



Original Research Article

Protective Effect of Co-Administration of Cow's Urine "Gomutra" and Antioxidants against Lindane Induced Genotoxicity in Swiss Mice (*Mus Musculus*)

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ABSTRACT

Effect of cow urine and combination of antioxidants against lindane induced genotoxicity in Swiss mice has been elucidated. Male healthy mice, 8 - 10 weeks old, weighing 30 ± 5 gms were randomly selected and divided into 8 groups, namely, control (C); lindane (L); antioxidant (A); antioxidant + lindane (A+L); cow urine (U); cow urine + lindane (U+L); cow urine + antioxidants (U+A) and cow urine + antioxidants+ lindane (U+A+L). Group C animals were administered vehicle only (olive oil); doses selected for other treatments were: lindane: 80 mg/kg bw; antioxidants: 125 mg/kg bw (vitamin C: 50 mg/kg bw, vitamin E: 50 mg/kg bw, α -lipoic acid: 25 mg/kg bw) and cow urine: 0.25 ml/kg bw. In group A+L and U+L antioxidants and cow urine were administered 1h prior to lindane administration and in group U+A and U+A+L cow urine was administered 10 min before antioxidants. All treatments were administered orally continuously for 96 hrs. Lindane brought about a non-significant increase in structural chromosomal aberrations and the number of micronucleated polychromatic erythrocytes and a significant depression in mitotic index. Pretreatment with cow's urine and combination of antioxidants showed decrease in chromosomal aberrations and micronucleated polychromatic erythrocytes whereas an increase in mitotic index.

Keyword: Chromosomal aberrations; micronucleated polychromatic erythrocytes; mitotic index; lipoic acid; vitamins; cow urine

INTRODUCTION

Pesticides used in the modern agricultural practices represent a very large input of toxic chemicals in our environment [1]. Their usage has increased manifolds in disease control

management without considering their harmful side effects on plants, animals and human beings [2]. The toxic effect of pesticides is not necessarily a result of direct application, some pesticides accumulate into the food to a toxic level and affect the public health [3 - 4].

Organochlorine insecticides are well known for their high stability and persistent nature in the environment [5] and have been extensively used in developing countries. Lindane, the gamma isomer of hexachlorocyclohexane (HCH) possesses the property of persistence, bioaccumulation and long term toxicity [6] and fulfills the criteria of POP's i.e., persistent organochlorine pesticides.

Genotoxic potential of lindane is suggestive, but not conclusive. IARC has classified lindane as a possible human carcinogen [7]. Some studies have shown that lindane was negative for mutagenicity and genotoxicity [8 - 14] and few manifest the genotoxicity [15 - 19]. Thus, in order to be conclusive, the present study on the lindane induced genotoxicity is undertaken.

Toxicity induced by lindane is attributed to oxidative stress as it induces the release of free radicals and generation of reactive oxygen species (ROS) [20 - 21]. Due to high chemical reactivity, the free radicals are able to induce cellular damage in a variety of ways. The most deleterious effects of free radicals are damage to DNA [22], which is associated with the process of carcinogenesis.

Antioxidants have gained immense importance in recent years. Antioxidants annul the effect of free radicals produced by toxicants by preventive, interceptive and repair mechanisms [23]. There is a paucity of information regarding the role of combination of antioxidants (vitamin C, vitamin E and alpha-lipoic acid) against the lindane induced genotoxicity.

Cow urine or Gomutra is considered sacred in Hindu mythology. From the ancient period in India, cow's urine has been used as a medicine. Cow urine is elaborately described in ancient scriptures like Charak Sanhita, Rajnighantu,

Brahad-Wagbhatt, Sushrut Sanhita and Amritsagar as bitter, pungent, piquant, warm and full of all the five types of elixirs. It is an anti-poisonous insecticide and a regulator for disorders like gas, acidity and cough. It promotes power of wisdom in human beings, acts like a universal medicine and is easily digested by all [24 - 25]. In Veda, cow's urine was compared to the nectar (Rigveda 10.15). In Sushrut several medicinal properties of cow's urine have been mentioned (45/221). It is mentioned in old Sanskrit script "*gavyam pavitram ca rasayanam ca pathyam ca hradyam balam buddhi syata aayuh pradam rakt vicar hari tridosh hrirog vishapaham syata*". It means that "cow urine panchgavya is great elixir, proper diet, pleasing to heart, giver of mental and physical health strength, and enhances longevity. It enhances bile, mucous and airs. It is a remover of heart diseases and effect of poison." In Ayurveda, clinical effects of cow's urine (Gomutra) were described to counter kapha(mucous) and pitta(bile) dosha. It also acts at cellular level and generates bioenergy [26]. The cow urine contains those substances, which are present in the human body and thus its consumption maintains the balance of these substances and cures incurable diseases [27]. Cow urine is also used alongwith herbs to treat various diseases like fever, epilepsy, anemia, abdominal pain, constipation etc. by the traditional healers [28].

According to our ancestors Cow Urine is considered to be the most effective medicine in the world which during their time was the elixir of many incurable diseases. Though there are numerous examples stating the efficiency of cow urine, scientific reports are few. Thus, to reveal the genoprotective potential of cow urine it was used in the present investigation. *In vitro* genoprotective role of cow urine has been reported [28 - 29] and recent studies have also shown the efficiency of "kamdhenu ark" against *in vivo* genotoxicity caused by organophosphate chlorpyrifos [30]. Hence, present study was

undertaken to investigate the genoprotective efficacy of fresh cow urine and its combined effect along with the various antioxidants.

MATERIALS AND METHODS

Chemicals

Lindane (γ -HCH) was obtained from Sigma chemicals St. Louis, Mo, USA (CAS No. 58-89-9 & purity 97%). Vitamin E, vitamin C and α -lipoic acid were obtained from Himedia, India. All other chemicals and solvents used were of analytical grade. **Cow urine:** Urine of young cow was collected from local cowshed and stored in an air tight bottle for further use.

Animals and treatment

Male Swiss mice, weighing 30 ± 5 g and 8 - 10 weeks old were procured from Cadila Health Care Institute, Ahmedabad. Animals were maintained on sterilized rice husk bedding in polypropylene cages and kept at a temperature of about $23 \pm 3^\circ\text{C}$ with 12 ± 1 h L:D cycle. Animals were fed on standard pelletal diet (Pranav Agro, Baroda). Food and water were *ad libitum*.

Experimental protocol was approved by the Institutional Animal Ethics Committee. Handling of animals was according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Govt. of India.

Dose selection

Dose for lindane was selected after conducting pilot experiments in our laboratory and oral dose of 80 mg/kg b.w. for 96 hrs was selected. This dose was selected on the basis of earlier report (17). There was no mortality in the exposure group during the study.

Doses for antioxidants were calculated keeping the doses prescribed for humans and also in accordance with the previous reports [31 – 33]. The combined dose of antioxidants selected was 125 mg/kg body wt. which included vitamin C 50 mg/kg body wt., vitamin E 50 mg/kg body

wt. and α -lipoic acid 25 mg/kg body wt. Cow urine was administered at a dose equivalent to the corresponding dose for human in ml/kg b.w. i.e., 0.25 ml/kg b.w. which was also used in earlier studies [31].

Doses of lindane, vitamin E and lipoic acid were prepared by dissolving in olive oil. Dose of vitamin C was prepared in distilled water. Cow urine was administered without any modification.

Experimental protocol

8 - 10 weeks old, healthy male Swiss mice weighing 30 ± 5 gms were used in the present study. These animals were randomly divided into eight experimental groups (with 8-10 animals in each group) according to the treatments given as follows:

- Group I: Control (C) group (given olive oil orally);
- Group II: Lindane (L) group (given 80 mg/kg b.w. dose of lindane dissolved in olive oil orally);
- Group III: Antioxidants (A) group (given combination of antioxidants at a dose of 125 mg/kg b.w. orally);
- Group IV: Antioxidants and lindane (A+L) group (given combination of antioxidants (125 mg/kg b.w.) followed by lindane (80 mg/kg b.w.) orally);
- Group V: Cow urine alone (U) group (given 0.25 ml/kg b.w. of cow urine orally);
- Group VI: Cow urine followed by lindane (U+L) group (given cow urine (0.25 ml/kg b.w.) followed by lindane (80 mg/kg b.w.) orally);
- Group VII: Cow urine followed by antioxidants (U+A) group (given cow urine (0.25 ml/kg b.w.) and combination of antioxidants (125mg/kg b.w.) orally)

Group VIII: Cow urine followed by antioxidants followed by lindane (U+A+L) (given combination of cow urine (0.25 ml/kg b.w.), antioxidants (125 mg/kg b.w.) and lindane (80mg/kg b.w.) orally)

In the group IV and VI the antioxidants and cow urine were administered 1 hr prior to lindane administration. In group VII and VIII cow urine was administered 10 min before the antioxidants administration. All the treatments were given continuously for a period of 96 hrs.

Euthanasia

The experimental animals were injected intraperitoneally with colchicine (4 mg/kg b.w.) 1.5 hrs prior to sacrifice. Immediately both the femora were removed *in toto*, by cutting through pelvis and tibia and the bones were then freed from muscle fibres. One of the femurs was used for bone marrow chromosomal assay and the other for micronucleus assay.

Mitotic Chromosome Preparations from Bone Marrow

The mitotic chromosome preparations from bone marrow were prepared according to the method of Preston et al [34]. One hundred metaphases per mouse for each dose were scored (at 1000X magnification) for evaluation of chromosome aberrations. Hence for each group a minimum of 600 metaphases were assessed.

Mitotic Index Determination

The mitotic index was used to determine the rate of cell division. The slides prepared for the assessment of chromosomal aberrations were also used for calculating the mitotic index. The slides were monitored to determine the number of dividing cells (metaphase stage) and the total number of cells. At least 1000 cells were examined per animal of each dose group.

The mitotic index was calculated as the ratio of the number of dividing cells to the total number of cells, multiplied by 100.

$$\text{MITOTIC INDEX} = \frac{\text{No. of Dividing Cells}}{\text{No. of Total Cells}} \times 100$$

Micronucleus Assay

The frequency of micro-nucleated cells in femoral bone marrow was evaluated according to the procedure of Schmid [35].

Scoring of slides: The frequency of micronucleated polychromatic erythrocytes (MnPCEs) was determined by scoring 500 PCEs per animal which accounted to 3000 PCEs per group.

Statistical Evaluation

Values are Mean \pm SD and the results obtained were analyzed using one way ANOVA. Inter group comparisons were performed by using the least significance difference (LSD) test. A probability value of $P < 0.05$, 0.01 was considered as statistically significant.

RESULTS

Chromosomal aberrations

The mean \pm S.D. values and changes in chromosomal aberrations are shown in Figure 1 and the chromosomal aberrations are shown in Figure 2.

Lindane intoxication resulted in a non significant increase in the total chromosomal aberrations as compared to control group. The frequency of aberrations was reduced significantly in groups A+L ($P < 0.05$), U+L ($P < 0.01$) and U+A+L ($P < 0.01$) as compared to lindane group.

An increase of 27.28% in the chromosomal aberrations was observed in lindane intoxicated animals as compared to animals of control group. This increase was not significant. A significant decrease of 24.97% in A+L group, 53.59% in U+L group and 66.13% in U+A+L

group was observed as compared to lindane group.

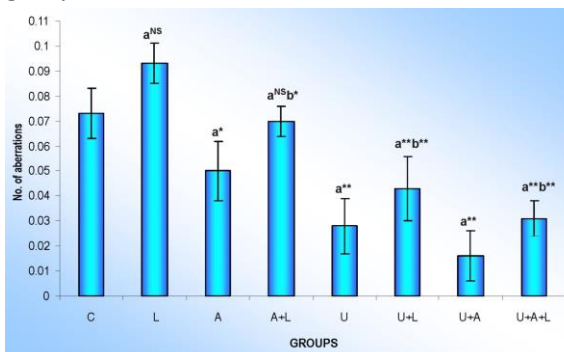


Fig.1: Mean ± S.D. values of total genotoxic effects in various groups (A 96 hr assessment)
a = when compared to control; *b* = when compared to lindane; NS = non significant; * = significant (*P* < 0.05); ** = highly significant (*P* < 0.01)

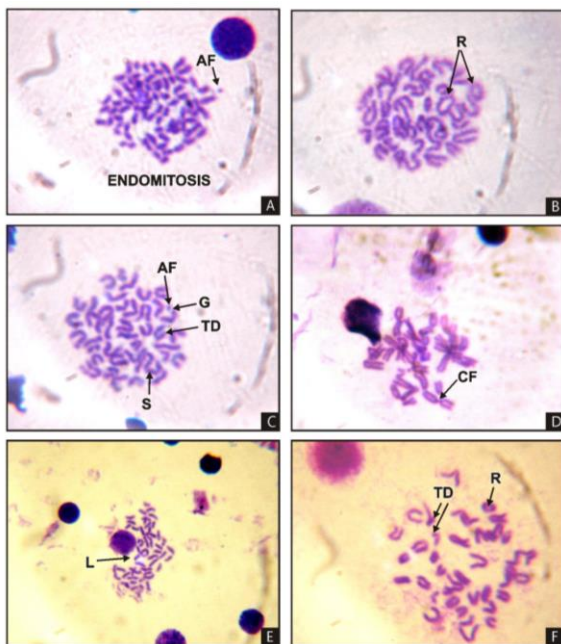


Fig. 2: Photomicrographs showing different chromosomal aberrations in lindane treated animals (1000 X)

AF: Acentric fragment; R: Ring formation; G: Gap; TD: Terminal deletion; S: Formation of S shaped chromosome may be due to joining of two chromosomes or acentric fragments at their sticky ends; CF: Centromeric fusion; L: Loop formation

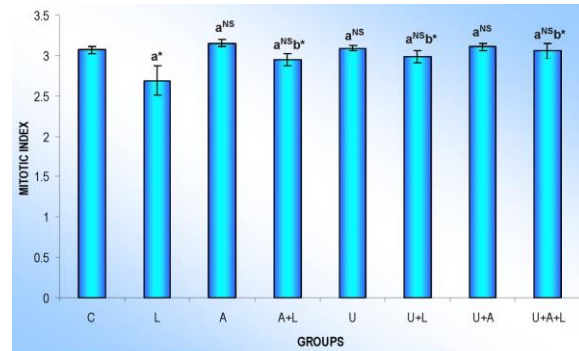


Fig.3: Mean ± S.D. values of mitotic index in various groups (A 96 hr assessment)

a = when compared to control; *b* = when compared to lindane; NS = non significant; * = significant (*P* < 0.05); ** = highly significant (*P* < 0.01)

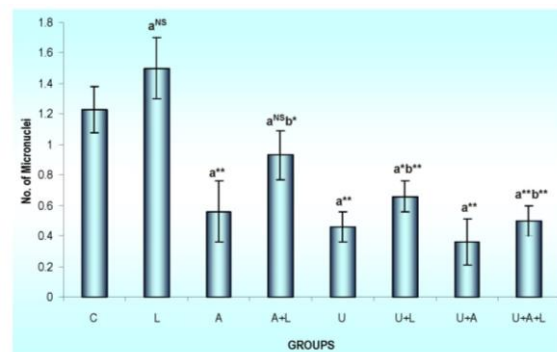


Fig.4: Mean ± S.D. values of % MnPCEs/3000 PCEs in various groups (A 96 hr assessment)

a = when compared to control; *b* = when compared to lindane; NS = non significant; * = significant (*P* < 0.05); ** = highly significant (*P* < 0.01)

The lindane treated animals revealed a few anomalies (Figure 2). The most conspicuous feature was endomitosis as seen in Fig. 2A. Ring chromosomes (R) were also observed (Fig. 2B, 2F). Few metaphase spreads revealed gaps (G) in the chromosomes which resulted in the formation of acentric fragment (AF) (Fig. 2A, 2C). Fig. 2D shows centromeric fusion (CF) of two chromosomes. Terminal deletion (TD) was also observed (2C, 2F) which results in formation of sticky ends. In Fig.2C, S-shaped chromosome structure is seen. This could have resulted due to the fusion of either acentric

fragments or whole chromosomes at the sticky ends formed as a consequence of terminal deletion. All these changes were non-significant. The pretreatment groups significantly reduced all such deformities suggesting that the lindane induced genotoxic effects were modulated by the various treatments.

Mitotic Index

The mean \pm S.D. values of mitotic indices in various groups are shown in Figure 3.

Lindane intoxication showed a significant depression ($P < 0.05$) in the mitotic index as compared to control group. The pretreatment groups A+L, U+L and U+A+L brought about a significant increase ($P < 0.05$) in the mitotic index as compared to lindane group.

As compared to control group, the mitotic index was depressed by 12.34% after lindane intoxication. With the different pretreatments the mitotic index was restored. An increase of 9.73% in A+L group, 10.92% in U+L group, and 13.56% in U+A+L group was observed as compared to lindane group. All these values were significant ($P < 0.05$). In comparison to control group, all the other groups showed non-significant change in the mitotic index.

Micronuclei induction

The mean \pm S.D. values and changes in MnPCEs/3000 PCEs are shown in Figure 4 and the micronucleated polychromatic erythrocytes are shown in Figure 5.

A non-significant increase in the number of micronucleated polychromatic erythrocytes (MnPCEs) was observed in lindane treated animals as compared to the control ones. The pretreatment groups A+L, U+L and U+A+L showed a significant decrease.

In terms of percentage, a non-significant increase of about 21.61% in the number of MnPCEs was observed in the lindane intoxicated group as compared to the control group. Group A+L showed a significant decrease

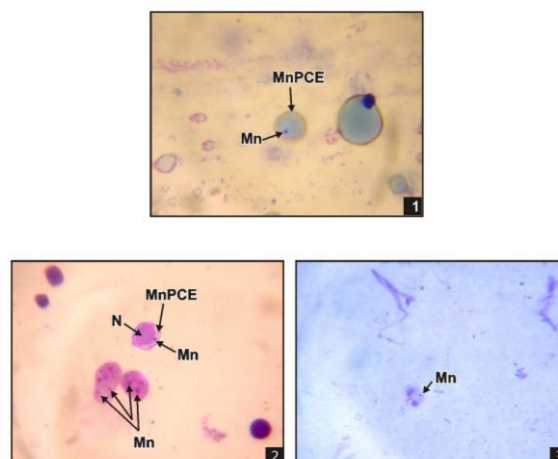


Fig. 5: Photomicrographs showing micronuclei (Mn) and micronucleated polychromatic erythrocytes (MnPCE) aberrations in lindane treated animals (1000 X)

1. Polychromatic erythrocyte (PCE) with micronuclei (MN).
2. A micronucleated polychromatic erythrocyte with the main nucleus (N).
3. A binucleated cell with micronuclei (Mn).

of about 37.77%, group U+L of 55.56% and group U+A+L showed a decrease of 66.66% in the number of MnPCEs as compared to lindane group. Thus the pretreatment groups showed significant protection against micronuclei formation. Micronucleated polychromatic erythrocytes (MnPCE) and micronuclei (Mn) are depicted in Figure 5.

DISCUSSION

Active oxygen species are known to be mutagenic and therefore playing an important role in cancer formation. The mutagenic capacity of free radicals is due to the direct interactions of hydroxyl radicals with DNA [36]. Oxidative stress plays an important role for the initiation of DNA damage.

Our results show that the animals treated subchronically at a dose of 80 mg/kg b.w. lindane for 5 days showed a significant decrease in the mitotic index as compared to the control group animals. Such findings have not been reported yet. A depression in mitotic index

points towards the cytotoxic nature of the chemical which will result in cell death. Lesser number of metaphase spreads also indicates that the toxic effect of the toxicant resulted in decrease in cell population. Thus, this depression in the mitotic index could be attributed to the cytotoxic and apoptotic effect of lindane [37 - 38]. The embryo exposure to lindane during early stages of gametogenesis severely impairs the number of germ cells in the fetal gonads thus affecting primary germ cell (PGC) survival through a direct proapoptotic action [37]. Similarly, it can be assumed that lindane might affect the bone marrow cells and may be responsible for the decrease in mitotic index. Moreover, decrease in the levels of GSH due to lindane toxicity could also have contributed to the cytotoxicity [31- 33, 39].

Ring chromosomes occur when the two ends of a chromosome fuse together and form a ring shape. There are several ways in which this can occur. Breaks in the chromosome arms and fusion of the proximal broken ends can lead to ring formation with loss of distal chromosomal material. Alternatively, rings can be formed by telomere dysfunction. Commonly, ring chromosomes form as spontaneous mutations in response to toxins, radiation, and similar types of exposure. The ring chromosome as observed in our study could be attributed to chromosomal breakage. Gap as seen in our results could have resulted in ring formation. As reported earlier free radicals can also cause oxidation of sulphhydryl groups in proteins and strand scission in nucleic acids [40]. This could be a possible reason for chromosomal gaps and ring formation. Chromosomal clastogeny and gaps as observed in the present study is in accordance with earlier reports [15, 18].

The presence of chromosomal fragment and gaps is further supported by the micronuclei observed in the polychromatic erythrocytes. These micronuclei are the chromosomal fragments which lag during the anaphase and appear as separate nuclei in the erythrocytes.

Micronuclei are formed from acentric fragments of chromosomes or entire chromosomes, which lag behind at anaphase of cell division. After telophase, these fragments may not be included in the nuclei of daughter cells and form single or multiple micronuclei in the cytoplasm. When a bone marrow erythroblast develops into a polychromatic erythrocyte, the main nucleus is extruded; any micronucleus that has been formed may remain behind in the otherwise anucleated cytoplasm. The visualization of micronuclei is facilitated in these cells because they lack a main nucleus. Moreover, in a blood smear the micronuclei are clearly visible as mature erythrocytes are non-nucleated and the micronuclei appear as small nuclei in the cytoplasm. In a blood smear the micronuclei are clearly visible as mature erythrocytes are non-nucleated. Such micronuclei induction by lindane has been reported earlier and thus the results of the present work are in concomitance with earlier reports [17, 19, 41]. At the same time the results of the present study are in disagreement with some of the earlier report [8, 12 - 13] where lindane was not found to induce micronuclei and was reported negative for mutagenicity.

The pretreatment groups namely, A+L, U+L and U+A+L, which were treated with combination of vitamin C, E and lipoic acid, cow urine and both together, respectively, lowered the genotoxic effects of lindane. Both micronuclei formation and chromosomal aberrations were minimized by these pretreatments. The advantage of vitamin C as an antimutagen and antioxidant has been extensively studied *in vitro* and *in vivo*, and it has been shown to decrease chromosomal damage *in vivo* [42]. The antimutagenic actions of different vitamins have been reported by many investigators [43 - 44]. Fawzia [45] found that vitamin C minimized the effect of lead acetate on the mitotic activities.

The role of vitamin C in reducing genotoxicity induced by many agents has been proved [46 - 56]. The efficacy of vitamin E against genotoxicity has also been extensively studied [46, 53, 57 - 59]. Lipoic acid administration also protects against genotoxic challenges [60 - 62]. Moreover, co-administration of vitamin C and E [46, 54], vitamin C, E and B-carotene [63], vitamins A, C and E [64], vitamin C, E and quercetin [65] and lipoic acid and L- carnitine [66] also showed effective genoprotection.

Studies have shown the immunomodulatory [67], antigenotoxic and antioxidant properties *in vitro* and *in vivo* [28, 30, 31], anticlastogenic [29] and chemoprotective [68] effects of distillate and redistillate of cow urine. Furthermore the effect of cow urine therapy has been evaluated on cancer patients [69]. Genoprotective efficacy of distillate and redistillate of cow urine has been studied *in vitro* [28 - 29] and of "kamdhenu ark" *in vivo* [30]. Moreover, it was reported that pretreatment with redistilled cow urine's distillate (RCUD) was found to be more effective than simultaneous administration [29]. It can be assumed that the antioxidant, antigenotoxic and anticlastogenic properties [28-31] of cow urine are responsible for reducing the lindane induced genotoxicity. Moreover, the alleviation in mitotic index due to cow urine pretreatment indicates its efficiency in preventing cytotoxicity.

CONCLUSION

It can be concluded that the given pretreatment of combination of antioxidants and cow urine works synergistically in reducing the genotoxic effects of lindane. Further studies are required to explore the potential of cow urine and its synergistic action along with various antioxidants which could help against pesticide toxicity.

CONFLICT OF INTEREST STATEMENT

None Declared

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