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Indian gooseberry and *Lycopodium* 200c can effectively reduce cadmium induced testicular damage in 40 days exposed mice

[*1] Mahasweta Chatterjee, [2] Gobinda Chandra Sadhukhan, [3] Jayanta Kumar Kundu
[1, 3] Genetics and Molecular Biology Unit, Department of Zoology, Vidyasagar University, Midnapore, West Bengal, India
[2] UGC – HRDC, Jadavpur University, Salt Lake, Kolkata, West Bengal, India

Abstract
Being a potential toxic metal cadmium is known to cause testicular damage by producing reactive oxygen species (ROS) resulting in infertility among male. The cadmium toxicity is due to its accumulation in different mammalian organs through food and drink from the environment. Its removal is not possible by most chelating agents as they are unable to pass through membrane. The present study was undertaken to see the effect of cadmium on mice testes and its remedies by herbal extracts and homeopathic medicines. Among different herbal products, Indian Gooseberry (amla) and the homeopathis medicine *Lycopodium* 200c are found to have antioxidant, anti-toxic and anti-tumorigenic properties. The study was designed to see the protective role of amla and *Lycopodium* 200c on cadmium induced testicular damage. To conduct this study, *Swiss* albino mice were divided into different sets with one control, one induced and other with treatments. Tests for sperm head anomalies (SHA), comet asaay and histopathological analysis of mice testes were performed. Result shows that toxicity and damages caused by cadmium can be effectively reduced with amla extracts and *Lycopodium* 200c.

Keywords: cadmium, toxic metal, ros, antioxidant, anti-tumorigenic activity, and sperm head anomalies, indian gooseberry, *Lycopodium* 200c

1. Introduction
Cadmium is a naturally occurring, soft, silver-white metal, but is not common in the environment. It is most often found in combination with other elements, such as oxygen (cadmium oxide), chlorine (cadmium chlorine) or sulfur (cadmium sulfide). Most cadmium used in our country is a soft, bluish metal or grayish powder obtained as a byproduct from the treatment of copper, lead and iron ores and is a probable carcinogen. There is evidence of prostate and kidney cancer in mammals due to cadmium induction, it has also been shown to cause lung and testicular damages in animals [1]. It has been suggested from previous studies that maximum cases of mammalian male infertility may be attributable to various environmental and occupational exposure to toxic metals [2]. Recently it has been found that increasing occupational and environmental exposures to toxic metals are directly related to declining sperm concentration and male infertility [3]. Even in low concentration this metal is toxic for cell [4]. Exposure to cadmium is known to induce the formation of reactive oxygen species (ROS) such as superoxide radical hydroxyl ion and hydrogen peroxide [5]. These reactive oxygen species reacts with cellular biomolecules by damaging several membrane proteins and DNA [6]. Various studies link cadmium with oxidative stress, since this metal can alter the antioxidant defense system in several tissues of many animals causing an alteration in the activity of antioxidant enzymes and changing the structure of cell membrane [7]. The most significant route of exposure to cadmium or cadmium compounds is through food, since food materials tend to take up and retain cadmium. For examples plants absorb cadmium from the soil, fish and shellfish take cadmium from the water, etc. This stored cadmium again enters in human body through food.

Besides, conventionally used treatments like surgery, radiotherapy and chemotherapy [8] in Cadmium (Cd) induced tissue damage; this study is looking forward for easily available herbal and homeopathic treatment with effective measures against carcinogenic environment. According to homeopathic literature *Lycopodium clavatum* (L.) in 200c potency has a clinical effect over cancer of different tissues. Scientists also found that *Lycopodium* 200c reduces cytogenetic damages yielding positive modulations of all biochemical, pathological and other risk factors including cell viability and expression of p53 protein as compared to controls [9].

Again it has been observed that amla or *Phyllanthus emblica* (L.) rendered protection against cadmium induced toxicity. It is a natural, efficacious antioxidant with richest natural source of Vitamin C. Amla (or Amlaka, Amlaki, or other variants) is one of the most frequently used Ayurvedic herbs.

The present study investigated the antagonistic role of amla and *Lycopodium* 200c on toxicological effect of cadmium chloride on testicular cells of *Swiss* albino mice. The work was done by performing a short term (40 days) Cd exposure and treatment series with amla and *Lycopodium* 200c on mice *in vivo*. Different assays including Histopathological tests, Sperm count, Sperm Head Anomalies (SHA) and comet assay were conducted for observing the effects of cadmium and other treatment series on the mammalian testis (*Mus musculus*).

2. Materials and Methods

The experiments were carried out in Genetics and Molecular Biology Research Unit of Department of Zoology, Vidyasagar University. The animals were handled and kept in normal laboratory condition of the concerned Department by maintaining all Animal Ethical Rules. The animals were provided with normal food per day along with purified drinking water through water *ad-libitum*.

Swiss albino mice (~20gm each; 4 mice in each set) were used as experimental model and divided into four different sets such as (1) SI or Normal Control (NC) set: daily administered with normal food and water and without any treatment, (2) SII or Cadmium induced (Cd) set: administered with cadmium chloride in addition with normal feeding, (3) SIII or Cadmium and Amla treated(Cd+A) set: administered with both cadmium and amla along with normal food and water, (4) SIV or Cadmium and *Lycopodium* 200c (Cd+L) treated set: administered with both cadmium and *Lycopodium* 200c along with normal food and water.

Cadmium Chloride was used as the source of cadmium and given with a high dose of 100 mg/kg body weight. Amla juice and *Lycopodium* 200c were administered at a dose of 0.5 µl/ gm body weight.

2.1 Sperm head anomalies (SHA)

Sperm Head Anomaly (SHA) is a special kind of test to observe sperm and to analyze their head structure. It was done to compare normal and abnormal head shaped sperm and for knowing the effects of cadmium as well as its remedies by amla and *Lycopodium* 200c over testicular follicle. To conduct this test first the testes were dissected out. Then the epididymes of each set was squeezed to bring out the sperms within 1 ml of double distilled water. Then 50 µl of these sperm suspension was taken in a grease free slide & smear was prepared. After air drying the slides were used for staining & SHA counting. Slides for SHA were stained with crude Giemsa (double filtered). Waited for 45 minutes; washed with distilled water and observed under microscope (1000X magnification).

2.2 Sperm count

Among the collected sperms (in 1 ml) 10 µl was taken on the Haemocytometer slide and then counting was performed under the microscope (at 400X magnification). The haemocytometer is 0.1 nm deep and the 25 large squares represent an area of 1 square mm. The volume above the 25 squares, we counted the sperms that were settled out of 0.02 µl (0.1/25 X 5 = 0.02). Therefore, we must multiply our count in 5 squares by 50,000 in order to determine how many sperms would have been in 1.0 ml (1000/0.02 = 50,000; we usually express sperm concentration in terms of numbers/ ml). To get the concentration of the original sperm sample we must however also multiply by the dilution factor [10]. The equation that follows would be used to convert the counts in 5 squares to concentrations/ ml →

Conc. / ml = (dilution factor)(count in 5 squares)($0.05X10^6$)

2.3 Comet assay

The comet assay or SCGE (Single Cell Gel Electrophoresis) was performed by following the procedure used by Singh *et al.*, 1996 and Raigosa *et al.*, 2007 [11, 12]. This test was conducted to detect the DNA damage in sperm cells after cadmium induction and it's recovery after the introduction of amla and *Lycopodium* 200c. In brief; two types of agarose solutions were prepared first: 1. 1% LMPA or Low Melting Point Agarose prepared by dissolving in Kenny's salt solution (0.4 M NaCl, 9 mM KCl, 0.7 mM K_2HPO_4, 2 mM $NaHCO_3$) 2. 1% NMPA or Normal Melting Point Agarose prepared by dissolving in TAE buffer; then grease free slides were marked properly; these slides were dip into molten 1% NMPA and then dried in an incubator for about 2 hours at 40˚C; Then the sperm cells were centrifuged at 200g for 3 minutes; The pellet was resuspended in 180 µl of 1% LMPA by gentle pipetting; Two drops (each of 85 µl) of the suspension were taken on slides pre-coated with NMPA; then covered with cover slips and incubate on ice for at least 10 minutes; After 10 minutes the cover slips were removed and the slides were placed in a coplin jar containing lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris Base, 1% SDS, NaOH to P^H 10) and incubate for 1 hour at 4˚C in dark to lyse the cell; After 2 hours the slides were removed from the lysing solution and rinsed in distilled water for 2 minutes; The slides were then transferred into electrophoresis chamber containing freshly prepared electrophoresis buffer (1N NaOH + 200 mM EDTA) and incubated in the buffer for 20 minutes to allow the DNA to unwind; then the electrophoresis was run for 30 minutes at 20 V and 300 mA; the slides were removed from the chamber after 30 minutes and 5 drops of neutralizing buffer (0.4 M Tris) were added and kept for 5 minutes; the same was repeated one more time; the slides were stored for analysis by dehydrating in 100% methanol for 5 minutes; before analysis the slides were rehydrate by applying 100 µl distilled water for 10 minutes; then the slides were stained by applying 40 µl ethidium bromide working solution (0.05 ml of 2 mg/ ml stock + 4.95 ml distilled water) to each replicate and covered with cover slips. Photos were taken under fluorescence microscope at 400X magnification.

2.4 Histopathological test

For histopathological analysis the tissues were collected and stored in 10% formaldehyde solution. Histological slides were prepared by double staining *(H&E)* method. It was performed to observe and compare the histological structure of mice testis from different sets (SI - SIV).

2.5 Statistical analysis

To draw significance between the collected data one way ANOVA was performed in combination with descriptive statistics. The data were analyzed at the significance level of 0.05.

3. Results

3.1 Sperm head anomalies (SHA): Fig 1a shows the percentage of sperm head abnormalities which is significantly higher in cadmium induced group and effectively lowers by amla and *Lycopodium* 200c treatments. This representation is based on percentage of sperms per field of slides prepared for SHA. Fig 1b and c show pictorial view of normal (a) and abnormal (b) sperms found throughout the experimental

period. Abnormalities are very prominent in fig 2b indicated by arrows. The mean difference between NC (SI) and CD (SII) groups are significant at 0.05 levels. Table 1 also contains concentrations of normal and abnormal sperms of different sets. The data are represented in mean±SEM values.

Table 1: Tabular representation of normal and abnormal sperm concentration per field and percentage of SHA.

Sets	Normal Sperms/ Field	Abnormal Sperms/ Field	% Of Sha/ Field
NC	4.51±1.914	0.10±0.006	2.49±0.911
CD	3.29±0.852	0.31±0.111	8.72±1.946*
CD+A	2.83±0.870	0.17±0.064	5.41±1.855
CD+L	3.38±0.116	0.21±0.046	5.98±1.389

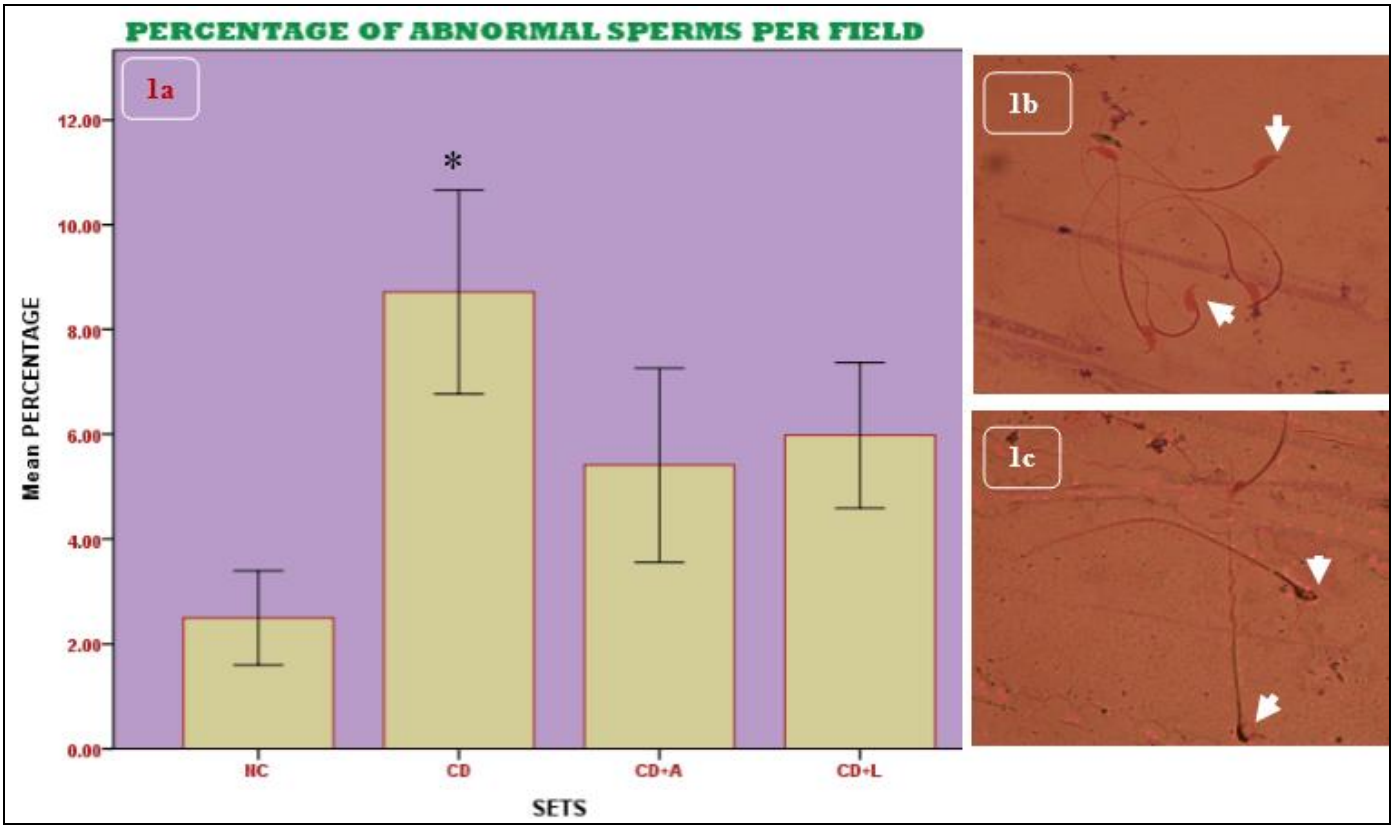


Fig 1: The graph in fig. 1a is prepared in SPSS software showing the mean percentage of sperm head anomalies (SHA) (DATA are represented in mean±SEM). The percentage is very low in SI or normal control group and very high in SII or cadmium induced group. Marking reduction is observed in SIII and SIV, i.e., the amla and *Lycopodium* treated group, respectively. Fig. 1b & 1c show the pictorial representation of normal and abnormal sperms. The 1b shows normal sperm cells with normal head structure (indicated by white arrow) while 1c shows abnormal sperm cells with damaged head (indicated by white arrows). * Data is significant at 0.05 level.

Sperm count: A tabular representation of sperm count can be seen in table 2. The sperm concentration in CD induced group is much less than other groups. Whereas the *Lycopodium* 200c treated group have highest sperm concentration. DATA are presented in mean±SEM values.

Table 2: Tabular representation of Sperm Count DATA

SETS	CONC./Ml
NC	$(39.57\pm5.553)x10^{6*}$
CD	$(1.5\pm0.1)x10^{6*}$
CD+A	$(3.42\pm0.448)x10^{6*}$
CD+L	$(99.3\pm12.41)x10^{6*}$

3.3 Comet assay: The damage in DNA level is clear from fig 2. The fig 2(a*)* represents a microscopic image of normal mammalian sperm cells with normal shape and size whereas, fig 2(b) represents the microscopic image of cadmium induced mammalian sperm cells; here the comet like tail of sperms proofs the occurrence of DNA damage due to the harsh effects of cadmium. Besides, the normal shapes of the sperms are also damaged due to the cadmium effect. In fig 2(c) and 2(d) the treatments with amla and *Lycopodium* 200c show drastic differences than that of the previous. These pictures show recovery of the cadmium induced damage.

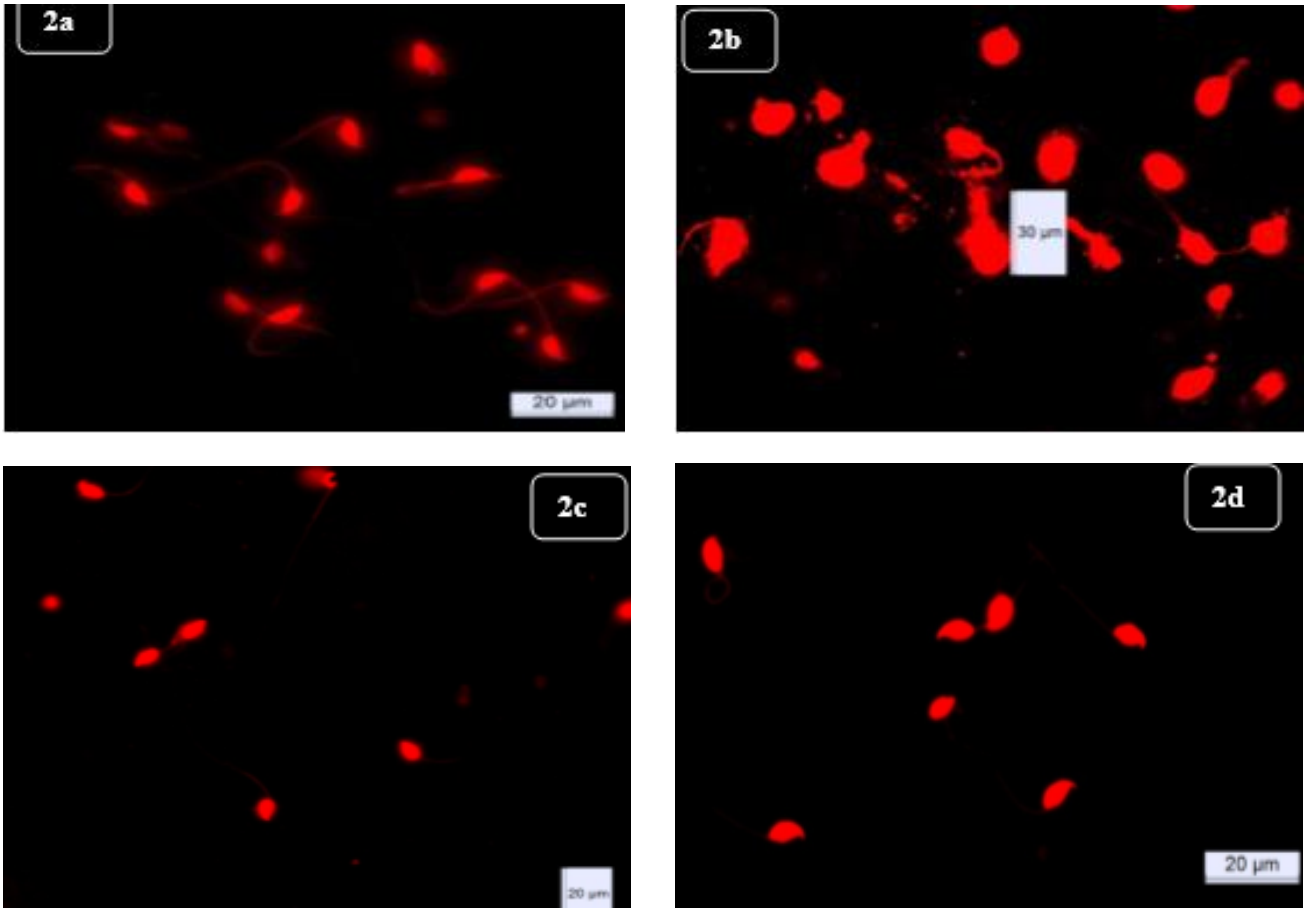
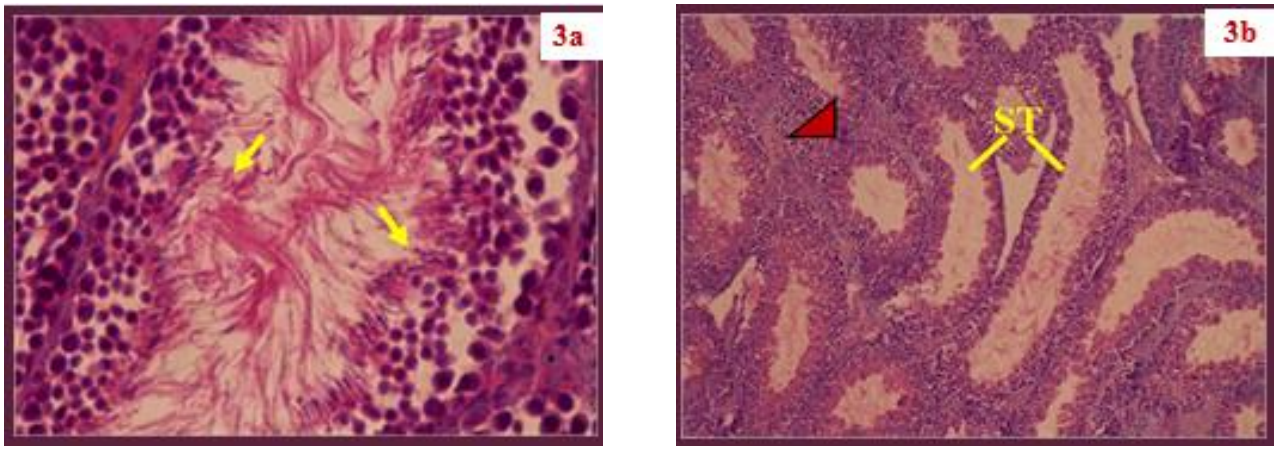


Fig 2: Pictorial representation of DNA damage and fragmentation of mice sperm cells of normal (2a) and cadmium exposed (2b) groups and group with amla and *Lycopodium* treated (2c & 2d), respectively after Cd exposure. The SI sperms (2a) show normal shape and structure while that of SII (2b) show broad and long comet tail proving DNA damage. SIII (2c) & SIV (2d) have more or less normal structures as SI.

3.4 Histopathological Test: The histological results of mice testes are shown in fig 3 (a – e). The fig 3(a) shows normal testis architecture (SI or NC set) in 500X with normal occurrence of spermatogenesis, normal seminiferous tubules (ST), spermatogonia, large number of spermatocytes and a huge number of mature sperm cells. Fig 3(b) shows the histoarchitecture of cadmium induced testis in 100X. Theis figure depicts that cadmium intoxication causes severe damage, such as, tubular elongation, lymphocytic infiltration, tissue disruption whereas fig 3(c) is the sectioning of the same in higher magnification (500X) and shows very little occurrence of spermatogenesis and a few mature sperm cells. On the other hand treatment with amla and *Lycopodium* 200c lead near about normal architecture of the mice testes showing little seminiferous tubular disruption and near normal number of sperm cells. These can be seen in fig 3(d) and fig 3(e).



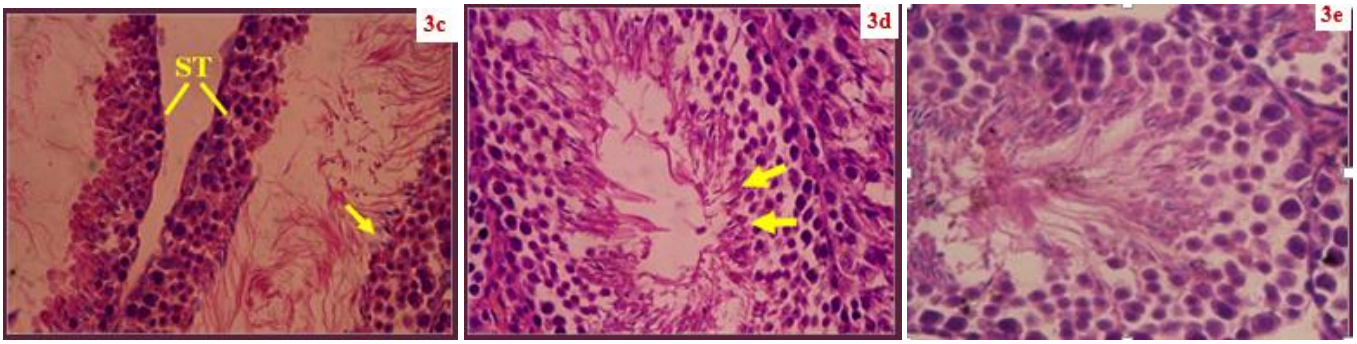


Fig 3: Histo-architecture of mice testis (3a – 3e) of normal, cadmium induced and amla or *Lycopodium* treated group. The normal group (3a) shows normal histo-architecture of mice testis, whereas the cadmium induced group shows marked differences with that of normal; amla treated group shows more or less equal architecture with that of the normal. In 3b and 3c the yellow arrows indicate elongated, narrow shaped tubules and little occurrence of spermatogenesis. The red arrow head in 3b shows lymphocytic infiltration due to Cadmium toxicity. The 3d & 3e show near about normal architecture and reoccurrence of spermatogenesis due to the anti-oxidative, anti-toxic effects of amla and *Lycopodium* (respectively). Here ST = Seminiferous tubules.

4. Discussion

From previous studies it has been observed that exposure to cadmium resulted in increased sperm head abnormalities and lowers the sperm count. In the cadmium treated group that is in SII of fig 3 (3b – c), maximum germ cells might have been destroyed either due to the membranous damage or macromolecular degradation which is possibly the result of the formation of reactive oxygen species (ROS) [13] In table 1 & figure 1a, a significant increase in sperm head abnormalities in cadmium treated group is found which emphasizes the possibility of gene alteration in germ cells induced by ROS which again causes DNA damage by forming breaks within DNA [14] which is clear from the results of comet assay (fig 2b). The abnormal headed sperms are also being seen in figure 1 (c). There are evidence from past that have showed the heavy metals induce gene mutation. The testicular germ cells carrying minor gene mutations are not eliminated but are manifested as morphologically deformed sperm. It is also found that certain metals like cadmium act as germ cell mutagens which affect specific gene loci in spermatogonial cells and increase the percentage of sperm abnormalities [15]. The sperm cell morphology is genetically controlled by many autosomal and sex linked genes [16]. Therefore it can be said that the formation of sperm head abnormalities in the present study is due to these mutagenic effects of cadmium induced ROS produced on specific gene loci of germ cell chromosomes and involved in the maintenance of normal sperm head structure. Thus ROS also induces sperm cell mortality which again reduces the sperm count number in cadmium treated group (table 2).

The number of spermatozoa is less in cadmium induced mice than other four groups. The structural changes of seminiferous tubules are caused by cadmium. Changes are also seen in the occurrence of spermatogenesis which is very less in cadmium treated group than others. Spermatogenesis is a sophisticated and complex differentiation process [17]. After a period of prenatal mitotic division, the germ cells present in the seminiferous cords of the testes at birth and remain quiescent until immediately before puberty, when they divide by mitosis to form spermatogonia. A population of these cells then enters the pre-stage of meiosis and become spermatocytes and after that, sperm cells. The remaining spermatogonial population in the seminiferous tubules serves as supporting cells, providing metabolic and physiologic functions. Degeneration of spermatogonia is an integral and important part of normal spermatogenesis. From previous works it can be seen that cell loss in the spermatogonial stages probably exceeds 75% in mammals like rat [18, 19, 20, 21]. This spermatogonial degeneration can result from exposure to toxic chemicals, heat and radiation, deficiencies of hormones or growth factors and immunodeficiency [21, 22, 23, 24]. Heavy metals like cadmium may cause irreversible testicular damage and lead to permanent spermatogonial loss and infertility.

Studies also showed that the testis is more sensitive to Cadmium than other important organs; in addition, it can interfere with testis function [25]. These support our result which revealed that cadmium administration decreases sperm numbers, and changes sperm morphology of the mice. Fig 3 shows that cadmium induced set contains elongated narrow shaped and thin walled seminiferous tubules with little tubular disruption along with less number of spermatozoa indicating abnormal spermatogenesis due to the adverse toxic effects of cadmium. The wall is almost lack of germ cells and covered with Sertoli's cells. It can be explained by the fact previously mentioned. Again the acute exposure to large doses of Cadmium induces gonadal necrosis whereas chronic treatment with low doses has been shown to produce testicular atrophy [26]. Cadmium decreases androgen biosynthesis, possibly by altering progesterone synthesis and metabolism through direct interaction of Cadmium with DNA and competitive inhibition of essential enzymes [27]. Cadmium is harmful even in very low dose, e.g. 10 mg/kg body weight and produces sperm abnormalities as proved in our previous studies [28].

Flavonoids such as quercetin and kaempferol, the active compounds of Indian gooseberry or amla, effectively impair with angiogenesis [29]. Besides, quercetin also proved that it could fight toxic elements [30] and thus reduces the adverse effects of cadmium. Amla also contains antioxidants such as emblicanin A and B and is a rich source of vitamin C, all of them together work against cadmium-induced toxicity [31, 32]. Besides, the homeopathic medicine *Lycopodium* 200c contains many active compounds such as lycopodine, clavatine, epigenin, clavatoxine, ferulic acid, selagine, lycoflexine, lycofoline [33], etc. The combined effect of all these active

compounds renders protection against such kind of toxic environment [34]. Their effects can be seen from the results. The protective effects of these two treatment elements used in this study are also prominent in other results including sperm count, SHA, histology and so on.

5. Conclusion

From the above study it can be concluded that the herbal extract of Indian Gooseberry or amla or *Phylanthus emblica* (L.) and the homeopathic medicine *Lycopodium* 200c made from *Lycopodium clavatum* (L.) or club moss are very effective in protecting the tissue, cellular and even DNA damages caused by severe toxicity of Cadmium. These results open a hope that these herbal extract and homeopathic medicine can also be used in case of other heavy metal toxicity or oxidative damages of different cells and organs of mammalian body. The present study is a gateway of many future studies which include many other herbal products or homeopathic medicines for treating such kind of injuries.

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