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Original Article

Dose-dependent effect of copper chloride on male reproductive function in immature rats

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Abstract
Background: Copper is essential as a trace element for metabolic processes. Exposure to copper in industries develops toxicity among the workers. Previous findings on adverse effects of copper on male reproductive function in adult albino rats led to investigate the effects of this metal on reproductive function of maturing male rats in the present experiment.
Methodology: To study these effects, immature (30 to 35 days old) Wistar strain albino rats weighing about 50-60 g were treated intraperitoneally with copper chloride at doses of 1000, 2000 and 3000 μg/kg body weight/day for 26 days.
Result: Significant fall in accessory sex organ weight and inhibition of testicular 17β-hydroxysteroid dehydrogenase activity along with degeneration of testicular growing spermatogenic cells and reduction in serum testosterone, FSH and LH level were observed at the doses of 2000 and 3000μg/kg/day. On the other hand, at the dose of 1000 μg/kg/day significant increase in testicular steroidogenic enzyme activity and stimulation of testicular spermatogenesis along with rise in serum testosterone and LH level were observed, though no significant change was observed in serum FSH level. This suggests that copper has got a dose-dependent effect on testicular steroidogenesis and spermatogenesis and serum testosterone and LH level in maturing male rats.

Keywords: Copper, male reproduction, gonadotropins, Immature rats

In spite of being an important biological trace element, necessary for different metabolic processes and enzyme activities[1], copper may have some adverse effects in different physiological systems. Occupational exposure to copper may lead to copper toxicosis in the industrial workers of algicides, fungicides, paints, alloys, construction materials and electroplating materials[2].

Among the different disorders, abnormalities in male reproductive function is being reported recently. Studies on workers exposed to electric welding reveal increased semen concentration of copper along with lowered sperm count, sperm viability and semen volume[3] . There are report that long term ingestion of copper chloride adversely affects sexual behaviour, fertility and testicular and accessory sex organ weight in adult male rats[4] . Chronic exposure of rats to copper chloride fumes show decreased concentrations of plasma FSH, LH and testosterone and dysfunction of virile gonads and disorders in spermatogenesis[5].

Recently it has been reported that chronic copper chloride administration in adult male rats caused inhibition of steroidogenic activity e.g., Δ^5-3β-hydroxysteroid dehydrogenase (Δ^5-3β-HSD) and 17β hydroxysteroid dehydrogenase (17β-HSD) and decreased level of serum testosterone along with fall in testicular and accessory sex organ weight[6]. Again, there are reports that during *in vitro* conditions copper stimulates the release of LHRH from median eminence area and thereby pituitary gonadotropins in adult and immature male rats[7]. So, the present study was undertaken to thoroughly investigate the effects of copper chloride on different andrological parameters in immature rats.

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Materials and methods

Experiments were carried out on immature male Wistar rats of 30-35 days of age having body weight of 50-60 g. They were maintained in a light (12L: 12D) and temperature (28 ±2°C ambient temp.) controlled animal house and given standard laboratory food and water *ad libitum*.

Copper chloride (assay 99%) was purchased from Sigma chemical Co., St. Louis, MD, USA and dissolved in sterile distilled water. Thirty-two rats were used, divided into four equal groups, and one group of rats was injected intraperitoneally (i.p) with 1 ml physiological saline / kg body wt./day for 26 days and designated as control animals (Group I). The other three groups of animals were injected with either 1000 μg or 2000μg or 3000 μg of copper chloride /ml sterile distilled water / kg body weight / day for 26 days (Groups II, III & IV). All the rats were killed on the 27th day between 8.00 to 10.00 hr., 24 hrs. after the last injection following protocols and ethical procedures. Blood samples for hormone assay were collected from the hepatic vein under light ether anaesthesia. The heparinized plasma was separated from the cells by centrifugation. Plasma samples were stored at –20°C until assayed. The body weight of all the rats were recorded on the first day of injection (initial) and on the day of sacrifice (final).

Testicular 17β-HSD was assayed following the method of Jarabak et al. (1960)[8]. Testis was homogenized in 20% spectroscopic grade glycerol containing 5mM potassium phosphate and 1mM 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 μmol. sodium pyrophosphate buffer (pH 10.2) and 0.3 μmol testosterone to make the volume 3ml. Enzyme activity was measured after adding 1.1 μmol 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.

Radioimmunoassay of serum testosterone was carried out following the method of Jacobs (1974)[9] using testosterone ^{125}I 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%.

Concentrations of serum LH and FSH were quantitated by RIA following 2nd antibody precipitation method. Carrier free ^{125}I 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 (1963)[10]. 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 μg/L for LH and 1 μg/L for FSH. All the samples were assayed in duplicate and the intraassay coefficient of variation was <7%. Hormone concentration were expressed in terms of National Institutes of Health (NIH) reference preparation RP-2.

For histological study of spermatogenesis testis was fixed in Bouin's fixative after a small excision at each of the two poles and processed for section cutting, embedded in paraffin and 5 mm sections routinely prepared using a microtome. Sections were stained by periodic acid Schiff technique and counterstained with haematoxylin. 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 spermatids (7Sd) were counted according to the method of Leblond and Clermont (1952)[11]. The nuclei of the different germ cells were measured using Leitz micrometer in 20 round tubular cross sections at stage VII in each rat. All crude counts were corrected for difference in tubular diameter by Abercombie's formula (1946)[12].

For statistical analysis to test for differences between control and treated experimental groups, a multiple comparison two tailed 't' test[13] was used. Differences were considered significant when $p < 0.05$.

Results

Copper chloride at the dose of 2000 μg/kg/day and higher doses caused a significant decrease and at 1000 μg/kg/day dose caused significant increase in accessory sex organ (seminal vesicle and ventral prostate) weight (Table-1). Testicular weight was significantly decreased at

the dose of 2000 µg/kg/day and higher doses but at the dose of 1000 µg/kg/day no significant change was observed (Table- 1).

Testicular 17β-HSD activity (Fig. 1) and serum testosterone level (Fig. 2) showed significant increase at 1000 µg/kg/day dose and significant reduction at 2000 µg/kg/day dose and higher doses, in comparison to control group.

Differential results were found for pituitary gonadotropins i.e., serum LH and FSH levels after copper treatment. Serum LH level showed significant rise after treatment of copper chloride at 1000 µg/kg/day dose and subsequently at the dose of 2000 µg/kg/day and higher doses show significant reduction when compared with control group (Fig. 3). On the other hand serum FSH level showed non-significant change at 1000 µg/kg/day dose and at 2000 µg/kg/day dose reduced significantly in comparison to control group (Fig. 4).

Though all the animals of control and treated groups were not yet pre-pubertal still, spermatogenic process has been initiated in the seminiferous tubules . Copper chloride at 1000 µg/kg/day dose caused stimulation of spermatogenic cycle with increased number of cells at different stages of spermatogenesis along with larger number of spermatozoa present in the tubular lumen. But treatment of copper chloride at 2000 µg/kg/day dose caused degeneration of growing spermatogenic cells at different stages and disintegration of maturing seminiferous tubules. Quantitative study of spermatogenesis showed significant increase in Type A spermatogonia (Asg), Mid-Pachytene spermatocytes (mPSc) and step 7 spermatids (7 Sd) counts at 1000 µg/kg/day dose and at the doses of 2000 and 3000 µg/kg/day significant decrease in different cell counts of seminiferous cycle in comparison to control group (Table-2). The ratio of mPSc : 7Sd were 1 : 2.47 and 1: 2.49 in controls and 1000 µg/kg/day group. But in the 2000 µg/kg/day and 3000 µg/kg/day of copper chloride treated rats this mPSc : 7Sd ratio were found to be 1: 2.27 and 1: 2.24 respectively. Thus the effective spermatid degeneration was much lower in the 1000 µg/kg/day dose of copper chloride in comparison to 2000 µg/kg/day and higher doses (Table- 3).

Table 1: Effect of copper chloride on testicular and accessory sex-organs' weight at different doses for 26 days in immature male rats

Treatment Group	Testicular weight (mg/100 g body wt.)	Seminal vesicle weight (mg/100 g body wt.)	Ventral prostate weight (mg/100 g body wt.)
Vehicle-treated Control	1291.61 ± 33.09^{a}	319.14 ± 15.66^{a}	180.48 ± 11.19^{a}
Copper Chloride (1000 µg/kg body wt./day)	1354.87 ± 32.16^{a}	354.48 ± 12.80^{b}	204.00 ± 9.24^{b}
Copper Chloride (2000 µg/kg body wt./day)	1216.54 ± 29.98^{b}	281.16 ± 14.54^{c}	151.12 ± 12.20^{c}
Copper Chloride (3000 µg/kg body wt./day)	1179.54 ± 27.89^{b}	270.52 ± 11.50^{c}	147.54 ± 12.20^{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.

Table 2: Changes in relative number of germ cell counts at stage VII of spermatogenesis after treatment of copper chloride at different doses for 26 days in immature rats.

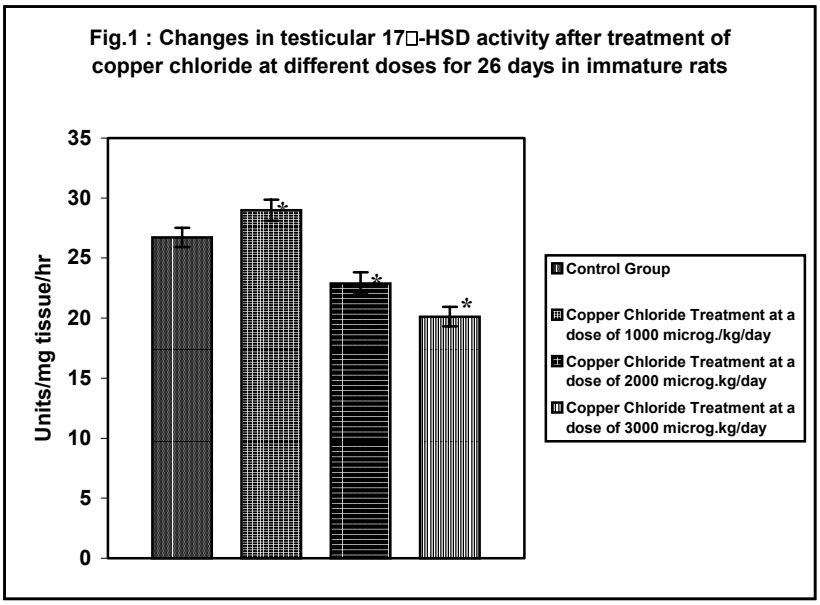
Treatment group	Type A sperma-togonia (ASg)	Preleptotene spermatocytes (pLSc)	Mid-Pachytene spermatocytes (mPSc)	Step 7 spermatids (7Sd)
Vehicle-treated Control	0.4 1± 0.03[a]	14.45 ± 0.78[a]	14.81 ± 0.59[a]	36.59 ± 0.58[a]
Copper chloride (1000 µg/kg /day)	0.47 ± 0.02[b]	15.39 ± 0.72[a]	16.58 ± 0.85[b]	41.35 ± 0.67[b]
Copper chloride (2000 µg/kg /day)	0.33 ± 0.04[c]	12.18 ± 0.57[b]	13.47 ± 0.54[c]	30.65 ± 0.82[c]
Copper chloride (3000 µg/kg /day)	0.31 ± 0.03[c]	11.09 ± 0.61[b]	12.89 ± 0.58[c]	28.96 ± 0.88[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.

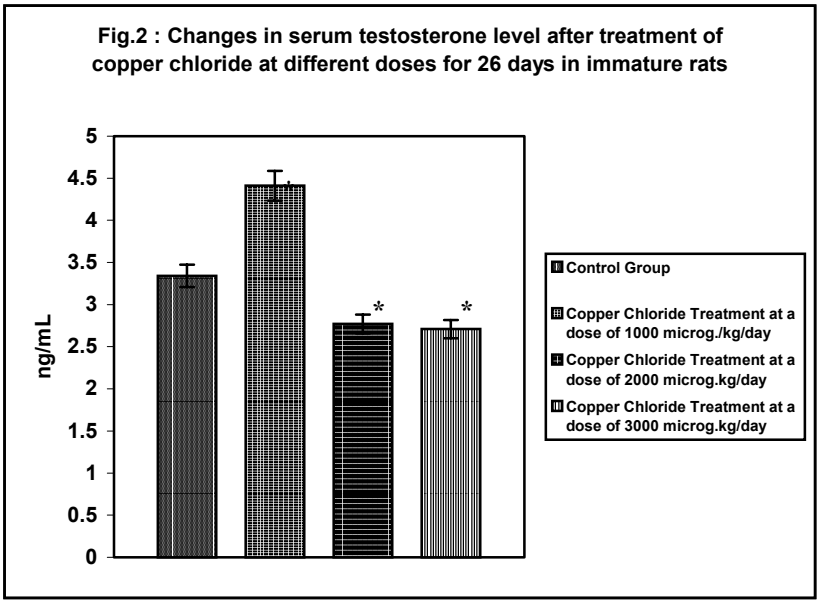
Table 3: Mid–Pachytene spermatocyte : Spermatid ratio at stage VII of the seminiferous cycle, percentage of spermatid degeneration and effective 7Sd degeneration after copper chloride treatment at different doses for 26 days in immature rats

Treatment group	Mid – Pachytene spermatocyte : Spermatid (mPSc : 7Sd)	% of Spermatid Degeneration	Effective 7Sd degeneration
Vehicle-treated Control	1 : 2.47[a]	38.25[a]	–
Copper chloride (1000 µg/kg /day)	1 : 2.49[a]	37.75[a]	– 0.50
Copper chloride (2000 µg/kg /day)	1 : 2.27[b]	43.25[b]	+ 5.00
Copper chloride (3000 µg/kg /day)	1 : 2.24[b]	44.00[b]	+ 5.75

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.



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.



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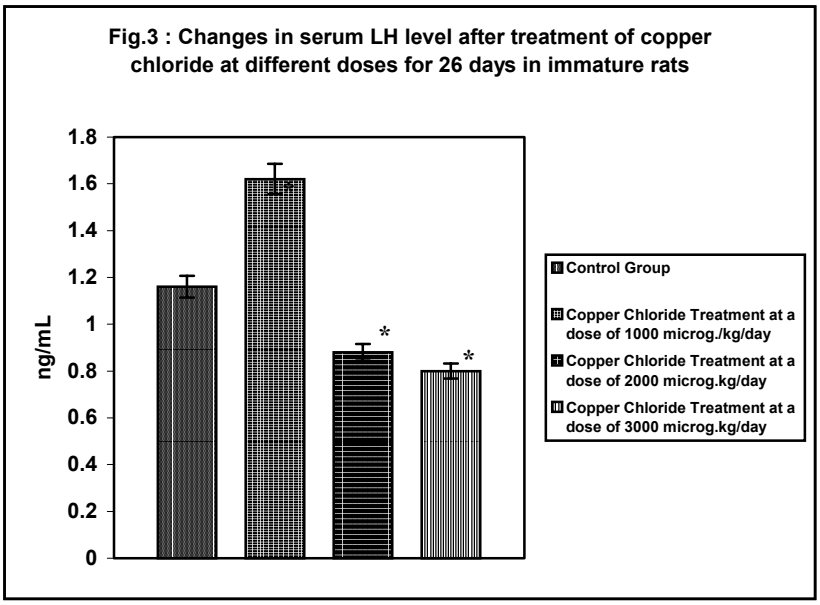

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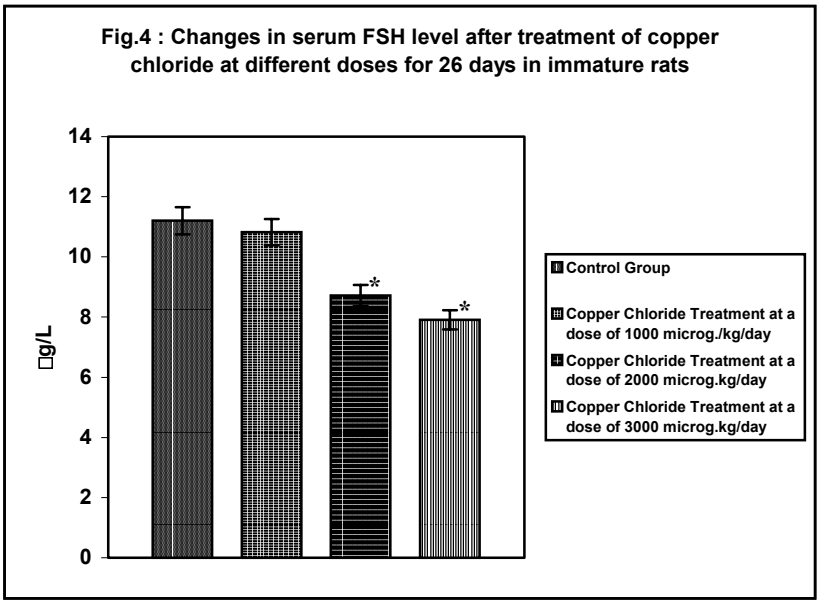

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Discussion

In our study accessory sex organs' weight (seminal vesicle and ventral prostate) were found to decrease significantly at the dose of 2000 μg/kg/day and higher doses and increase at 1000 μg/kg/day dose. These data correspond with testicular 17β-HSD activity and serum testosterone concentrations. As circulating testosterone plays a major, if not sole role in the maintenance of structural integrity and functional activities of the accessory sex organs[14], decrease and increase in accessory sex organs' weight is a reflection of reduction and elevation of serum testosterone level after copper treatment at different doses. As testicular 17β-HSD is the key steroidogenic enzyme responsible for testosterone biosynthesis[15], increased and decreased activities of testicular 17β-HSD resulted in elevated and reduced concentrations of testosterone in the blood. In immature rats of our study, the pituitary LH level of blood has been found to be elevated at lower dose of copper treatment (1000 μg/kg/day) and reduced at higher doses (2000 & 3000 μg/kg/day). Since, testicular 17β-HSD is a gonadotropin-dependent enzyme[15] the increased and decreased activities of 17β-HSD is therefore, a result of increased and decreased pituitary gonadotropin secretion, as LH plays a major role in testicular androgenesis[16].

There may be two possible ways by which different doses of copper affect pituitary gonadotropin secretion. First, copper may act at the pituitary receptors of GnRH which controls the release of pitutiary gonadotropins[17]. It has been observed that hypothalamic neurons that release GnRH have copper-interactive sites for copper, and their interaction stimulates the GnRH release from median-eminence explants *in vitro*[18]. Again, the maturational process of these hypothalamic neurons is dependent on androgens and it has been observed *in vitro* that presence of testosterone in the vicinity increases the affinity of copper-interactive sites for copper on GnRH-secreting neurons[7]. In our experiments, at lower dose copper chloride probably increases serum testosterone, circulating levels of which in hypothalamic area increases interaction of copper with its binding sites and resultant GnRH secretion that ultimately leads to increased LH and testosterone level via Hypothalamo-Pituitary-Testicular (H-P-T) axis.

As already mentioned, chronic inhalation of copper chloride fumes leads to lowered gonadotropin and testosterone level in adult rats[5] and chronic i.p injection of copper chloride at the dose of 2000 μg/kg/day and higher doses caused decreased testicular 17β-HSD activity and serum testosterone level in adult rats[6]. Similar results may be extrapolated in case of immature rats for higher doses of our present experiment. At these doses increased level of serum corticosterone may play some role in reducing testicular steroidogenesis.

Although the exact mechanism of action of high doses of copper chloride cannot be explained from the present study, secondly, there may be important involvement of adrenal corticoids in our experiment. It has been shown very recently that in immature rats copper chloride at low dose (1000 μg) caused significant reduction in serum corticosterone level and at higher doses (i.e. 2000 and 3000 μg/kg/day dose) caused significant elevation of serum corticosterone level[19] . High corticosterone level may reduce testosterone level either directly by inhibiting testicular steroidogenesis[20] or by decreasing the pituitary responsiveness of gonadotrophins to GnRH or altered secretion of hypothalamic GnRH[21] thereby reducing testosterone through reduced activity of H-P-T axis. So, in our experiment, higher doses of copper probably caused higher serum corticosterone level that ultimately leads to decreased levels of serum LH, FSH and testosterone.

Though still now there is no report of reproductive stimulation caused by inhibition of adreno-cortical activity in maturing or immature male rats, it has been reported that hypocorticalism (adreno-cortical inhibition) resulting from dexamethasone treatment caused significant increase in testicular weight and well-organised interstitium along with stimulation of spermatogenic process with spermatogoneal proliferation and stimulation in testicular 3β-HSD and 17β-HSD activities in maturing white leg-horn chicks[22]. This antagonistic relationship between adrenocortical activity and reproduction has been supported by similar other reports in toads[23].Thus, in immature rats of the present experiment, copper chloride at the dose of 1000 μg/kg/day caused inhibition of adrenocortical steroidogenesis[19], which possibly resulted in reproductive stimulation by increasing serum levels of LH and testosterone. Moreover, as at this dose of copper chloride serum corticosterone level is significantly reduced the suppressive influence of glucocorticoids (corticosterone) on hypothalamic GnRH release[21] is probably withdrawn which activates H-P-T axis and thereby serum LH and testosterone levels are elevated. But this low dose of copper failed to elevate serum FSH level. This finding probably supports the earlier proposition of separate regulatory mechanisms of FSH and LH by glucocorticoids[24].

According to that proposition, LH response to GnRH can be suppressed by glucocorticoids but not the FSH response. Our finding thus resembles the withdrawal of corticosterone suppression to produce higher LH level but no change in FSH level in case of low dose of copper treatment (1000 µg/kg/day)in immature rats.

Furthermore, quantitative study of spermatogenesis in these immature rats revealed an increased germ cell counts at different steps of spermatogenesis at stage VII of seminiferous cycle after copper chloride treatment at 1000 µg/kg/day dose. This stimulation in spermatogenesis is possibly because of increased serum testosterone level as testosterone can initiate and maintain spermatogenesis in hypophysectomized rats[25] Though at this dose serum FSH level showed no significant change, probably higher serum testosterone along with this unaltered serum FSH level were sufficient to stimulate the spermatogenic process in these immature rats[26] .Again at 2000 and 3000 µg/kg/day doses of copper chloride , serum levels of FSH,LH and testosterone – all were significantly reduced that caused arrest of growing spermatogenesis and degeneration and disintegration of seminiferous tubules. Thus quantitative study showed significant reduction in ASg,pLSc,mPSc and 7Sd counts along with higher effective spermatid degeneration in comparison to control or low dose of copper chloride treated group.

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