

Original Article

Testosterone Supplemented Protection On Inhibition Of Testicular Function Induced By Copper Chloride

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Abstract:

Though being an importance trace element required for normal metabolic activities, copper has some adverse effects in various organ-systems including testis. Occupational exposure to copper in different industries may result in abnormal rise in plasma copper level which can bring about spermatogenic inhibition. Our findings on inhibition of spermatogenesis and decrease in testosterone production after copper chloride treatment in a dose and duration dependent manner, led us to investigate whether exogenous supplementation of testosterone can have any reversible effect on this reproductive inhibition by copper exposure in male rats. Treatment of copper chloride at the dose of 2000 µg/kg/day for 26 days along with simultaneous supplementation of testosterone at the dose of 100 µg/kg body weight per day for last 14 days of treatment resulted in significant recovery of spermatogenesis (as evidenced by quantitative evaluation of spermatogenesis at stage VII), plasma testosterone level and weights of accessory sex organs. Conversely, testicular weight, plasma FSH & LH levels and testicular steroidogenic enzyme activities remained inhibited. Testicular copper content also found to remain elevated after testosterone supplementation. Though complete recovery of testicular dysfunction, elevated testicular copper content and reduced gonadotropin levels induced by copper could not be achieved, almost complete protection of spermatogenesis was observed after testosterone supplementation in copper treated rats. Thus it can be concluded that copper exposure affects hypothalmo-pituitary-testicular axis and thereby testosterone production rather than directly affecting testis. Further studies are required to elucidate the exact mechanism of this recovery after testosterone supplementation in copper exposed testis.

Keywords: Copper chloride; Spermatogenesis; Steroidogenesis; Gonadotropin; Testosterone; Supplementation.

Introduction:

In spite of being an important biological trace element required for different metabolic activities, copper can bring about adverse effects in various organ-systems [1] including testis [2] copper toxicosis has been observed in farm animals and in human beings with occupational exposure to copper in the industries of pesticides, fungicides, paints, alloys, construction materials and electroplating materials [3], observation on copper poisoning show vomiting tendencies, diarrhoea leading to coma and death [4].

The inhibitory effect of copper in male reproduction shows decreased concentrations of plasma FSH, LH and dysfunction of virile gonads and disorders in spermatogenesis [2]. We have also reported the inhibitory effect of copper chloride on testicular steroidogenesis and spermatogenesis. Plasma testosterone level and accessory sex organs' weight were also decreased following copper treatment in albino rats [5]. It was found that the testicular weight and motility of spermatozoa decreases, while the number of dead and defective spermatozoa increases following copper chloride treatment [2].

Since, copper chloride treatment at the dose of 2000 μ g/kg body weight/day for 26 days resulted in inhibition of the above mentioned parameters related with male reproductive activity [5] and the level of testosterone in the male has been suggested to play a role in the severity of copper deficiency [6], present study was undertaken to investigate whether supplementation of testosterone can have any protective effect on copper induced inhibition of testicular function.

Materials & Methods:

Experiments were carried out on matured male albino rats of Wistar strain weighing 140-160 g. They were fed standard rat diet and water *ad libitum* and were maintained under standard laboratory conditions at $28\pm 2^{\circ}\text{C}$ with constant light-dark cycle. The maintenance of the animals was in accordance with the ethical procedures and approved by the institutional animal ethical committee (CU-IAEC). Twenty four rats were taken for the experiment and were divided into three equal groups and subjected to the following experimental schedule:

- (a) Vehicle—treated control (animals treated with mammalian physiological saline 0.1 mL/100 g body weight/day for 26 days).
- (b) Copper chloride treated animals at the dose of 2000 μ g/kg body weight/day for 26 days [5].
- (c) Animals treated with copper chloride at the dose of 2000 μ g/kg body weight/day for 26 days and simultaneously supplemented with testosterone at the dose of 100 μ g/mL 10% ethanol/kg body weight/day for last 14 days of copper treatment.

All the animals were sacrificed on 27th day of experiment, 24 hrs after the last copper chloride injection following protocols and ethical procedures and their body weights were noted. Blood was collected from all the rats in separate tubes. Plasma was collected and stored at -20°C and used for radioimmunoassay of testosterone, LH and FSH [7-8]. Weights of testes and accessory sex organs (seminal vesicle and ventral prostate) were taken. One testis from each animal was fixed in Bouin's fluid for histological studies while the other one was used for the biochemical assay of testicular 17β -HSD and Δ^5 - 3β -HSD activities [9, 10]. Testicular 17β -HSD was assayed following the method of Jaraback *et al.* [9].

Testis was homogenized in 20% spectroscopic grade glycerol containing 5 mM potassium phosphate and 1 mM EDTA at a tissue-concentration of 100 mg/ml of homogenizing mixture. It was centrifuged cold at 10000 rpm for 30 minutes. The supernatant (1 ml) was mixed with 400 μ mol sodium pyrophosphate buffer (pH 10.2) and 0.3 μ mol testosterone to make the volume 3 ml. Enzyme activity was measured after adding 1.1 μ mol NAD⁺ to the mixture in a spectrophotometer against a blank (without NAD⁺). One unit of enzyme activity is equivalent to a change in absorbance of 0.001/min at 340 nm.

Biochemical assay of testicular Δ^5 -3 β -hydroxysteroid dehydrogenase (HSD) activity was measured following the method of Talalay [10]. The same supernatant was mixed with 100 μ L sodium pyrophosphate buffer (pH 8.9) and 30 μ g dehydroepiandrosterone, making the incubation volume 3 ml. Enzyme activity was measured after adding 0.5 μ mol of NAD⁺ to the mixture in a spectrophotometer against a blank (without any NAD⁺). One unit of enzyme activity is equivalent to a change in absorbance of 0.001/min at 340 nm.

Radioimmunoassay of plasma testosterone was carried out following the method of Jacobs [7] using a testosterone ¹²⁵I RIA Kit (ICN Biochemical Inc., Diagnostic Division, Costa Mesa, CA 92626, USA). Radioactivity was determined using the gamma counter (Model No. IC-4702, Electronic Corporation of India, Hyderabad, India). All samples were run in duplicate in a single assay to avoid interassay variation. The intraassay coefficient of variation was 6.5%.

Concentration of plasma LH and FSH were quantitated by RIA following 2nd antibody precipitation method. Carrier free ¹²⁵I for hormone iodination was obtained from Bhaba Atomic Research Centre (Mumbai, India). Pure rat LH (NIADDK-LH-1-6) and FSH (NIADDK-FSH-1-6) were iodinated using chloramine-T (Sigma Chemical Co., St. Louis, MD, USA) according to the method of Greenwood, Hunter and Glover [8]. The antisera to LH and FSH, NIADDK anti-r-LH-S-9, and NIADDK anti-r-FSH-S-11 were used at a tube dilution of 1:150,000 and 1:100,000 respectively. The sensitivities of the assays were 0.75 μ g/L for LH and 1 μ g/L for FSH. All the samples were assayed in duplicate and the intraassay coefficient of variation was <7%. Hormone concentration was expressed in terms of National Institute of Health (NIH) reference preparation RP-2.

After fixation testis was dehydrated in graded alcohol, cleared with benzene and finally embedded in paraffin. The sections (5 μ m thick) of the tissues were stained with Eosin-hematoxylin sequence. Quantitative study of the seminiferous epithelium was performed on the basis of the relative number of germ cell nuclei/cross-section of seminiferous tubule at stage VII of the cycle. The relative number of each variety of Germ Cells at Stage VII of the cycle of seminiferous epithelium, i.e., Type A spermatogonia (ASg), preleptotene spermatocytes (pLSc), midpachytene spermatocytes (mPSc), step seven spermatid (7Sd) were counted according to the method of Leblond and Clermont [11]. All nuclear counts were corrected for differences in tubular diameter by Abercrombie's formula [12] and tubular shrinkage by Sertoli cell correction factor [13].

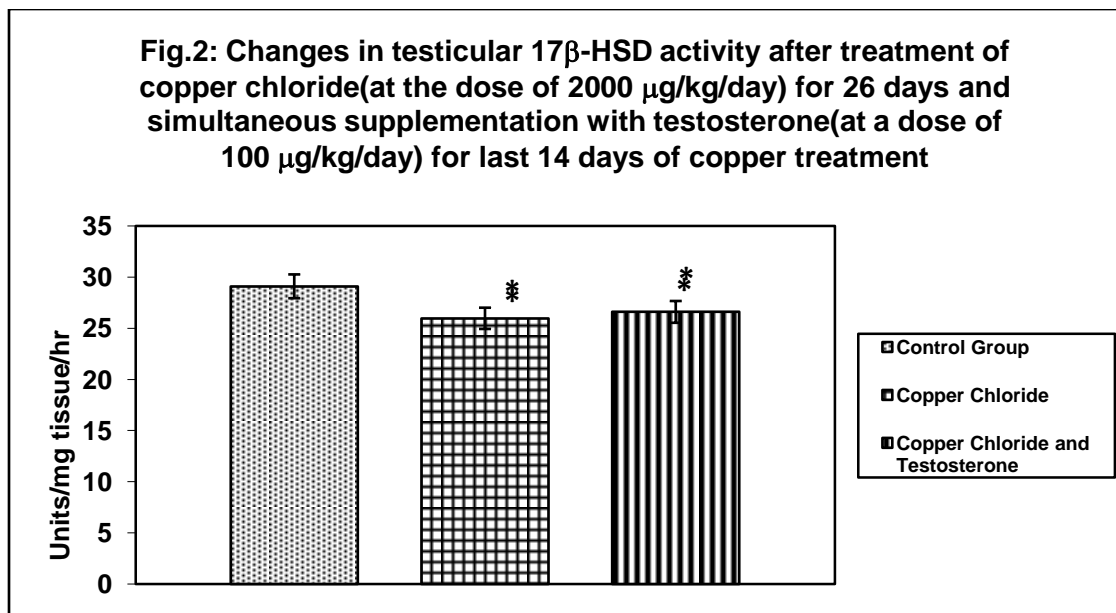
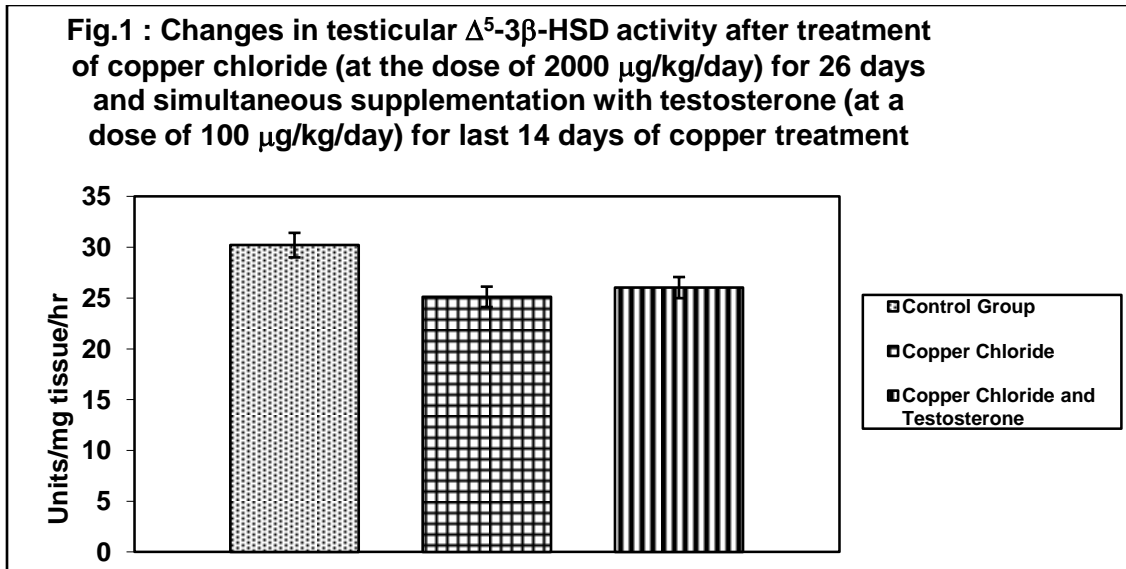
For statistical analysis to test for differences between control and treated experimental groups, a multiple comparison two tailed 't' test was used. Differences were considered significant when p<0.05 [14].

Results and Discussion:

Copper chloride treatment significantly reduced the testicular weight and weights of accessory sex organs. Supplementation with testosterone in copper chloride treated rats showed significant recovery in accessory sex organs' (seminal vesicle and ventral prostate) weight but no significant alteration was observed in testicular weight of testosterone supplemented copper treated rats compared with solely copper treated rats (Table-1).

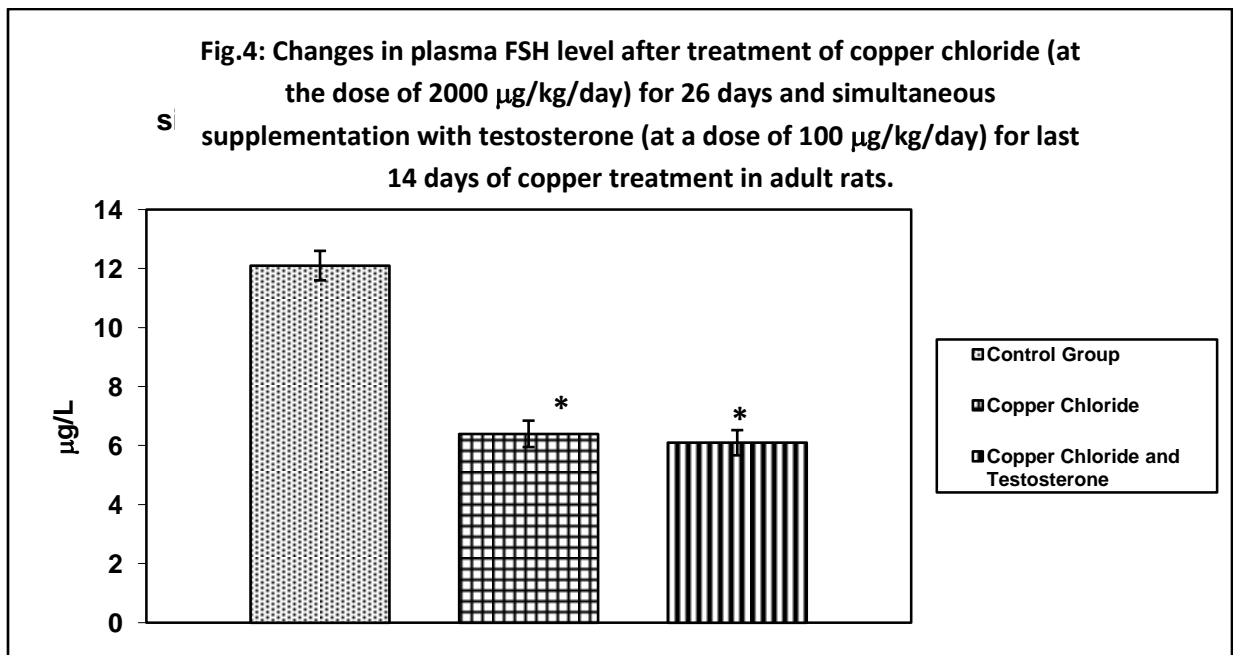
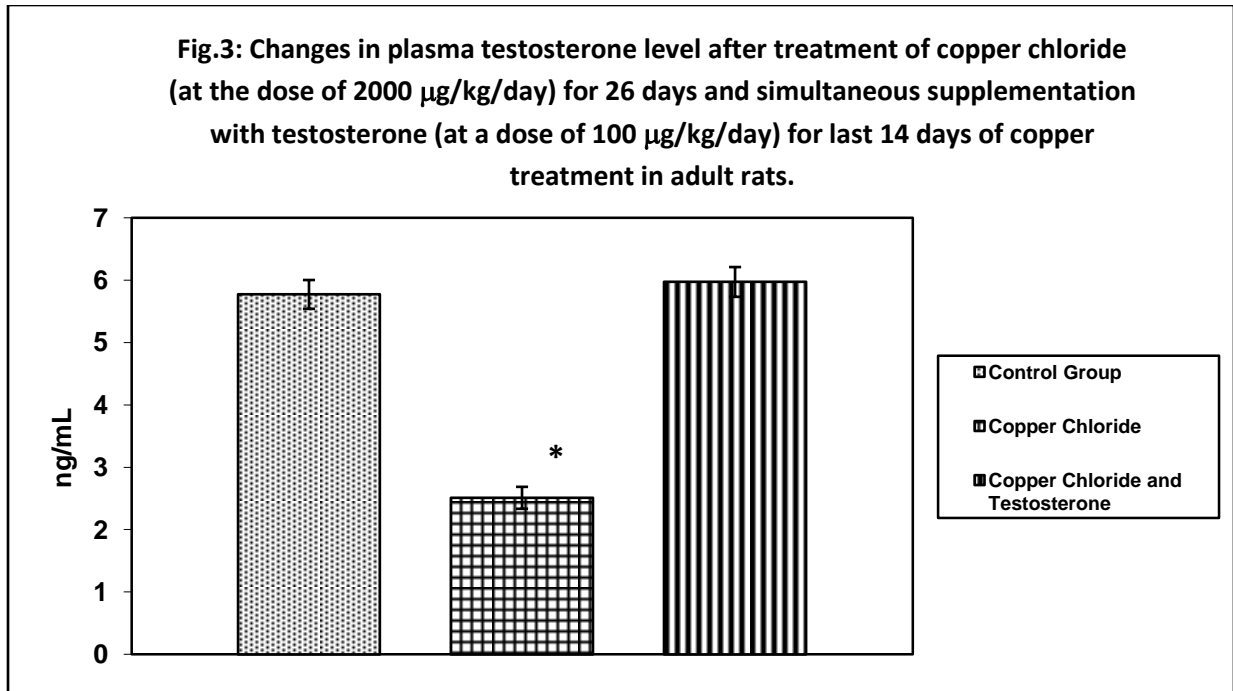
Table – 1			
Changes in testicular and accessory sex organs' weight after copper chloride treatment at the dose of 2000 µg/kg/day for 26 days and simultaneous supplementation of testosterone (at a dose of 100 µg/kg/day) for last 14 days of copper treatment in adult rats			
Treatment group	Testicular Weight (mg/100 g body wt.)	Ventral prostate weight (mg/100 g body wt.)	Seminal vesicle weight (mg/100 g body wt.)
Vehicle-treated Control	1468.75 ± 27.92	215.71 ± 18.68	469.15 ± 21.17
Copper chloride	1290.18 ± 19.87*	140.83 ± 17.55*	334.96 ± 22.62*
Copper chloride & testosterone	1321.12 ± 23.46*	183.23 ± 23.10	441.26 ± 19.33
Values marked with asterisks are significantly different from corresponding control values. Values are mean ± SEM of 8 rats/group. ANOVA followed by multiple comparison 't'-test where *p<0.05 was considered to be significant.			

Testicular Δ^5 -3 β -HSD and 17 β -HSD activities were significantly reduced in copper treated rats in comparison to vehicle – treated control rats. Testosterone supplemented copper treated rats showed no significant recovery in either of these testicular enzymes and these enzyme activities were found to be significantly reduced in testosterone supplemented rats compared with vehicle – treated control rats (Figs. 1,2).

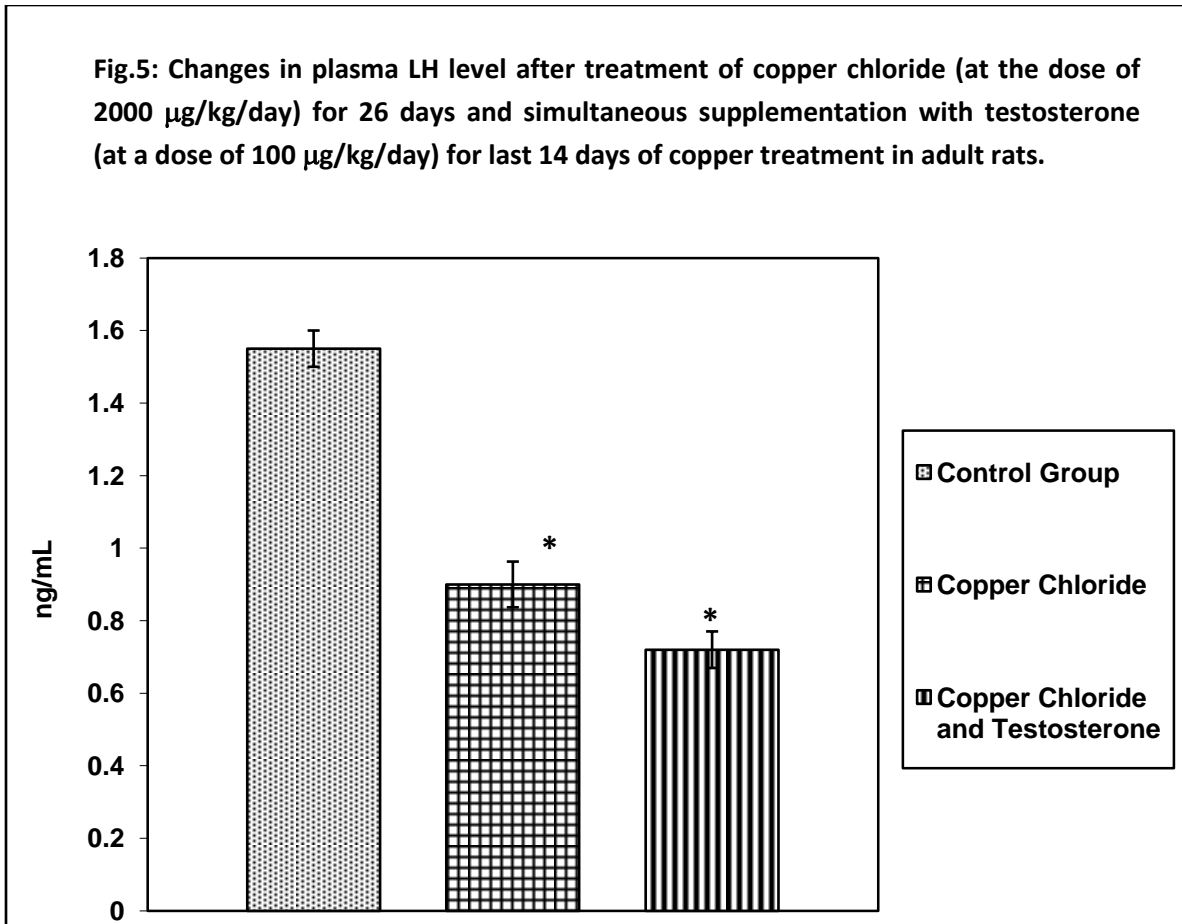


Values marked with asterisks are significantly different from corresponding control values. Values are mean \pm SEM of 8 rats/group. ANOVA followed by multiple comparison 't' test where * p <0.05 was considered to be significant.

Plasma levels of FSH, LH and testosterone showed a significant diminution in copper chloride treated rats in comparison to vehicle-treated control rats, but supplementation of testosterone in copper chloride treated rats showed significant recovery in plasma testosterone level and there was no significant difference in the values of plasma testosterone level between testosterone supplemented copper treated group and vehicle treated control group (Fig.3). On the other hand plasma levels of FSH (Fig. 4) and LH (Fig. 5) showed no significant recovery in testosterone supplemented copper treated group in comparison to solely copper treated group.



Values marked with asterisks are significantly different from corresponding control values. Values are mean \pm SEM of 8 rats/group. ANOVA followed by multiple comparison 't' test where $*p < 0.05$ was considered to be significant.



Values marked with asterisks are significantly different from corresponding control values. Values are mean \pm SEM of 8 rats/group. ANOVA followed by multiple comparison 't' test where $*p < 0.05$ was considered to be significant.

Quantitative analysis of germ cells at stage VII of seminiferous tubular cycle revealed that copper chloride treatment significantly reduced the number of ASg, pLSc, mPSc and 7Sd and also reduced the number of matured spermatozoa in tubular lumen (Plates 1,2). Significant recovery in ASg and 7Sd counts were noted after supplementation with testosterone in copper treated rats (Table 2, Plate-3) when compared with solely copper treated rats. Though trends in recovery in spermatocytes counts were also seen (Table 3), this recovery in pLSc and mPSc counts were statistically non-significant (Table-2). The number of matured spermatozoa in tubular lumen was also recovered to some extent (Plate-3). Effective degeneration of spermatids was also reduced after testosterone supplementation in copper treated rats (Table-3).



Plate-1: Spermatogenesis at stage VII of seminiferous cycle; vehicle treated control (NX400)

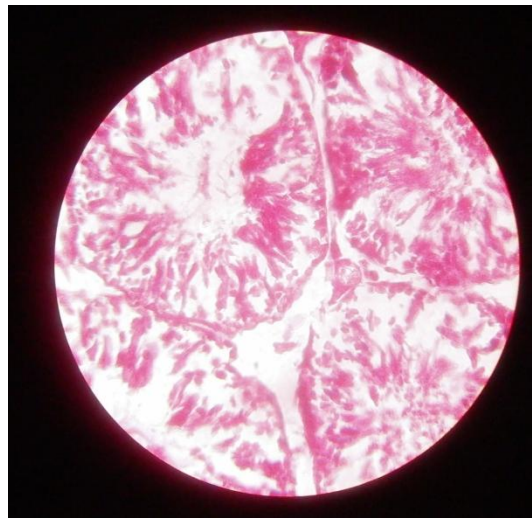


Plate-2: Spermatogenesis at stage VII of seminiferous cycle; copper chloride treatment at the dose of 2000 µg/kg/day for 26 days (NX400)

Table – 2				
Changes in relative number of germ cell counts at stage VII of spermatogenesis after treatment of copper chloride (at the dose of 2000 µg/kg/day for 26 days) with simultaneous supplementation of testosterone (at a dose of 100 µg/kg/day) for last 14 days of copper treatment in adult rats				
Treatment group	Type A spermatogonia (ASg)	Preleptotene spermatocytes (pLSc)	Mid-Pachytene spermatocytes (mPSc)	Step 7 spermatids (7Sd)
Vehicle-treated Control	0.63 ± 0.04 ^a	18.44 ± 0.69 ^a	19.32 ± 0.72 ^a	64.39 ± 1.68 ^a
Copper chloride	0.41 ± 0.04 ^b	12.89 ± 0.64 ^b	12.83 ± 0.84 ^b	34.73 ± 1.52 ^b
Copper chloride & testosterone	0.52 ± 0.03 ^c	12.61 ± 0.51 ^b	13.49 ± 0.62 ^b	43.19 ± 1.11 ^c

Values are mean ± SEM of 8 rats/group. ANOVA followed by multiple comparison 't' – test where *p<0.05 was considered to be significant. In any vertical column, the means with same superscript do not differ from each other significantly.

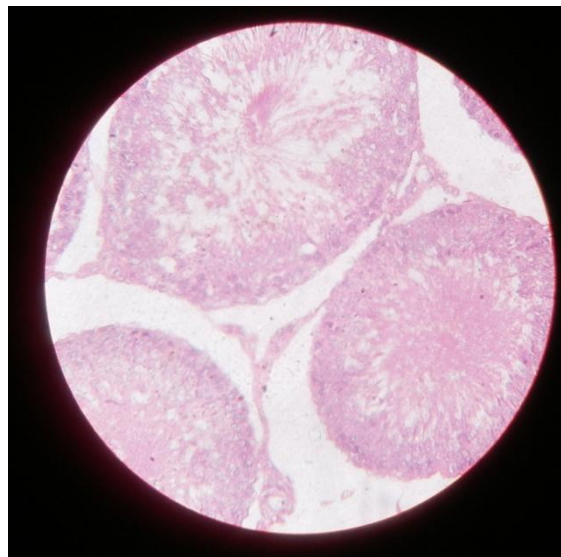


Plate-3: Spermatogenesis at stage VII of seminiferous cycle; copper chloride treatment at the dose of 2000 µg/kg/day for 26 days and simultaneous supplementation of testosterone at the dose of 100 µg/kg/day for last 14 days (NX400)

Table – 3			
Mid–Pachytene spermatocyte : Spermatid ratio at stage VII of the seminiferous cycle, percentage of spermatid degeneration and effective 7Sd degeneration in adult rats after copper chloride treatment (at the dose of 2000 µg/kg/day) for 26 days and simultaneous supplementation of testosterone (at a dose of 100 µg/kg/day) for last 14 days of copper treatment			
Treatment group	Mid – Pachytene spermatocyte : Spermatid (mPSc : 7Sd)	% of Spermatid Degeneration	Effective 7Sd degeneration
Vehicle-treated control	1 : 3.33 ^a	16.75 ^a	–
Copper chloride	1 : 2.71 ^b	32.25 ^b	+ 15.50
Copper chloride & testosterone	1 : 3.20 ^a	20.00 ^a	+ 3.25
Values are mean ± SEM of 8 rats/group. ANOVA followed by multiple comparison 't' test where *p<0.05 was considered to be significant. In any vertical column, the means with same superscript do not differ from each other significantly.			

The study has demonstrated that copper chloride treatment decreased testicular and accessory sex organs' weight, testicular steroidogenic enzyme activities, plasma levels of LH, FSH and testosterone and spermatogenesis. As testosterone can regulate the spermatogenic process in a dose and duration dependent manner, testicular androgenesis being reduced after copper chloride treatment, the effect of this androgen in copper treated rats was shown in this experiment.

The results indicated that supplementation with testosterone in copper chloride treated rats caused no significant alteration in testicular weight and Δ^5 -3 β -HSD and 17 β -HSD activities, but weights of accessory sex organs were significantly recovered. Seminal vesicle and ventral prostate – both of these accessory sex organs are directly dependent on testosterone [15], and thus with exogenous supplementation of testosterone in copper treated rats, these organs significantly regained their weights.

Exogenous supplementation of testosterone in copper treated rats resulted in complete recovery of plasma testosterone level in this group, but plasma gonadotropins (LH and FSH) levels remained reduced in this testosterone supplemented copper treated group. This result cannot be explained from the present study but possibly recovered plasma testosterone level might have played

some negative feedback role in hypothalamic GnRH release or anterior pituitary gonadotropins release [16,17]. As testicular Δ^5 -3 β -HSD and 17 β -HSD are gonadotropin dependent enzymes reduced levels of gonadotropins in the testosterone supplemented copper treated group may probably be an explanation for non-recovery of these enzyme activities in this group. Though testicular weight showed a trend of improvement in testosterone supplemented copper treated group no significant change in testicular weight was observed. Possibly testosterone alone was not sufficient for complete recovery of testicular weight and reduced level of serum gonadotropins mainly FSH was probably the required factor for significant improvement in testicular weight in this group.

Quantitative analysis of germ cells at stage VII of seminiferous cycle revealed that number of type A spermatogonia and step7 spermatids were increased after supplementation of testosterone in copper chloride treated rats. The partial recovery in the ASg after testosterone supplementation was possibly due to its effect on primary spermatocytes, as testosterone is required for the conversion of primary spermatocyte prophase to metaphase and for maturation of spermatids. Thus the percentage of 7Sd degeneration that was 32.25% after copper treatment became 20.00% with testosterone supplementation. The result also indicated that testosterone was capable of initiating and maintaining the spermatogenic process in copper chloride treated matured rats and this was in agreement with previous findings where it has been shown that this steroid can initiate and maintain the first wave of spermatogenesis in hypophysectomised rats [18].

Conclusion:

It can be concluded from this experiment that though complete recovery of testicular degeneration and dysfunction (including steroidogenic function) induced by copper chloride could not be found by simultaneous testosterone supplementation, almost complete protection of spermatogenesis can be achieved after testosterone supplementation in copper exposed rats.

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