8^{a}	83.40±1.47^{a}	16.6±1.49^{a}	75.25±8.06^{a}
1month stress exposure	69.96±3.74^{de}	62.60±1.36^{d}	37.4±1.36^{d}	120.00±4.49^{b}
Recovery after 1 month exposure	101.78±5.16^{c}	72.80±2.35^{bc}	27.3±2.35^{bc}	84.00±7.11^{a}
4 months control	161.34±14.18^{a}	82.60±0.93^{a}	17.4±0.93^{a}	83.75±6.82^{a}
4 months stress exposure	72.70±2.39^{de}	64.60±2.50^{cd}	35.4±2.5^{cd}	235.00±22.94^{d}
Recovery after 4 months exposure	87.64±3.26^{cd}	57.20±6.24^{d}	42.8±6.24^{d}	155.50±5.84^{c}
6 months control	159.40±6.79^{a}	78.40±2.89^{ab}	21.6±2.89^{ab}	94.75±3.07^{ab}
6 months stress exposure	67.12±4.46d^{e}	61.00±4.59^{d}	39.0±4.59^{d}	254.5±19.64^{d}
Recovery after 6 months exposure	48.05±3.07^{f}	56.40±2.48^{d}	43.6±2.48^{d}	222.50±7.12^{d}
10 months control	148.75±3.69^{b}	77.40±2.35^{ab}	22.6±2.36^{ab}	97.50±2.96^{ab}
ANOVA **F-value** **df = 40, 9**	**51.017** **P≤0.05**	**13.297** **P≤0.05**	**13.297** **P≤0.05**	**40.82** **P≤0.05**

All the values are mean ±SEM

Mean values with same superscript letters in the given column are not significantly different, whereas those with different superscript letters are significantly (P≤0.05) different as judged by Duncan's multiple test.

Histomorphology of the testis:

The testis of control rats revealed normal structure and seminiferous tubules were replete with germ cells at different stage of spermatogenesis and abundant spermatozoa (Figure 1a, b & c). The seminiferous tubules of 2 months stressed rats were shrunken and contained fewer spermatozoa compared to controls and there was some vacuolization in the seminiferous epithelium (Figure 1d). The severity of damage increased with increase in duration of exposure i.e 4 and 6 months exposure period (Figure 1e and f). The seminiferous tubules of recovery group rats after 1 month stress exposure resembled controls (Figure 1g) whereas those of the recovery group rats of 4 and 6 months stress exposure did not show improvement compared to the testis of respective stress group rats and many degenerated germ cells were present (Figure 1h and i).

Counts of germ cells in stage VII of spermatogenesis:

There was a significant duration of exposure dependent decrease in the counts of A spermatogonia, preleptotene, midpachytene and elongated spermatids following 1, 4 and 6 months stress exposure compared to controls (Table 4). Round spermatids in 1 month stressed rats did not differ from controls whereas they were significantly lower than controls in 4 and 6 months stressed rats (Table 4). Recovery group rats after 1 month stress exposure contained A spermatogonia, round spermatids and elongated spermatids similar to controls whereas number of preleptotene and mid pachytene spermatocytes did not differ from stressed rats. The counts of all categories of germ cells in recovery groups of 4 and 6 months exposure did not differ from respective stress group (Table 4).

Activities of antioxidant enzymes and MDA levels:

The activities of testicular CAT, SOD, GPx, GST and GR were significantly decreased following exposure to 1, 4 and 6 months stress compared to controls (Table 5). In the recovery group of rats after 1 month stress exposure SOD and GST activities did not differ from controls and were significantly higher than stress group. CAT, GPx and GR

activities were significantly higher than the respective stress group but significantly lower than controls. Activities of these enzymes in recovery group of 4 or 6 months stress exposed rats did not differ from the respective stress group rats and were significantly lower than controls (Table 5).

The concentration of MDA in the testis was significantly increased following 1, 4 or 6 months stress exposure compared to respective controls whereas that of recovery group rats of 1 month stress exposure did not differ from controls but in recovery group rats of 4 and 6 months stress exposure it was significantly higher than control group and did not significantly differ from respective stress groups (Table 6).

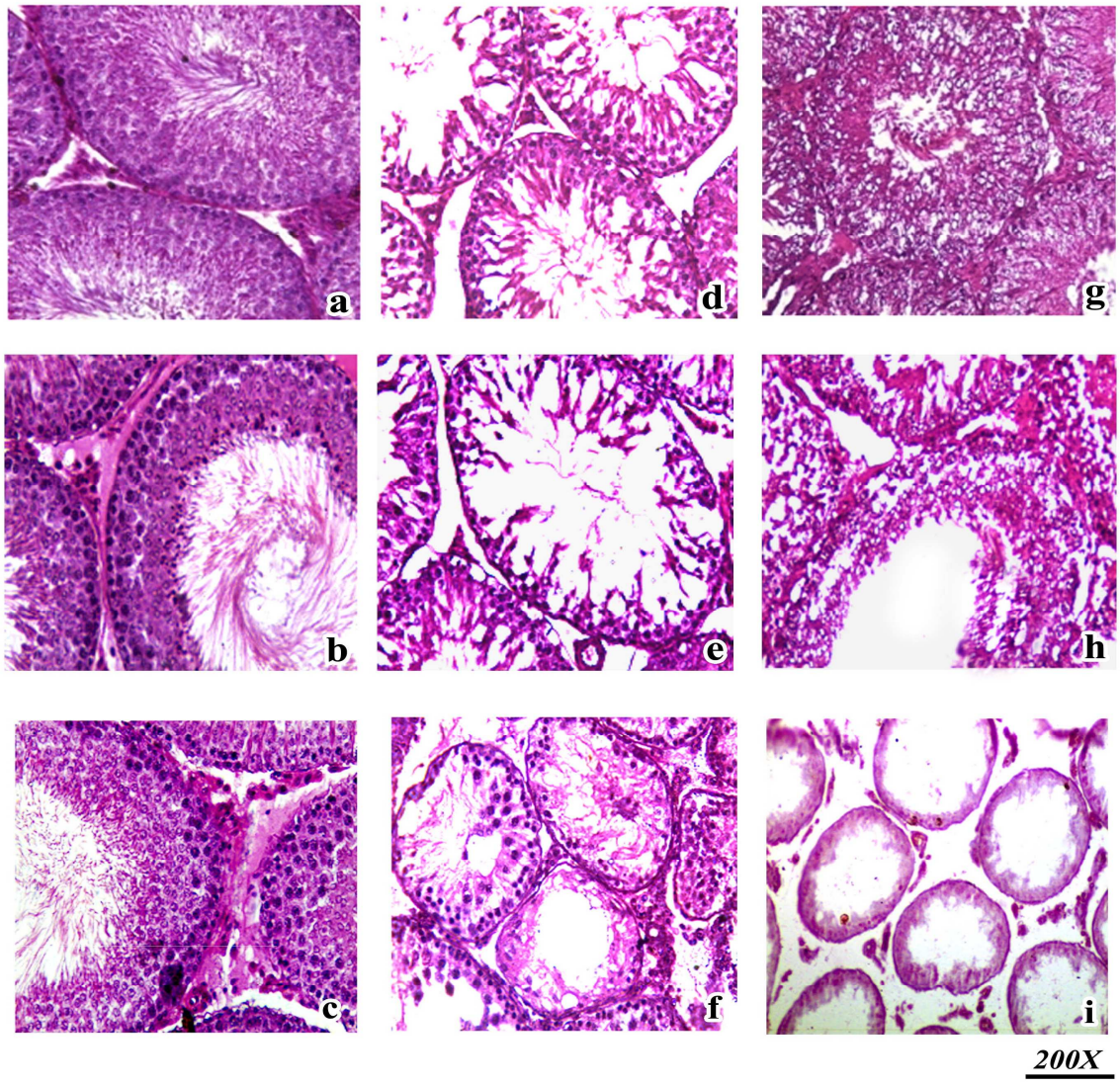

Figure 1: Photomicrographs of cross sections of the testis of controls after 1(a), 4(b) and 6(c) months duration, stressed rats (d, e, f) and recovery group rats (g, h, i)

Note the presence of seminiferous tubules repletes with germcells in controls (a,b & c) in contrast to shrinkage of seminiferous tubules, vacuolization and accumulation of debris in seminiferous tubules of 1(d), 4(e)and 6(f) months stressed rats. The seminifrous tubules of recoverygroup rats after 1 month exposure show normal appearance (g) similer to controls whereas those of recovery group rats of 4(h) or 6 (i) months exposure resembles stressed rats. (H & E)

Table 4: Effects of chronic stress exposure for different periods on counts of different categories of germ cells in stage VII of seminiferous epithelial cycle

Groups	Mean number of germ cells/category/testis ± SEM)				
	Type A spermatogonia	Preleptotene spermatocyte	Midpachytene spermatocyte	round spermatids	Elongated spermatids
1month control	3.53±0.14^{a}	17.69±0.84^{a}	34.46±0.81^{a}	106.14±2.72^{a}	127.2±1.07^{a}
1month stress exposure	2.96±0.19^{b}	15.49±0.14^{b}	31.94±1.07^{ab}	98.03±0.56^{a}	96.2±1.83^{b}
Recovery after 1 month exposure	3.23±0.06^{ab}	14.96±0.43^{b}	30.52±1.45^{b}	96.57±1.305^{a}	124.2±2.52^{a}
4 months control	3.59±0.11^{a}	16.13±0.94^{ab}	34.71±1.08^{a}	103.89±4.39^{a}	128.8±3.46^{a}
4 months stress exposure	1.44±0.24^{c}	6.41±1.27^{c}	16.54±1.25^{c}	45.43±4.64^{b}	81.8±1.93^{c}
Recovery after 4 months exposure	1.31±0.04^{cd}	5.43±0.71^{c}	10.39±0.92^{d}	46.94±6.49^{b}	71.8±2.67^{d}
6 months control	3.57±0.07^{a}	17.12±0.68^{ab}	34.55±0.51^{a}	104.81±2.02^{a}	124.61.43^{a}
6 months stress exposure	1.08±0.06^{cd}	4.41±0.41^{c}	11.43±0.62^{d}	45.01±3.17^{b}	56.00±3.39^{e}
Recovery after 6 months exposure	0.97±0.07^{d}	5.82±0.31^{c}	9.92±0.43^{d}	41.80±2.34^{b}	57.0±4.32^{e}
10 months control	3.31±0.09^{ab}	15.85±0.42^{ab}	33.68±0.44^{a}	104.19±1.76^{a}	121.6±1.60a^{b}
ANOVA F-value df = 40, 9	**82.80** **P≤0.05**	**64.99** **P≤0.05**	**146.81** **P≤0.05**	**77.52** **P≤0.05**	**131.94** **P≤0.05**

Mean values with same superscript letters in the given column are not significantly different, whereas those with different superscript letters are significantly (P≤0.05) different as judged by Duncan's multiple test.

Table 5: Effects of chronic stress exposure for different periods on the activities of catalase(CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione S transeferase (GST) and glutathione reductase (GR) activities in testis of rat

Groups	CAT (nm/mg/min)	SOD (unit/mg protein)	GPx (µmol/mg/min)	GST (µmol/mg/min)	GR (unit/mg)
1month control	0.0058±0.001^{a}	2.38±0.09^{a}	0.0065±0.001^{a}	34.24±3.46^{a}	24.43±1.44^{a}
1month stress exposure	0.0024±0.002^{c}	1.08±0.04^{b}	0.0027±0.001^{cd}	16.88±1.07^{bcd}	14.76±0.56^{c}
Recovery after 1 month exposure	0.0044±0.002^{b}	2.11±0.11^{a}	0.0052±0.001^{b}	31.87±2.23^{a}	20.46±0.49^{b}
4 months control	0.0057±0.001^{a}	2.22±0.19^{a}	0.0056±0.002^{a}	36.28±3.24^{a}	23.44±0.74^{ab}
4 months stress exposure	0.0022±0.002^{c}	0.954±0.073^{b}	0.0025±0.001^{cd}	20.41±1.098^{bc}	11.25±0.09^{de}
Recovery after 4 months exposure	0.0029±0.002^{c}	0.838±0.057^{b}	0.0030±0.001^{c}	20.68±4.01^{b}	15.7±0.75^{cd}
6 months control	0.0063±0.002^{a}	2.320±0.16^{a}	0.0058±0.001^{a}	32.68±1.47^{a}	24.26±1.69^{ab}
6 months stress exposure	0.0021±0.002^{c}	1.036±.092^{b}	0.0021±0.001^{de}	13.21±1.17^{cd}	9.21±0.54^{ef}
Recovery after 6 months exposure	0.0023±0.001^{c}	0.903±0.122^{b}	0.0013±0.002^{e}	10.19±1.65^{d}	7.43±0.40^{f}
10 months control	0.0054±0.001^{ab}	2.129±0.17^{a}	0.0055±0.001^{b}	30.79±2.33^{a}	22.11±3.08^{ab}
ANOVA F-value df = 40, 9	**19.87** **P≤0.05**	**31.66** **P≤0.05**	**30.35** **P≤0.05**	**15.73** **P≤0.05**	**27.912** **P≤0.05**

All the values are mean ±SEM

Mean values with same superscript letters in the given column are not significantly different, whereas those with different superscript letters are significantly (P≤0.05) different as judged by Duncan's multiple test.

Table 6: Effects of chronic stress exposure for different periods on the testicular concentration of MDA, ascorbic acid and tocopherol in rat

Groups	MDA(nmol/g protein)	µmol/mg protein	
		Ascorbic acid	Tocopherol
1month control	5.72±0.16^{a}	2.56±0.09^{ab}	2.59±0.21^{a}
1month stress exposure	13.98±1.79^{c}	1.38±0.12^{d}	1.27±0.84^{d}
Recovery after 1 month exposure	7.64±1.51^{a}	2.40±0.06^{b}	2.27±0.04^{ab}
4 months control	5.78±0.45^{a}	2.87±0.09^{a}	2.42±0.11^{a}
4 months stress exposure	15.56±1.21^{c}	1.01±0.12^{e}	0.66±0.14^{e}
Recovery after 4 months exposure	10.74±0.31^{b}	1.43±0.12^{d}	0.79±0.15^{e}
6 months control	5.25±0.42^{a}	2.42±0.12^{b}	2.03±0.14^{bc}
6 months stress exposure	15.22±1.33^{c}	0.89±0.14^{e}	0.58±0.11^{e}
Recovery after 6 months exposure	16.18±1.38^{c}	0.66±0.15^{e}	0.67±0.13^{e}
10 months control	5.66±0.45^{a}	2.43±0.18^{b}	1.77±0.08^{c}
ANOVA F-value df = 40, 9	**19.19** **P≤0.05**	**42.04** **P≤0.05**	**39.49** **P≤0.05**

All the values are mean ±SEM

Mean values with same superscript letters in the given column are not significantly different, whereas those with different superscript letters are significantly (P≤0.05) different as judged by Duncan's multiple test.

Non enzymatic antioxidants:
There was a significant duration dependent decrease in the concentrations of ascorbic acid and tocopherol in 1, 4 and 6 months stressed rats compared to controls. The concentration of ascorbic acid was significantly increased in recovery group rats of 1 month exposure compared to stress group and did not significantly differ from controls whereas that of recovery groups of 4 and 6 months stress exposed rats was significantly lower than controls (Table 6). The concentration of tocopherol following recovery after 1 month exposure did not differ from controls whereas that of the recovery group rats of 4 and 6 months exposure did not differ from respective stress groups and was lower than controls (Table 6).

DISCUSSION

Stress induced adrenal hypertrophy is a well established phenomenon [45] and strong stimulation of the adrenal gland during prolonged stress situation is known to cause adrenal hypertrophy [46]. In addition, CRH released during stress suppresses appetite [47, 48]. Hence, in our present study, a significant increase in the adrenal gland weight coupled with an increase in the activity of adrenocortical key steroidogenic enzyme, 3β HSDH and reduction in gain in body weight following chronic exposure to restraint and forced swimming exercise every day for 1, 4 or 6 months indicated stress experienced by these rats and concomitant degenerative changes in the testes were stress responses. In the present study, chronic exposure to stress resulted in a significant duration of exposure dependent decrease in weight of the testes, counts of germ cells in stage VII of spermatogensis, concentration of non enzymatic antioxidants (ascorbic acid and tocopherol) in the testis and duration dependent increase in the number of abnormal spermatozoa accompanied by derangement of seminiferous epithelium. In addition a significant uniform decrease in testicular 3β HSDH, serum testosterone levels, different antioxidant enzyme activities, total sperm count and percentage of motile spermatozoa and a significant increase in MDA levels were found after chronic stress exposure for 1, 4 and 6 months. Though the results of our present study are in agreement with our earlier study [49] and others [4-15] several novel facts are evident with reference to stress and testicular functions.

Earlier studies exposed experimental animals to a single stressor for a single short or long duration ranging from 30 to 90 days and found degenerative changes in the testis and impaired secretion of reproductive hormones [50, 51, 4-15]. Hence, whether the testicular damage remains at the same level or further deterioration occurs with increase in number of days of stress exposure could not be ascertained from these studies. In the present study testicular weight, counts of different categories of germ cells in stage VII of spermatogenesis and testicular concentrations of non-enzymatic antioxidants (Ascorbic acid and tocopherol) showed a significant decrease following 1 month exposure and further significant decrease after 4 months and 6 months exposure, whereas the number of abnormal spermatozoa increased significantly as the duration of exposure increased from 1 month through 6 months. Concomitantly, there was steady degeneration of histoarchitecture of seminiferous tubules as the days of exposure to chronic stress increased, reaching intense histopathological state after 6 months exposure as shown by accumulation of cellular debris, fibers and degenerated germ cells in the seminiferous tubules. It is interesting to note that, while these parameters showed duration of exposure dependent alterations, alterations in other parameters, viz. decreases in activity levels of testicular 3β-HSDH, serum testosterone levels, and antioxidant enzymes, total count of epididymal spermatozoa and percentage of motile spermatozoa and increase in concentration of MDA did not differ among different exposure periods. It is evident from this study that some of testicular parameters especially sperm count and antioxidant enzymes are most sensitive to stress effects as they showed maximum decrease within shorter exposure period of 1 month, and remained at that level throughout the exposure period of 6 months.

The earlier studies on stress and testicular activities [4-15] did not focus on whether chronic stress effects are transient and the testis reverts to normalcy after the cessation of exposure and if so, whether shorter or longer periods of exposure influence the reversibility of stress effects. Hence, the present study addressed this aspect by allowing the rats to recover after different stress exposure periods i.e., 1, 4 and 6 months. It is to be noted that all the recovery group rats were allowed a uniform recovery period of 4 months, irrespective of their stress exposure period. The recovery period approximately corresponds to duration of two spermatogenic cycles in rats. This recovery period was chosen for complete replacement of affected germ cells by new waves of spermatogenesis, so that any permanent damage of germ cells only can result in decreased spermatogenesis. In the recovery group rats after 1 month exposure, majority of parameters, i.e., testes weight, counts of abnormal spermatozoa, counts of A spermatogonia and spermatids , activities of SOD, GST and MDA and tocopherol concentrations were completely restored to control levels, whereas 3β-HSDH activity, serum testosterone levels, total sperm count, percentage of

motile spermatozoa, number of spermatids, concentration of ascorbic acid and activity of CAT showed significant increases over stressed rats, although they were lower than controls, indicating that they were in process of recovery. On the other hand all these parameters of recovery group rats after 4 or 6 months exposure did not significantly differ from respective stress group, despite elapse of 4 months recovery period. In our earlier study [49] wherein rats were exposed to similar stressors (restraint followed by forced swimming) everyday for 2 months, also did not show restoration of testicular activity to control level. Hence, the results of our study put together clearly demonstrate that, the recovery of testicular function after chronic stress exposure depends on duration of exposure, the shorter duration permits recovery whereas longer, i.e., more than a month stress exposure might result in irreversible change in testicular function. The results gain importance in the present context of increasing rate of infertility in men due to exposure to variety of factors including stress in modern life.

The fact that increasing duration of stress exposure, results in irreversible changes indicates the possibility of permanent damage to spermatogenic and steriodogenic cells of the testis. Indeed it is reported that chronic stress leads to apoptotic loss of germ cells and the Leydig cells [52, 53] and also glucocorticoid which is secreted at higher concentrations under stress causes apoptosis of spermatogonia [54]. Since spermatogonia are the stem cells that generate new waves of spermatogenesis the loss of these might lead to decrease sperm output. In our present study, there was a drastic decline in total sperm count following 1, 4, and 6 months exposure which did not show improvement after recovery periods in 4 or 6 months stress exposed rats whereas it showed partial recovery after 1 month exposure. Similarly, the testicular 3β HSDH and serum testosterone concentration also showed decline after 1, 4 or 6 months stress exposure whereas a partial recovery was found in 1 month stressed rats but not in 4 or 6 months stressed rats. Since stress or elevated glucocorticoid is known to cause apoptotic loss of germ cells and the Leydig cells, it is plausible that stress induced loss of stem spermatogonia and the Leydig cells causes irreversible decline in sperm production and testosterone secretion. A short exposure period might have caused less damage allowing recovery, which is evident in durational effects of stress, as counts of spermatogonia A and testosterone concentration after 1 month exposure were higher than those after 4 or 6 months exposure. Weight and size of the testis and spermatogenesis [13] are known to be dependent on testosterone. The stage VII of spermatognic cycle in rat is androgen sensitive as highest level of androgen receptors are expressed in this stage and testosterone is required for conversion of round spermatids into elongated spermatids [55]. Stress induced reduction in testosterone secretion due to suppression of hypothalamus-pituitary-testis axis by activation of hypothalamus-pituitary-adrenal axis has been reported [3]. Stress induced reduction in gonadotrophin levels consequently suppress the testicular androgen biosynthesis [56]. In addition stress induced hyper secretion of glucocorticoids reduces the response of testis to gonadotriphin [54, 56] and also induces apoptosis of the Leydig cells resulting in reduced testicular testosterone biosynthesis [57]. Therefore, altered steroidogenic activity of the testis and reduced testosterone secretion appears to be a major factor in arrest of spermatogenesis under stress.

In the present study stress induced suppression of testicular steroidogenesis was evident as shown by a significant decrease in the activity of a key steroidogenic enzyme, 3β-HSDH and serum testosterone levels concomitant with decrease in weight of testis and counts of germ cells, including most androgen sensitive germ cells, the elongated spermatids in stage VII of spermatogenic cycle. These results indicate that reduced testosterone secretion led to drop in sperm count. The important outcome of the study is that first time it is demonstrated that after a long term stress exposure neither the serum testosterone levels nor the germ cell counts and sperm counts are restored to normalcy. It is suggested that this phenomenon might be the basis of infertility observed in captive animals and humans exposed to threats.

In addition to impaired secretion of reproductive hormones and direct interference of glucocorticoids with testicular steroidogenic cells causing disruption of testicular activities, another possible mechanism is the oxidative damage caused by the stress resulting in testicular dysfunction. Antioxidants protect germ cells against oxidative damage [58] which is a major factor that induce germ cell apoptosis in the testis [59]. Increased production of ROS than the antioxidant capacity may lead to oxidative damage which can be assessed by membrane lipid peroxidation, which is the most frequently cited in support of the involvement of oxidative stress in tissues [27]. Stressors are known to increase ROS production [47], which may be due to increased concentration of glucocorticoid hormones that intensify ROS generation in body [60]. Hence, the present study examined whether stress induced functional involution of testis is accompanied by oxidative damage. It is evident that during all the exposure periods, there was reduction in the activity levels of different antioxidant enzymes viz CAT, SOD, GPx, GR and GST and the

concentration of non enzymatic antioxidants (ascorbic acid and tocopherol) indicating reduced antioxidant capacity of the testis resulting in the oxidative damage. Indeed the oxidative damage was evident as shown by a significant increase in MDA levels (lipid peroxidation) in the testis. These changes were accompanied by the suppression of steroidogenic and gametogenic activities of the testis. Thus, our results strongly point to role of oxidative damage in stress induced testicular dysfunction. Though there were reports on oxidative damage impairing the testicular activity, those studies used factors other than stress viz. environmental pollutants [59, 61] , smoking [62, 63] alcohol [64,65] to induce oxidative damage. Our present study shows that stress can cause imbalance in oxidant-antioxidant system leading to testicular damage. In addition, first time it is shown that short term exposure (1 month) to chronic stress can result in reversible damage to antioxidant system whereas longer duration (4 or 6 month) results in irreversible change, as majority of antioxidant enzyme activities were not restored to normalcy after a recovery period accompanied by irreversible reduction in sperm count and testosterone levels.

CONCLUSION

The present study provides experimental evidence that short term stress exposure though impairs the testicular activity, it can be reversed, whereas long term exposure causes irreversible damage.

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