Annexure

Papers so far published/accepted for publication:


Effect of a homeopathic drug, Chelidonium, in amelioration of p-DAB induced hepatocarcinogenesis in mice

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Abstract

Crude extracts of Chelidonium majus, and also purified compounds derived from crude extracts of this plant, have been reported to exhibit anti-viral, anti-inflammatory, anti-tumor and anti-microbial properties both in vitro and in vivo. Chelidonium is a homeopathic drug routinely used against various liver disorders including cancer in humans. Two potencies of Chelidonium (Ch-30, Ch-200) have been tested for their possible anti-tumor and enzyme modulating activities in liver and anticlastogenic effects during p-DAB-induced hepatocarcinogenesis in mice compared to suitable controls.

Methods

Several cytogenetic and enzymatic protocols were used at three fixation intervals; at 60 days, 90 days and 120 days of treatment. Different sets of healthy mice were fed: i) hepatocarcinogen, p-DAB plus phenobarbital (PB), ii) only PB, iii)
neither p-DAB nor PB (normal control). One set of mice fed with p-DAB plus PB was also fed Ch-30 (iv) and another set Ch-200 (v). All standard currently used methods were adopted for cytogenetical preparations and for the enzyme assays.

**Results**

All group (i) mice developed tumors in liver at all fixation intervals, while none of group (ii) and (iii) mice developed any tumors. About 40% mice in group (iv) and group (v) did not show tumor nodules in their liver. Feeding of Chelidonium to group (iv) and (v) mice reduced genotoxic effects to a significant extent (p < 0.05 to p < 0.001).

**Conclusion**

The homeopathic drug Chelidonium exhibited anti-tumor and anti-genotoxic activities and also favorably modulated activities of some marker enzymes. Microdoses of Chelidonium may be effectively used in combating liver cancer.

**Outline**

- Abstract
- Background
- Materials and methods
- Results
- Discussion
- Conclusion
- Acknowledgements
- References
- Pre-publication history

**Background**

*Chelidonium majus* L. (Papaveraceae) is a plant of great interest for its use in various diseases in European countries and in Chinese herbal medicines. Crude extracts of various parts such as the root, shoot and leaves have been reported to have several isoquinoline alkaloids, such as, sanguinarine, chelidonine, chelerythrine, berberine and coptisine. Both crude extracts of *C. majus* and purified compounds derived from it have been reported to exhibit interesting anti-viral, anti-inflammatory, anti-tumor and anti-microbial properties both *in vitro* and *in vivo*[1-3]. Besides, inhibitory effect of *Chelidonium majus* herb extract has been reported on growth of keratinocytes in human, and on lipoxygenase activity in mice [4] while stimulatory effect has been reported on bile acid independent flow in isolated perfused rat liver [2].

In the homeopathic mode of treatment, various micro doses (potencies) of Chelidonium herb extract are routinely used against several forms of liver disorders, including liver cancer [5] with good effect. But, to our knowledge, whether ultra-low doses of *Chelidonium majus*, namely, Chelidonium-30 (Ch-30) and Chelidonium-200 (Ch-200), could also have similar anti-tumor or anti-genotoxic activities had not been experimentally tested so far in mice *in vivo*. The present investigation was therefore undertaken primarily to examine if Ch-30 and Ch-200, prepared as per homeopathic procedure, could show i) anti-tumor activity in liver, ii) anti-clastogenic effect in bone marrow cells, iii) protective/repair ability on sperm heads, and iv) ameliorating effects in the activities of some marker enzymes like acid and alkaline phosphatases, and peroxidase in various tissues during azo dye induced hepatocarcinogenesis in mice.
**Outline**

- Materials and methods
  - Materials
    - An inbred strain of Swiss albino mice (*Mus musculus*), reared and maintained in the animal house of the Department of Zoology, (under the supervision of the Animal Welfare Committee), Kalyani University, served as materials for the present study. Mice were provided food and water *ad libitum*. The food was made of wheat, gram and powdered milk without any animal protein supplement. With due permission from the Animal Welfare Committee, Kalyani University, which also oversees ethical issues of animal experimentation, the present investigation was undertaken. A group of 25 healthy mice weighing between 25-30 grams were used for each of the three long term fixation intervals viz. 60, 90 and 120 days. Each group was further divided into five different sets consisting of five mice each. The first set of mice were allowed normal low protein diet mixed with 0.06% p-DAB (Sigma, D-6760), a known "initiator" of hepatocarcinoma, and water *ad libitum*, till 30 days after which the water was replaced with 0.05% aqueous solution of PB, a known "promoter", till they were sacrificed. The second set of mice were provided with low protein diet without p-DAB and 0.05% aqueous solution of PB instead of pure water after one month as in group (i) till they were sacrificed. For the third set of mice the low protein diet was neither mixed with p-DAB nor water was replaced with PB. The third set served as negative control. The fourth set of mice were given p-DAB and PB in the same way as that of the first group but were also fed 0.06 ml of stock solution of the drug-Ch-30 thrice a day (6 A.M, 12 Noon, 6 P.M) from first day onward of p-DAB feeding, for seven days, and then twice a day (6 AM, 6 PM) till they were sacrificed. In the fifth set of mice the feeding of p-DAB, PB and Ch-200 followed the same manner as that of the fourth set of mice, except that the drug was fed twice a day (6AM, 6PM) all along till they were sacrificed.

- Preparation of the potentized homeopathic drug
  - The two potencies of Chelidonium, procured from "HAPCO", 165, Bipin Behari Ganguli Street, Kolkata, were prepared as per the standard procedure of homeopathic drug preparation. The dry drug material of *Chelidonium majus* (whole plant) was extracted in 44% ethyl alcohol (i.e. the "mother tincture"). 1 ml of the mother tincture was subsequently diluted with 99 ml HPI approved solvent (IP 96/HPI grade ethyl alcohol) and "succussed" 10 times to make the potency 1. The potency 2 was similarly made by diluting 1 ml of potency 1 with 99 ml of ethyl alcohol and giving 10 jerks, and the procedure was repeated to get the microdoses of Ch-30 and Ch-200.

- Preparation of stock solution of the drug
  - 1 ml each of Ch-30 and Ch-200 was finally diluted separately with 20 ml of double distilled water to make the stock solution of Ch-30 and Ch-200, respectively.
Feeding procedure and dose

Each mouse was fed 1 drop (0.06 ml) of either Ch-30 or Ch-200 from the stock solutions at a time with the aid of a fine pipette.

Cytogenetic assay

Mice were intra-peritoneally injected with 0.03% colchicine @ 1 ml/100 gm body weight 1 hr and 30 min before sacrifice. Marrow of the femur was flushed in 1% sodium citrate solution at 37°C and fixed in acetic acid/ethanol (1:3). Slides were prepared by the conventional flame drying technique followed by Giemsa staining for scoring bone marrow chromosome aberrations. A total of 500 bone marrow cells were observed, 100 from each of 5 mice of a set.

For micronucleus (MN) preparation, a part of the suspension of bone marrow cells in 1% sodium citrate was smeared on clean grease free slides, briefly fixed in methanol and subsequently stained with May-Grunwald followed by Giemsa. Approximately 5000 bone marrow cells, comprising both polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) were scored and the ratios between PCE and NCE were calculated.

The mitotic index (MI) was determined from the same slide which was scanned for MN. The non-dividing and dividing cells were recorded and their ratios ascertained.

For sperm head anomaly (SHA), the epididymis of each side of mouse of both (control and treated) sets was dissected out and its inner content squeezed out into 10 ml of 0.87% normal saline separately. It was made free of fats, vas deferens and other tissues. The content was thoroughly shaken, filtered through a silken cloth and dropped on grease free clean slides. The slides were allowed to air dry and then stained by dilute Giemsa (1 ml Giemsa in 10 ml distilled water).

Biochemical assays

Mice were sacrificed and their liver, spleen and kidney were quickly isolated. The tissues were homogenized with cold 0.87% normal saline, followed by centrifugation at 3000 g for 20 minutes in cooling centrifuge (C24, Remi Instruments). Before carrying out the enzymatic estimations the quantitative estimation of total protein was made by the method of Lowry et al[7]. To 0.1 ml of the sample, 0.9 ml of 0.1 (N) NaOH was added. Then 5 ml of alkaline solution was added to the sample solution followed by 0.5 ml of Folin-Phenol reagent and after 30 minutes the extinction was read at 750 nm against appropriate blank in spectrophotometer (Shimadzu, Double beam Spectrophotometer UV-180, Japan).

Estimation of Lipid Peroxidase:

The lipid peroxidation was estimated from the supernatant by the method of Buege and Aust [8]. 1 ml of sample (homogenate containing 0.1-0.2 mg of protein) was mixed thoroughly with 2
ml of TCA-TBA-HCl (15% w/v TCA and 0.375% w/v TBA in 0.25 N HCl). The absorbance of the sample was determined at 535 nm in a double beam spectrophotometer against a suitable blank. The malonaldehyde concentration of the sample was calculated by using extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$.

**Estimation of Acid and Alkaline Phosphatases**

For the study of acid and alkaline phosphatases method of Walter and Schutt [9] was followed. For acid phosphatase, to 0.2 ml tissue homogenate 1 ml of acid buffer was added to make the volume 1.2 ml. It was mixed and incubated at 37°C for 30 minutes. Then 2 ml of 0.1 (N) NaOH was added. The absorbance was measured at 405 nm against a blank. Then the activity of acid phosphatase was calculated as mM phenol liberated per mg of protein after 30 min of incubation at 37°C using suitable standard curve.

For alkaline phosphatase activity the 0.05 ml homogenate was mixed with 2 ml alkaline buffer so that the volume always stood at 2.05 ml. It was incubated at 37°C for 30 minutes, then 10 ml of 0.05 N NaOH was added and the absorbance was measured at 405 nm against a blank. The activity of alkaline phosphatase was calculated as mM phenol liberated per mg of protein after 30 min of incubation at 37°C using suitable standard curve.

**Statistical analysis and scoring of data**

The significance test between different series of data was conducted by student’s t-test. During preparation of slides for cytogenetical observation and biochemical estimation of the different enzymes, the “observer” was kept “blinded” in order to remove any “bias” in observation and to keep uniformity in scoring data of both treated and control sets of mice.

**Outline**

- Abstract
- Background
- Materials and methods
- Results
- Discussion
- Conclusion
- Acknowledgements

**Results**

Out of the total number of 45 mice fed with p-DAB plus PB and sacrificed at three fixation intervals, livers in some 27 mice showed distinct sign of tumor formation in the form of pale reddish multiple nodules (some other relevant histo-pathological data of liver in p-DAB+PB treated and untreated control mice also provided in table-1), while the remaining ones did not develop such nodules. All mice fed p-DAB plus PB but no homeopathic drug developed tumorous nodules in liver and also had appreciably enlarged spleen. However, every 2 out of 5 mice that received either Ch-30 or Ch-200 alongside p-DAB plus PB did not show tumorous nodules, although they had slightly
enlarged spleen. Thus, in the present experiment there was an overall 40% decline in the occurrence of tumors owing to the treatment of Ch-30 and Ch-200.

**Cytogenetical Studies**

As compared to normal metaphase plates (Fig. 1a) which did not normally reveal any aberrations, various types of chromosome aberrations of both major (Figs. 1b, 1c, 1d, 1e, 1f, 1g) and minor nature (Fig. 1f, 1g) were encountered in certain metaphase plates of mice that received p-DAB and/or PB treatments (see Table 2). The total frequencies of aberrations were found to be maximum in the p-DAB and PB fed mice and the aberrations were considerably reduced in both the drug fed series (Fig. 2). However while Ch-30 appeared to protect the bone marrow cells at a higher scale at 60 and 90 days, Ch-200 showed greater protection at 120 days (p < 0.001; see Table 2). The mice fed PB alone had less number of chromosome aberrations than in the p-DAB + PB treated series and differences in the % of the CA, when compared with that of the normal controls were found to be statistically significant at 90 (p < 0.05) and 120 days (p < 0.01).

**Micronucleated erythrocytes**

Data on occurrence of micronuclei in polychromatic (Fig. 1m) erythrocytes (PCE) and normochromatic (Fig. 1l) erythrocytes (NCE) have been provided in Table 2. The percentages of MN were highest in the p-DAB and PB fed mice. Both Ch-30 and 200 feeding reduced the occurrence of MN. Ch-200 showed more pronounced action (p < 0.05) at 60 and 120 days (Fig. 3). PB itself produced a few micronucleated erythrocytes not significantly different from that of normal controls (Table-2).

**Mitotic index**

In both Ch-30 and 200 fed mice, the MI was much less than in the p-DAB plus PB fed mice and the protection was statistically significant (p < 0.05 through p < 0.001). The mitotic index in the PB fed mice was only slightly more than in the normal control series (Table-2, Fig. 4).

**Sperm head anomaly**

As against sperm with normal head morphology (Fig. 1l), quite high incidence of sperm showing some form of abnormal head morphology (Fig. 1j, 1k) has been recorded in the different treatment series (see Table 2, Fig. 5). Both Ch-30 and Ch-200 reduced considerably the percentages of sperm with abnormal head morphology and the differences were statistically significant (p < 0.05 through p < 0.001). The feeding of PB alone also produced abnormal sperm in greater number than in the normal control and the differences were statistically significant at 60 days (p < 0.05) and at 120 days (p < 0.01).

**Lipid peroxidase activity**

The lipid peroxidase activity was the highest in all the three
tissues at all fixation intervals in the p-DAB+ PB treated series except at 90 and 120 days in the liver where the activity was the highest in the p-DAB + PB+Ch-200 fed mice (Fig. 6). However, interestingly enough, while the lipid peroxidase enzyme activity was generally much reduced in the Ch-200 fed mice as compared to Ch-30 fed mice in spleen and kidney, the lower micro dose i.e. Ch-30 appeared to reduce the activity in liver (p < 0.05) more than that of Ch-200 at all fixation intervals (p < 0.01 to p < 0.001, see table 3). The feeding of PB alone produced similar or marginally increased activity in spleen, kidney and liver at 60 and 90 days but the activity became appreciably higher in the liver and kidney at 120 days but not in the spleen.

**Alkaline phosphatase activity**

The alkaline phosphatase activity (AlkPA) in the p-DAB + PB fed mice was highest in spleen and liver at all the three fixation intervals while the activity declined in both Ch-30 and Ch-200 fed mice (p < 0.05, p < 0.001, see table-3, Fig. 7). Ch-200 showed greater efficacy in reducing AlkPA than that of Ch-30 fed mice except at 90 days in case of spleen. In the kidney, however, although p-DAB + PB showed, except for a few cases, a high degree of AlkPA at all fixation intervals, the activity could not be reduced by Ch-200 till at 120 day (p < 0.05) while the activity level became actually higher at 60 and 90 days. However, Ch-30 could reduce the activity to some extent at all the fixation intervals although the differences were not statistically significant.

**Acid phosphatase activity**

The acid phosphatase activity (AcPA) in spleen, liver and kidney was very high in p-DAB + PB fed mice and the activity was slightly declined in the Ch-30 and Ch-200 fed mice except at some fixation intervals where actually it was higher than in the p-DAB + PB fed mice. The differences were not statistically significant in most cases and only some favorable modulation was noted in liver and kidney at 120 days (p < 0.05, p < 0.001; see table 3, Fig. 8).
Figure 8

Showing acid phosphatase activity in liver of experimental mice at different fixation intervals.

Tables

Table 1

Showing some salient changes in liver histology of p-DAB+PB treated mice (showing tumors) as compared to untreated controls

Table 2

Showing frequency distribution of mitotic indices (MI) in 5000 cells, chromosome aberration (CA) in 500 cells, micronuclei (MN) in polychromatic (PCE) and normochromatic (NCE) erythrocytes in 5000 cells and sperm head anomaly (SHA) in 5000 sperm in mice treated with p-Dimethylaminoazobenzene (DAB) + Phenobarbital (PB), DAB + PB + Chelidonium (Ch)-30 and DAB + PB + Chelidonium (Ch)-200 and their Phenobarbital treated and normal controls. Equal number of cells/sperrms observed from each of 5 mice of a set.

Table 3

Mean activities of lipid peroxidase (nMol MDA/gm wet tissue), acid and alkaline phosphatase activities (in terms of mM Phenol liberated/100 mg protein after 30 min of incubation at 37°C), in different organs of mice treated with p-DAB+PB.
It had earlier been conclusively demonstrated that feeding of carcinogenic azo dyes produced liver damage followed by regeneration of parenchymal cells, proliferation of bile ducts and connective tissue, and at later stages tumors developed from liver parenchyma that ended up with neoplastic characteristics \[10\]. Further, it was also demonstrated that dietary PB had positive carcinogenic effect only when fed with the azodye 2-methyldiaminoazobenzene, but neither of these two when fed alone showed positive hepatocarcinogenesis in both mice and rat \[11,12\]. It is generally accepted that covalent binding of the metabolites of p-DAB (e.g. MAB, AAB etc.) with DNA is a major carcinogenic factor \[13\]. In the present study, the only PB fed mice also did not develop tumors in liver, while those fed with p-DAB + PB developed tumors. However, interestingly enough, every 2 out of 5 mice, that is, about 40% of mice that received both p-DAB and PB along with either Ch-30 or Ch-200 did not develop tumors in liver while all mice fed p-DAB plus PB, but no Chelidonium developed tumors. This seems to be a dramatic finding as neither of these potentized drugs Ch-30 or Ch-200 had literally a single molecule of original drug substance in their diluted forms and were yet capable of reducing/delaying tumor growth in mice. Such agents that can antagonise or render protection at various levels of carcinogenesis are always considered very important, particularly so when they can be administered in micro doses and they do not have any ill-effects/side-effects of their own. When microdoses of either of these drugs were fed alone to healthy mice in similar doses, and dissected at corresponding fixation intervals, no tumor was found in their liver, nor was any genotoxic effect found from the assay of their chromosomes, sperm heads, or micronuclei. Earlier, Roberfroid et al\[14\] reported that micro doses of PB 9C positively reduced the incidence of tumors and mortality in rats chronically fed with another carcinogen Acetylaminofluorine along with PB. However, these authors did not consider any of the protocols used in the present study. Fisher \[15\] reported that the DNA repair mechanisms of cultured mammalian cells in vitro could be stimulated by very small doses of mutagens while working with human lymphocytes.

Extensive toxicological investigations have now established that increase in lipid peroxidation, alkaline and acid phosphatase activities along with decreased level of glutathione actually denote cytotoxicity and hepatocellular dysfunction \[16-19\]. The favorable modulations of some of these enzymes, chromosomal and sperm head damages noted in the Chelidonium fed mice as compared to drug unfed p-DAB plus PB fed mice were very significant. Thus it was suggestive that the micro doses of Ch i.e. 30 and 200 had positive protective effects against tumors induced by p-DAB plus PB, and that Ch-200 appeared to have marginally better effects at the longer intervals. Incidentally, the
efficacy of serial agitated dilutions of homeopathic drugs in experimental toxicology had also been convincingly advocated by meta-analysis done by Linde et al.[20].

p-DAB and its metabolites have been reported to cause oxidative DNA damage [13], which could also be attributable to the various types of chromosome aberrations encountered in the present investigation. The formation of adducts, DNA-copper-hydroperoxo complexes, etc as suggested by Ohnishi et al.[13], could also play an important role in the carcinogenic processes of p-DAB. Therefore antagonism in an unknown manner to either formation of various metabolites of p-DAB or else in their formation of adducts in DNA could have been one of the major ways by which the potentized Chelidonium acted in the mice treated with the azo dye to reduce the chromosomal damage.

It is difficult to understand precisely at the present state of our knowledge how the ultra low doses of Chelidonium could achieve such spectacular protective changes which were amply demonstrable in the present study with the different protocols used. One hypothesis to explain the possible mechanism of action of the micro doses could be as follows. Since these low doses of the medicines were administered orally, their actions could possibly be mediated through the receptor systems located on tongue and the oral cavity. The drugs must have emanated specific signals in the receptor cells that could activate specific region of brain (presumably hypothalamus) in a manner that could possibly help elicit further signals to activate or repress certain transcriptional activities of specific regions of DNA meant for restoration of the damages caused due to the carcinogenic interaction. One way to test this hypothesis can be either to measure the activity of the signal transduction system through estimation of secondary messenger (i.e cyclic AMP) or else to block the pathway selectively after use of any drug in such microdoses. Incidentally, when some potentized homeopathic drugs were used along with Actinomycin D, a transcription blocker, the homeopathic drugs failed to elicit the desired protective levels against chromosomal and other cytogenetical damages produced by arsenic intoxication and by ultrasonic sound waves [21,22] as compared to when the drugs were administered alone (i.e. without Actinomycin-D). Thus the repair mechanisms were essentially mediated through active gene action. Khuda Bukhsh [23] proposed a hypothesis to explain the possible mechanism of action of such micro doses based on many circumstantial evidences [21-33] that the micro doses act through the regulation of relevant gene expression by eliciting impulses similar to that of some hormones and enzymes. That can suitably explain the mechanisms involved in the repair of damaged chromosomes or sperm head by the application of the micro doses. This can also explain the antagonizing action against tumorgenesis/carcinogenesis which is essentially a multi-gene and multi-step process in majority of cancer including hepatocarcinoma [34]. Since the activation of proto-oncogene to oncogene is the key event for the transformation of the normal hepatocyte to a malignant liver tumor cell and that this process is controlled by the interactions of many tumorigenic and tumor suppressor genes (e.g. p53 gene), it may be speculated that the micro doses of the drug might have interfered with the process of carcinogenesis either by actively modifying action of certain of these genes responsible for the transformation of cells to
cancerous ones by release of specific transcription factors, or else by activating certain tumor-suppressor genes in an unknown manner. A careful study of telomerase activity in these experimental mice can also be helpful in understanding the underlying genetic mechanism. In fact the modulating effects of the drug on restoration of damage caused to several gene regulated phenomena like enzyme activities, chromosome and sperm structure etc would further strengthen the contention that these drugs possibly acted through regulatory actions on a number of key genes, related not only to the structure and normal functioning of liver hepatocytes, but also to the ones meant for maintaining integrity of bone marrow chromosomes and sperm head.

Outline  Conclusion

From the present investigation, it becomes evident that ultra-low doses of the homeopathic drug Chelidonium are also capable of rendering anti-tumor and anti-genotoxic activities against azo-dye induced hepatocarcinoma in mice. This would encourage their use even in cancerous liver disorders with greater degree of assurance of their efficacy. Further, it would hopefully open up new vistas for understanding the little known mechanism of action of the homeopathic microdoses which in many instances do not even contain a single molecule of the original drug substance.

Outline  Acknowledgements

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Cytogenetic Effects of Sonication on Spathosternum prasiniferum (Grasshopper), Anabas testudineus (Fish), and Mus musculus (Mammal)


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Because of its superiority and several advantages over other methodologies like X-rays in pinpointing location/site of abnormalities in internal anatomy of human beings without causing any apparent injury to them, the use of ultrasonic sound waves is being increasingly made in modern diagnostics e.g. for detection of various anatomical disorders, blockage or overgrowths (tumours etc.), position of foetus, appendicitis etc. (Simonovsky 2000; Princi et al. 2000) and therapies (Kremkau 1979; Marmor 1983), thereby inviting attention to test whether its use as a medical tool is otherwise absolutely safe. Contrary to the reports of several workers that ultrasonic irradiation have certain undesirable biological effects, producing enzymatic, physiological, biochemical and genomic (DNA) changes (Hedges and Leeman 1979; Leibeskmd et al. 1979, Haupt et al. 1981) a large number of workers failed to confirm such effects particularly by testing effects on DNA of lymphocytes or sperm chromosomes in vitro of subjects exposed to ultrasonic irradiation and therefore, advocated its safe use as a tool in medical and therapeutic practices (Miller et al. 1983, Ciaravino et al. 1985, 1986, Becher et al. 1983; Au et al. 1982, Lundberg et al. 1982, Tateno et al 2000) This controversy prompted us to re-examine the cytogenetic effects in vivo, if any, in three different test models viz. grasshopper (invertebrate), fish (lower vertebrate) and mice (higher vertebrate)

MATERIALS AND METHODS

Live adult specimens of grasshopper (Spathosternum prasiniferum) of male sex, of fish (Anabas testudineus) and mammal (Mus musculus) of both sexes served as the test materials for the present study.

Ultrasonic Sound Exposure. For ultrasonic irradiation, the machine used was an ultrasonic cell disrupter (LSI SECFROID SA Microson Model XL 2005, Switzerland) which generated sound waves of 20-23 KHz. The microprobe (tuned to vibrate at 20 KHz) was inserted into water slightly above the experimental specimens submerged in water in a suitable glass jar (containing just enough water to enable fish specimen to submerge its dorsal fin under water and enough water to submerge mice specimens leaving its head free of water) For grasshoppers, the microprobe was directly touched onto the dorsal thoracic part of the specimens. During sonication, ice was placed around the glass jar containing water and the specimen, preventing water inside to become too hot during sonication.

Acute ultrasound exposure series: Half of total number of live specimens were selected randomly and exposed to sonication (treatment group) for 1 minute, given a rest period of 1 minute and then again subjected to 1 minute irradiation at an output percentage of 70. Then they were sacrificed at 2hr, 24hr and 48 hr. A total number of 30 specimens each of grasshoppers and fish and 50 specimens of mice were used for the present investigation.

Methodologies To study the meiotic chromosomes the routine squash preparation of
asshoppers' testes was made at 2, 24 and 48 hrs after sonication and stained with ematoxylin. The conventional citrate-flame-drying-Giemsa stain schedule was opted for preparation of somatic chromosomes from bone marrow cells of mice and kidney and gill cells of fish, and germinal chromosomes from testis of mice.

r study of micronucleus induction, routine smear preparations of peripheral blood mfish and bone marrow cells from mice were made at 24 and 48 hr after sonication and were stained with May-Grunwald Mitotic index was ascertained from the marrow preparations of mice.

study of sperm shape anomaly, smeared preparation of sperm of mice sacrificed 24 and 48 hr after sonication were made from epididymis and stained with emsa.

embryotoxic study, 15 females were exposed to ultrasonic irradiation only once 2nd, 4th, 6th, 8th or 10th day after conception and they were dissected on the 15th of gestation to examine any possible embryotoxic effect by examining the presence of any foetus or presence of any abnormal foetus or scar-mark indicating the presence of foetus or scar-mark indicating the presence of foetus.

onic ultrasonic sound exposure series. In another set of experiment, a total number of 40 specimens of mice were used to examine the chronic or "cumulative" effect, if any, of ultrasonication, mice were subjected to repeated exposure for a total of 2 min each at an interval of 20 days. In the same way in acute exposure series, so that mice sacrificed at 30, 60, 75 and 90 days after the initial dose actually lived 2, 3, 4 and 5 such doses of sonication, respectively. In this series bone marrow chromosome aberration, micronucleus induction and sperm shape abnormalities were studied. 20 specimens each were used as treatment and control lots.

Trol Series In case of both mice and fish models, specimens identical in number of treatment series were sham exposed to the chambers but not actually treated and sacrificed at different fixation intervals served as controls for each p of treatment. Similarly, normal grasshoppers collected from the wild and mated in laboratory condition for different fixation intervals served as controls. Actual numbers of cells examined for different parameters for different species have been mentioned in the respective tables.

ULTS AND DISCUSSION

ultrasonic sound exposed series, structural chromosomal damages (breaks, 13ents, sticky-bridges etc.) as well as damages of lesser significance (stickiness, consonance etc.) were observed in different meiotic divisional stages (Figs 1-4) of grasshoppers and their frequency distribution has been summarized in Table-1. In

1. Frequency distribution of major aberration types observed in grasshopper autotocytic chromosomes after exposure to ultrasonic sound (T) and in control grasshoppers (C), at different fixation intervals.

<table>
<thead>
<tr>
<th>Series</th>
<th>% of aberrations in different meiotic stages ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dikinesis</td>
</tr>
<tr>
<td>C1</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>T1</td>
<td>10.7 ± 2.3^a</td>
</tr>
<tr>
<td>C2</td>
<td>3.2 ± 2.1</td>
</tr>
<tr>
<td>T2</td>
<td>5.3 ± 3.6</td>
</tr>
<tr>
<td>C3</td>
<td>1.0 ± 1.0</td>
</tr>
<tr>
<td>T3</td>
<td>2.7 ± 1.1</td>
</tr>
</tbody>
</table>

* /tension includes break, constriction,acentric fragment, sticky bridge, dicentric bridge, used etc., a = p<0.05, b = p<0.01, c = p<0.001.

Individuals examined in each case =15; cells scored per individual =100
Figures 1-18. Photomicrographs of spermatocyte complements of grasshoppers showing: break and acentric fragment (Fig.-1), stretching (Fig.-2), constriction (Fig.-3) and a sticky bridge (Fig.-4); somatic metaphase complements of Anabas showing a normal plate (Fig.-5) and plates with aberrations like stickiness (Fig.-6), C-mitotic effect (Fig.-7), and somatic metaphase complements of mice showing polyploidy (Fig.-8), c-mitotic effect (Fig.-9) and crumpledness (Fig.-10); stickiness (Fig.-11), poly-chromatic (Fig.-12) and normochromatic erythrocytes (Fig.-13) showing MN, sperm showing abnormal head shapes (Figs.-14 & 15); germinal plates of male mice showing, ring (Fig.-16), multivalent association (Fig.-17) and abnormal spermatogonial metaphase with chromatid break (Fig.-18).
general, the frequencies of aberrations were found to be maximum at 2 hr, which declined with the lapse of time. No aberration was noted in the diplotene, either in the sonicated or in normal grasshoppers. In fact, the maximum aberrations were contributed by condensed chromosomes, possibly due to excessive spiralization effect. On the other hand, in case of somatic chromosomes of both fish and mice (Table 2), no break type or any other major type aberrations such as gaps, changes, fragment etc. could be encountered on somatic metaphase spreads. However, clastogenic effects of lesser significance such as stickiness, crumpledness, indensation, c-mitotic effect, polyploidy etc. could be encountered in quite a good number of metaphase spreads, of both fish (Figs. 5-7) and mice (Figs. 8-11) particularly at the early intervals, which again declined with the lapse of time. There was statistically insignificant increase of micronuclei (Figs. 14-15) in the sonicated series vis-a-vis controls (Table 3) in mice, the mitotic index of bone marrow cells is also found to be slightly enhanced by the ultrasound exposure (Table 3). However, the number of abnormal sperm heads (Figs. 12-13) was more in the sonicated series at both 24 and 48 hr which was statistically significant as compared to controls (Table 3). There were also elevated frequency of aberrations in the

Table 2. Frequency distribution of different types of aberrations examined in sonicated fish (T2) and mice (T3) and in their respective controls (C2 and C3).

<table>
<thead>
<tr>
<th>Fixation time intervals (hr)</th>
<th>Series</th>
<th>% of aberration ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fish</td>
<td>Mice</td>
</tr>
<tr>
<td></td>
<td>C2</td>
<td>C3</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>T3</td>
</tr>
<tr>
<td>2</td>
<td>0.8 ± 0.2</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>17.4 ± 1.1*</td>
<td>29.8 ± 0.4*</td>
</tr>
<tr>
<td>24</td>
<td>0.6 ± 0.3</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>12.0 ± 1.1*</td>
<td>19.0 ± 0.9*</td>
</tr>
<tr>
<td>48</td>
<td>1.2 ± 0.2</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>7.6 ± 0.9*</td>
<td>18.0 ± 0.7*</td>
</tr>
</tbody>
</table>

Aberrations include polyploidy, aneuploidy, stickiness, c-mitotic effect, pyknosis, condensation, crumpled etc. No of Individuals examined in each series/fixation intervals = 5; cells scored per individual = 500.

Table 3. Frequency distribution of MI, MN in NCE and PCE in sonicated mice (T3) against their respective controls (C3) at different fixation intervals.

<table>
<thead>
<tr>
<th>Fixation time intervals</th>
<th>Series</th>
<th>% of MN in NCE</th>
<th>% of MN in PCE</th>
<th>Total % of MN in NCE and PCE ± SE</th>
<th>P/N ratio</th>
<th>MN NCE% ± SE</th>
<th>MI % ± SE</th>
<th>SHA % ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hr</td>
<td>C3</td>
<td>0.06 ± 0.05</td>
<td>0.06 ± 0.02a</td>
<td>0.5 ± 0.04 ± 0.6 ± 0.08</td>
<td>0.8 ± SE</td>
<td>0.4 ± 0.06</td>
<td>0.2 ± 0.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>0.35 ± 0.63</td>
<td>0.44 ± 0.02a</td>
<td>0.8 ± 0.21 ± 1.8 ± 0.09a</td>
<td>0.8 ± SE</td>
<td>0.4 ± 0.05</td>
<td>0.2 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>48 hr</td>
<td>C3</td>
<td>0.09 ± 0.08</td>
<td>0.08 ± 0.02</td>
<td>1.0 ± 0.04 ± 0.2 ± 0.04</td>
<td>0.8 ± SE</td>
<td>0.2 ± 0.14</td>
<td>1.5 ± 0.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>0.49 ± 0.39</td>
<td>0.44 ± 0.05a</td>
<td>0.8 ± 0.21 ± 1.8 ± 0.09a</td>
<td>0.8 ± SE</td>
<td>0.4 ± 0.05</td>
<td>0.2 ± 0.04</td>
<td></td>
</tr>
</tbody>
</table>

MN= Micronucleus, NCE= Normochromatic Erythrocytes, PCE= Polychromatic Erythrocytes, MI= Mitotic Index, SHA= Sperm Head Anomaly

* p < 0.05

No of Individuals examined in each study/series/fixation intervals = 5; cells scored per individual = 5000.
Table 4. Frequency distribution of different types of aberrations examined in meiotic chromosomes of sonicated mice (T\textsubscript{3}) and in controls (C\textsubscript{3}).

<table>
<thead>
<tr>
<th>Fixation time intervals</th>
<th>Series</th>
<th>% of aberration ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hr</td>
<td>C\textsubscript{3}</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>T\textsubscript{3}</td>
<td>1.8 ± 0.3\textsuperscript{c}</td>
</tr>
<tr>
<td>48 hr</td>
<td>C\textsubscript{3}</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>T\textsubscript{3}</td>
<td>0.9 ± 0.1\textsuperscript{c}</td>
</tr>
</tbody>
</table>

Aberration includes multivalent association, ring, polyploidy, stickiness, pulverisation, chromatid break, terminal association etc.

Table 5. Frequency distribution of CA, MN in NCE and PCE and SHA in sonicated mice (T\textsubscript{3}) against their respective controls (C\textsubscript{3}) at different (long term) fixation intervals.

<table>
<thead>
<tr>
<th>Fixation time intervals</th>
<th>Series</th>
<th>% of CA ± SE</th>
<th>% of MN in NCE</th>
<th>% of MN in PCE</th>
<th>% of MN in NCE and PCE ± SE</th>
<th>P/N ratio</th>
<th>% of SHA ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 d</td>
<td>C\textsubscript{3}</td>
<td>0.40 ± 0.25</td>
<td>0.06</td>
<td>0.07</td>
<td>0.06 ± 0.03</td>
<td>0.45</td>
<td>0.18 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>T\textsubscript{3}</td>
<td>35.90 ± 0.23\textsuperscript{e}</td>
<td>0.25</td>
<td>0.52</td>
<td>0.40 ± 0.09\textsuperscript{a}</td>
<td>1.14</td>
<td>5.56 ± 0.11\textsuperscript{a}</td>
</tr>
<tr>
<td>60 d</td>
<td>C\textsubscript{3}</td>
<td>0.38 ± 0.25</td>
<td>0.06</td>
<td>0.06</td>
<td>0.07 ± 0.03</td>
<td>0.45</td>
<td>0.19 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>T\textsubscript{3}</td>
<td>23.00 ± 0.77\textsuperscript{e}</td>
<td>0.22</td>
<td>0.74</td>
<td>0.42 ± 0.10\textsuperscript{b}</td>
<td>0.61</td>
<td>0.88 ± 0.10\textsuperscript{b}</td>
</tr>
<tr>
<td>75 d</td>
<td>C\textsubscript{3}</td>
<td>0.42 ± 0.25</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06 ± 0.03</td>
<td>0.50</td>
<td>0.16 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>T\textsubscript{3}</td>
<td>25.60 ± 0.51\textsuperscript{c}</td>
<td>0.39</td>
<td>0.65</td>
<td>0.49 ± 0.11\textsuperscript{b}</td>
<td>0.60</td>
<td>0.98 ± 0.02\textsuperscript{b}</td>
</tr>
<tr>
<td>90 d</td>
<td>C\textsubscript{3}</td>
<td>0.40 ± 0.25</td>
<td>0.06</td>
<td>0.06</td>
<td>0.05 ± 0.03</td>
<td>0.45</td>
<td>0.18 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>T\textsubscript{3}</td>
<td>29.40 ± 0.50\textsuperscript{b}</td>
<td>0.55</td>
<td>0.74</td>
<td>0.06 ± 0.09\textsuperscript{b}</td>
<td>0.37</td>
<td>1.48 ± 0.11\textsuperscript{c}</td>
</tr>
</tbody>
</table>

Chromosome aberrations (CA) include gap, break, centric fusion, translocation, fragment, pulverisation, ring, terminal association, polyploidy, aneuploidy, stickiness, C-mitotic effect, etc.

Table 4 includes multivalent association, ring, polyploidy, stickiness, pulverisation, chromatid break, terminal association etc.

No. of Individuals examined in each series/fixation intervals = 5; cells scored per individual = 1000.

Embryotoxic effect could not apparently be found in any of the 15 female pregnant mice that received sonication on different days of the conception, because all the mice had intact foetuses as found in their control counterparts. However, further studies are warranted before coming to a definite conclusion.

In the present study, ultrasonic sound waves of moderate intensity, when directly applied on the body surface of grasshoppers, were found to produce more drastic effect on germinal chromosomes of grasshoppers than when they were indirectly given on fish and mice kept under water. However, still some clastogenic effect, of
lesser significance though, was found to occur in the somatic metaphase chromosomes of both fish and mice. Interestingly, the frequencies of aberrations in single dose experiments, declined along with the lapse of time, implying that some of the aberrations had possibly been restituted or repaired. But in the "repeated dose" experiment the effects of CA, MN and SHA, appeared to increase with the lapse of time and with the increase in number of exposure. Thus, it seemed that like that of X-ray, the ultrasonic irradiation effect may also be "cumulative" in nature. Further, the increase in abnormal shapes of spermheads and chromosome aberrations in germinal cells of male mice exposed to ultrasonic sound is an important finding and should be viewed with some concern. However, the apparent lack of embryotoxic effect found in pregnant mice exposed to ultrasonic sound may not exclude the possibility of any genetic disorder to follow in the progeny, an aspect which has not been included in this study but needs more attention and further experimental studies.

The biophysical effects of ultrasound in aqueous solutions can be characterized as thermal effects, cavitation, and direct effects (Hill 1968). The mechanism of ultrasonic action in vivo and in vitro appears to be complex due to the interaction of the three types of modes. In aqueous media, the nonthermal effects of ultrasound have been attributed mainly to cavitation. The collapse of cavitation bubbles produces a variety of free radical species and induces local shock waves, while the oscillation of cavitation bubbles causes hydrodynamic shearing stress (Hill 1968, Baker and Dalrymple 1978). The more drastic effects observed in grasshopper spermatocytic chromosomes may be due to the thermal and direct effects while the fish and mice showing lack of breaktype aberrations may therefore partly be due to the cavitation effect and partly be due to the formation of free radicals within them (Jana et al 1990a, b 1995). Since ultrasonic sound waves are now being extensively used in various diagnostic procedures, physiotherapy, deep-sea fishing and in hyperthermia treatments for cancer (Kremkau, 1979) for both induction of localized hyperthermia (Varmor 1983) and non-invasive thermometry of internal tumours (Christensen 1983), the possible effects on the genetic system should be carefully studied. More so because degradation of DNA in aqueous solutions and destruction of cells have already been reported to be induced by ultrasound, mostly by the shearing stress of cavitation (Miller and Brayman 1997). Thus it is quite possible that the shearing stress as well as the chemical effects of the free radicals produced during the collapse of ultrasound-induced cavitation bubbles might possibly be responsible for the origin of the clastogenic effects observed in both fish and mice in the present study. Further, the failure to observe induction of SCE at enhanced rate in in vitro cultured lymphocytes (Lundberg et al 1982, Miller et al 1991) may not reflect the same picture in in vivo system, as the possibility of differential response in in vitro and in vivo systems cannot be totally ruled out (Liebeskind et al 1979, Ciaravino et al 1985, 1986). Therefore, although the present study should not discourage the use of transonication totally as a medical tool, certainly its indiscriminate and overuse would be of some risk to the genome and therefore, avoided as far as practicable.

Acknowledgements We are thankful to Prof. S. N. Chatterjee of Bose Institute, Calcutta, and to Prof. S. P. Sen, Department of Botany, Kalyani University, for their appreciation and encouragement of the work, to Prof. G. K. Manna, Emeritus Professor, Department of Zoology, Kalyani University for kindly permitting the use of the Sonicator.

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123
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Tateno H, Kimura Y, Yanagimachi R (2000) Sonication per se is not as deleterious to sperm chromosomes as previously inferred Biol Reprod 63:341-346
THE CYTOGENETIC EFFECTS OF REPEATED EXPOSURE TO ULTRASONIC SOUND WAVES IN MICE AND THEIR ALTERATIONS BY A HOMOEOPATHIC DRUG, ARNICA MONTANA.

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ABSTRACT

Separate sets of healthy mice were directly exposed to sonication (with the aid of ultrasonic cell disrupter at a frequency of 23 kHz at energy output level of 70) for 2 min, 1 min at a time with an interval of 1 min. This dose of sonication was repeated at an interval of 20 days, so that mice sacrificed at 30, 60, 75 and 90 days after the initial dose actually received 2, 3, 4 and 5 such doses of sonication, respectively. The genotoxic effects in sonicated mice were assessed through the study of chromosome aberrations (CA), sperm head anomaly (SHA), and micronucleated erythrocytes (MNE) as against suitable unsonicated controls. Further, a group of sonicated mice were orally administered with Arnica Montana-30, a homoeopathic drug commonly used against shock and injury and the results were compared with another set of succussed alcohol fed controls (the "vehicle" of the drug being ethyl alcohol). In the sonicated mice, elevated frequencies of CA (comprising mainly of physiological types), MNE and SHA were noted as compared to that of unsonicated controls. Correspondingly, the cytogenetical effects in sonicated and drug-fed (combined) series appeared to be relatively less as compared to succussed alcohol fed sonicated control, thereby indicating that the homoeopathic drug had positive modifying effect on genotoxicity produced by ultrasonication. The implications of the results have been discussed.

INTRODUCTION

The ultrasonic sound waves are being increasingly used in biology, medicine for diagnostic purposes, in physiotherapy, in hyperthermia for cancer therapy and in non-invasive thermometry of internal tumors (Kremkau 1979, Hahn et al. 1981, Christensen 1983, Marmor 1983). Pregnant women are also often exposed to periodic ultrasonographic tests. Although some amount of work has been done for understanding the biological effects of ultrasonication in mammal (Hill 1968; Baker et al. 1978; Jana et al. 1990), the effect on mammalian genetic system in vitro appears to be inadequately studied and the results have been inconclusive; some authors suggested no significant effects in in vitro system (Ciaravino et al. 1985, Lundberg et al. 1982, Dooley 1983) while others claimed some positive genotoxic changes in in vivo (Kondo 1985, Jana et al. 1986, Khuda-Bukhsh and Chakraborti 1998) systems. In the present investigation an attempt was made to assess the extent of genotoxic
effects of ultrasonication, if any, in the mammalian model *Mus musculus*, in vivo. The other objective of the study was to examine if the potentized homoeopathic drug Arnica montana-30, which showed antigenotoxic action against X irradiation (Khuda-Bukhsh *et al.* 1982), could also favourably modify the harmful effects of sonication, if any.

**MATERIALS and METHODS**

Healthy Swiss albino mice (*Mus musculus*) weighing between 25-30 grams of both sexes served as materials for the present study.

**Experimental design**

Batches of 5 mice each were subjected to whole-body ultrasonic sound waves with the help of an ultrasonic cell disrupter machine (LSL, SECFROID, Switzerland) operating at a frequency wave of 23KHz, and at an energy output level of 70 for a period of 2 min (twice for 1 min each with an interval of 1 min in between). This dose of sonication was repeated at an interval of 20 days, so that mice sacrificed at 30, 60, 75 and 90 days after initial dose actually received 2, 3, 4 and 5 such doses of sonication, respectively. One batch of sonicated mice was orally administered with dilute potentized homoeopathic drug, Arnica Montana-30 (procured from HAPCO, Calcutta), normally used against external and internal shock and injury. One drop (0.06 ml) of the drug in 90% alcohol medium was diluted with 10 ml of double distilled water for making the "stock solution" of the drug, from which experimental mice were fed 0.06 ml twice at an interval of 12h.

Another batch of sonicated mice which served as controls was fed with dilute "succussed alcohol-30" (alcohol 30 prepared as per homoeopathic potentization procedure of giving 10 succussions to the 90% alcohol "vehicle" of the drug and diluting 1 ml each time with 99ml of fresh alcohol to increase one potency); further dilution of alcohol-30 was followed in the same manner as that of Arnica montana-30.

**Chromosome aberration study**

Mice at all fixation intervals were injected intraperitoneally with 0.03% colchicine solution @ 1 ml/100g body weight one and a half hours prior to sacrifice. The conventional citrate-flame-drying-Giemsa technique was followed for the bone marrow chromosome preparation.

**Micronuclei testing**

A part of suspension of bone marrow cells in 1% sodium citrate solution was smeared
on clean, grease free slides. The slides were briefly fixed in methanol and subsequently stained with May-Grunwald solution followed by Giemsa staining (Schmid, 1976).

**Sperm head anomaly**

Epididymis of each side of the control and treated male mice was dissected and taken out separately into 10 ml of 0.87% normal saline. It was made free of fats, vas deferens and other tissues. The inner contents were taken out and it was thoroughly shaken to make the sperm suspend in saline solution. This suspension was filtered through a silken cloth to remove debris and was dropped on clean grease free slides uniformly. The slides were allowed to air-dry and then stained in dilute Giemsa as per the routine procedure (Wyrobek et al. 1984).

**OBSERVATIONS**

The frequency distribution of various chromosome aberrations at different fixation intervals encountered in unsonicated mice, sonicated mice, sonicated mice fed with alcohol 30 (positive control) and in sonicated mice fed with the homoeopathic drug Arnica Montana 30 have been summarised in Table-1 and representative photomicrographs provided (PM 1 to 7). An analysis of the data would reveal that there was an increase in total aberration frequencies from 30 days through 90 days in the sonicated series as compared to unsonicated control, and in general the homoeopathic drug fed mice showed the lowest frequency of aberrations as compared to that in sonicated mice and in alcohol-30 fed sonicated mice, in respect of both the "major" and "other" types of aberrations (Table 1). Further, in the alcohol-30 fed sonicated mice there was an abrupt increase of total aberrations at 90 days. The data on induction of micronucleated erythrocytes, both NCE and PCE, have been summarised in table 2 and representative photomicrograph provided (PM 11); an analysis of the data would also reveal that the effects of sonication increased with the lapse of time and that in the drug fed series the incidence of total number of micronuclei was the lowest as compared to the other two sonicated series. The same trend of apparently "cumulative effect" of sonication was also reflected in the data of the sperm head anomaly (Table 3, PM9, 10).

**DISCUSSION**

Khuda-Bukhsh and Chakrabarti (1998) reported that even a single dose of ultra-sound radiation could produce genotoxic effects in mice as compared to normal unirradiated controls. In the present investigation, in addition to confirmation of the earlier findings, it would be revealed that repeated exposure to ultrasonic sound waves yielded a fairly appreciable number of chromosome aberrations, enhanced the induction of micronucleated...
Table 1. Frequency distribution of chromosome aberrations in 500 bone marrow cells examined (100 cells from each of 5 individual) at different fixation intervals.

<table>
<thead>
<tr>
<th>Fixation Time intervals (Days)</th>
<th>Series</th>
<th>Major aberration types</th>
<th>% of other aberration types</th>
<th>Total aberration</th>
<th>Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GS</td>
<td>BS</td>
<td>CF</td>
<td>TR</td>
</tr>
<tr>
<td>Unsonicated control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>Sonic</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Sonic + Drug</td>
<td>0</td>
<td>2</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Sonic + Alcohol</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>Sonic</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Sonic + Drug</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Sonic + Alcohol</td>
<td>1</td>
<td>3</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>75</td>
<td>Sonic</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Sonic + Drug</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Sonic + Alcohol</td>
<td>1</td>
<td>5</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>90</td>
<td>Sonic</td>
<td>0</td>
<td>5</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Sonic + Drug</td>
<td>0</td>
<td>4</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Sonic + Alcohol</td>
<td>7</td>
<td>5</td>
<td>13</td>
<td>3</td>
</tr>
</tbody>
</table>

"Major" types: GS= Gap; BS= Break; CF= Centric fusion; TR= Translocation; F= Fragment; PUL= Pulverisation; RS= Ring; TA= Terminal association; PP= Polyploidy; AP= Aneuploidy.

"Other" types include: Precocious centromeric separation, centromeric stretching, Stickiness, C-mitotic effect.

n= non-significant, *p<0.05, **p<0.01, ***p<0.001.
Table 2. Frequency distribution of micronucleated erythrocytes in approximately 5000 bone marrow cells (1000 cells from each of 5 individuals) examined at different fixation intervals

<table>
<thead>
<tr>
<th>Fixation Time intervals (days)</th>
<th>Series</th>
<th>NCE Total No.</th>
<th>NCE %</th>
<th>PCE Total No.</th>
<th>PCE %</th>
<th>P/N</th>
<th>MNE in NCE and PCE</th>
<th>Unsonicated control vs Sonication + Drug</th>
<th>Sonication vs Sonication + Drug</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unsonicated Control</td>
<td>3457</td>
<td>2</td>
<td>1543</td>
<td>1</td>
<td>0.065</td>
<td>0.446</td>
<td>0.06 ± 0.025</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sonication</td>
<td>2371</td>
<td>6</td>
<td>2695</td>
<td>14</td>
<td>0.519</td>
<td>1.136</td>
<td>0.40 ± 0.087</td>
<td>0.135***</td>
</tr>
<tr>
<td></td>
<td>Sonication + Drug</td>
<td>2770</td>
<td>7</td>
<td>2230</td>
<td>6</td>
<td>0.260</td>
<td>0.805</td>
<td>0.26 ± 0.051</td>
<td>0.200*</td>
</tr>
<tr>
<td></td>
<td>Sonication + Alcohol</td>
<td>3303</td>
<td>13</td>
<td>1776</td>
<td>9</td>
<td>0.506</td>
<td>0.537</td>
<td>0.43 ± 0.042</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sonication</td>
<td>3115</td>
<td>7</td>
<td>1895</td>
<td>14</td>
<td>0.739</td>
<td>0.608</td>
<td>0.42 ± 0.099</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sonication + Drug</td>
<td>2950</td>
<td>4</td>
<td>2050</td>
<td>4</td>
<td>0.195</td>
<td>0.694</td>
<td>0.16 ± 0.016</td>
<td>0.259*</td>
</tr>
<tr>
<td></td>
<td>Sonication + Alcohol</td>
<td>2593</td>
<td>29</td>
<td>892</td>
<td>17</td>
<td>1.900</td>
<td>0.344</td>
<td>1.32 ± 0.254</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sonication</td>
<td>3055</td>
<td>12</td>
<td>1840</td>
<td>12</td>
<td>0.652</td>
<td>0.602</td>
<td>0.49 ± 0.105</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sonication + Drug</td>
<td>3165</td>
<td>10</td>
<td>1895</td>
<td>9</td>
<td>0.474</td>
<td>0.598</td>
<td>0.38 ± 0.045</td>
<td>0.315***</td>
</tr>
<tr>
<td></td>
<td>Sonication + Alcohol</td>
<td>3768</td>
<td>18</td>
<td>1259</td>
<td>49</td>
<td>3.800</td>
<td>0.334</td>
<td>1.33 ± 0.662</td>
<td>0.115*</td>
</tr>
<tr>
<td></td>
<td>Sonication</td>
<td>3652</td>
<td>20</td>
<td>1348</td>
<td>10</td>
<td>0.742</td>
<td>0.369</td>
<td>0.60 ± 0.093</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sonication + Drug</td>
<td>3085</td>
<td>7</td>
<td>1915</td>
<td>5</td>
<td>0.261</td>
<td>0.621</td>
<td>0.24 ± 0.040</td>
<td>0.180**</td>
</tr>
<tr>
<td></td>
<td>Sonication + Alcohol</td>
<td>2730</td>
<td>44</td>
<td>2270</td>
<td>21</td>
<td>0.925</td>
<td>0.832</td>
<td>1.30 ± 0.139</td>
<td></td>
</tr>
</tbody>
</table>

n = non-significant, *=p<0.05, **=p<0.01, ***=p<0.001.
Table 3. Frequency distribution of sperm with abnormal heads in 5000 sperm (1000 sperm in each of 5 individuals at different fixation intervals)

<table>
<thead>
<tr>
<th>Fix time intervals (days)</th>
<th>Sperm observed</th>
<th>Sperm with abnormal heads No.</th>
<th>%±SE</th>
<th>Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Unsonicated control</td>
<td>5000</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sonication</td>
<td>5000</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sonication + Drug</td>
<td>5000</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sonication + Alcohol</td>
<td>5000</td>
<td>30</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>Sonication</td>
<td>5000</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sonication + Drug</td>
<td>5000</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sonication + Alcohol</td>
<td>5000</td>
<td>83</td>
</tr>
<tr>
<td>60</td>
<td></td>
<td>Sonication</td>
<td>5000</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sonication + Drug</td>
<td>5000</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sonication + Alcohol</td>
<td>5000</td>
<td>130</td>
</tr>
<tr>
<td>75</td>
<td></td>
<td>Sonication</td>
<td>5000</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sonication + Drug</td>
<td>5000</td>
<td>32</td>
</tr>
<tr>
<td></td>
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<td>Sonication + Alcohol</td>
<td>5000</td>
<td>115</td>
</tr>
<tr>
<td>90</td>
<td></td>
<td>Sonication</td>
<td>5000</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sonication + Drug</td>
<td>5000</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sonication + Alcohol</td>
<td>5000</td>
<td>83</td>
</tr>
</tbody>
</table>

*=p<0.05, **=p<0.01, ***=p<0.001.

erythrocytes and number of sperm with anomalous head shapes; and that there was a "cumulative action" for repeated exposure to ultrasonic sound waves. However, several workers (Au et al. 1982; Miller et al. 1983; Lundberg et al.1982; Brulfert et al.1984; Barnet et al. 1987) did not get elevated frequencies of SCE in cultured lymphocytes of human being exposed to ultrasonic sound waves. On the other hand, several other workers (Hedges and Leeman 1979; Haupt et al.1986; Jana et al. 1986) reported positive effects of ultrasonic sound waves in lymphocytes of human beings and in egg lecithin.

The biophysical effects of ultrasound in aqueous solutions can be categorised as thermal effects, cavitation, and direct effects (Hill 1968). The mechanism of action of ultrasound is quite complex, in aqueous media the non-thermal effects of ultrasound is mainly due to
cavitation. The degradation of the cavitation bubbles produces free radicals (Edmonds et al. 1983) and induces temporary local shock waves, on the other hand the "to and fro" motion of the cavitation bubbles produce hydrodynamic shearing stress (Hill 1968; Baker and Dalrymple, 1978). This results in degradation of DNA in aqueous solution and even destruction of cells (Coakley and Nyborg 1978; Miller and Brayman 1997). Chatterjee and his coworkers (Jana et al. 1990; Jana and Chatterjee 1995) also documented positive changes in enzymes related to lipid peroxidation and strongly held the view that ultrasonic irradiation caused cytotoxicity. In the present investigation the administration of the homeopathic drug Arnica montana-30 reduced the genotoxic effects to a considerable extent, for which its use may be recommended in patients who have to undergo repeated ultrasonographic tests, either for diagnostic purpose or as a therapy.

Legends for Photomicrographs (PM):

Photomicrographs of some chromosome aberrations: PM 1-break (BS), PM 2- ring (RS), PM 3- terminal association (TA). PM 4- precocious centromeric separation (PCS) and centric fusion (CF). PM 5- stretching (STR), PM 6-polyploidy (PP), PM 7- stickiness; PM 8-normal sperm and PM 9 to 10- sperm with abnormal head shape; PM 11- erythrocyte with micronucleus Magnification (Bar represents 10 μm).
ACKNOWLEDGEMENTS

Grateful acknowledgement is due to University of Kalyani for financial assistance for the work.

REFERENCES


THE CYTOGENETIC EFFECTS OF REPEATED EXPOSURE TO ULTRASONIC SOUND WAVES IN MICE

Cytogenetical effects of sonication in mice and their modulations by actinomycin D and a homeopathic drug, Arnica 30

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Received 11 April 2001; revised 17 August 2001

Experiments were designed to examine if Actinomycin D, an antibiotic, and Arnica 30, a homeopathic drug used against shock and injury, can ameliorate cytogenetic damage induced by single or multiple exposures to ultrasonication. Separate sets of healthy mice were directly exposed to sonication for two minutes either once or they received multiple exposures at an interval of 20 days. The mice were then assessed at different intervals, against suitable controls, using parameters like chromosome aberrations (CA), mitotic index (MI), sperm head anomaly (SHA) and micronucleated erythrocytes (MNE). Separate groups of sonicated mice were either orally administered with Arnica 30 (alcohol 30 in control) or injected intramuscularly with Actinomycin-D (AMD). Elevated frequencies of CA, MI, MNE and SHA were noted in sonicated mice. AMD had genotoxic effects of its own and also had additive effects on sonication induced genotoxicity. Sonicated mice fed with Arnica 30 showed appreciably reduced genotoxicity as against alcohol 30 and distilled water fed controls, thereby showing ameliorating effect which may have human application.

The effect of sonication on mammalian genetic system appears to be inadequately studied and the results have been rather inconclusive. While some authors suggested no significant effects in vitro system1,3, hers claimed some positive genotoxic changes in vivo in vivo test4. However, since recent work also demonstrated some positive cytogenetical changes in vivo in three test models, grasshoppers, fish and mice, objected to whole-body ultrasonication10, it was of interest to search for any agent that would have no cytogenetical ill effects of its own, but could protect against ultrasound induced cytogenetical damages. This seemed important because the use of ultrasonic sound waves as a tool in medicine can not be objected to for its superiority and several advantages over other methodologies like X-rays in pin-pointing location/site of abnormalities in internal anatomy of human beings without causing any apparent injury to them. Of various antibiotics, Actinomycin-D has been reported to have anti-radiation activity11, but it was so reported to have a great deal of genotoxic effect12,13. On the other hand, the homeopathic drugs, which are used in ultra-low doses and are known to have no toxic side-effects, are becoming increasingly popular in both developing Asian countries and developed European countries14 after their initial efficacy was supported by many well-conducted research publications including two major meta-analysis15,16. Keeping the above in view the homeopathic drug, Arnica Montana, commonly used against shock and injury has been examined for its possible protective role against ultrasonication. Incidentally, the potentized form of the homeopathic drug, Arnica Montana 30, had earlier been reported to have anti-clastogenic activities against X-ray induced cytogenetical damage17,19.

Materials and Methods

Experimental design

Control series

Unsonicated healthy mice were examined for their baseline chromosome aberrations and other protocols used (S). Another set of unsonicated healthy mice were fed with Arnica 30 (S2) alone in doses described below and were scanned for the data at the same fixation intervals as in sonicated lot.

Exposure to sonication

Single exposure series

For homeopathic drug series, 9 batches of 5 mice each were subjected to whole-body ultrasonic sound waves from an ultrasonic cell disrupter machine (LSL, SECFROID, Switzerland) operating at a frequency...
frequency wave of 23 KHz, and at an energy output percentage of 70 for a total period of 2 min (twice for min each with an interval of 1 min in between). ice receiving this two min exposure comprised the itials for the single exposure study.

Mice were immobilised during sonication in a thin cloth bag and the lower part of the body submerged in water in glass beaker. The beaker was surrounded with ice before start of sonication to avoid y possible rise in temperature of water inside aker during sonication. Out of the 9 batches, 3 were sacrificed at 2, 3 at 24 and the other 3 at 48 hr. mong these 3 batches, 1 batch of mice was orally administered with potentized homeopathic drug, Arnica 30 [HAPCO, Kolkata,(0.06 ml of liquid Arnica diluted with 20 ml double distilled water (ddw) to take the stock solution of the drug, from which 1 op, i.e. 0.06 ml was fed thrice at an interval of 30 in to mice which were sacrificed at 2 hr and thrice a y at an interval of 8 hr to mice sacrificed at 24 and 48 hr)]. Another batch of mice was fed with diluted succussed alcohol (alcohol 30) (positive control 1)pared as per homeopathic potentization procedure mlarly at corresponding intervals of time and the 3rd batch of mice were neither fed with the homeopathic drug, nor with the diluted alcohol measured as r homeopathic principle (i.e. alcohol 30), but was lowered to take only ddw at corresponding intervals ositive control 2). Another set of unsonicated healthy mice served as negative control.

Similarly, in Actinomycin D (AMD) treated series, batches of mice, unsonicated and sonicated, were tranmuscularly injected with 0.0005% Actinomycin @ 1ml/100 g body weight and were sacrificed at 2, 4 and 48 hr. Controls were maintained for sonicated ice injected with distilled water (AMD dissolves in ater). In another set of experiments, sonicated and MD injected mice were either fed with the homeopathic drug, Arnica 30, or with alcohol 30 (as control 'homeopathic drug, Arnica, the “vehicle” of the drug being ethyl alcohol). As AMD itself showed a low amount of clastogenic effect, which could only partially reduced by the feeding of the homeo-thic drug (Table 1), further experimentation with MD was not continued.

Multiple exposure series

Four sets of mice (each set comprising 5 mice) ere subjected to repeated exposures as mentioned above for 2 min each at an interval of 20 days, so that mice sacrificed at 30, 60, 75 and 90 days after the ini-
Table 1—Frequencies of chromosome aberrations in 500 bone marrow cells examined (100 cells from each 5 individuals) at different fixation intervals in single exposure series: Unsonicated, AMD treated, Sonicated + AMD treated, Sonicated + AMD treated + Arnica-30 fed and Sonicated + AMD treated + alcohol 30 fed

<table>
<thead>
<tr>
<th>Fixation Interval</th>
<th>Series</th>
<th>% ± SE</th>
<th>% of Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hr</td>
<td>Unsonicated</td>
<td>0.40 ± 0.25</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>AMD</td>
<td>23.6 ± 0.75</td>
<td>23.20&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Sonicated + AMD</td>
<td>28.00 ± 1.30</td>
<td>4.40</td>
</tr>
<tr>
<td></td>
<td>Sonicated + Arnica-30</td>
<td>21.25 ± 0.51</td>
<td>4.75&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Sonicated + alcohol 30</td>
<td>26.00 ± 0.86</td>
<td></td>
</tr>
<tr>
<td>4 hr</td>
<td>AMD</td>
<td>20.80 ± 0.66</td>
<td>20.40&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Sonicated + Arnica-30</td>
<td>17.40 ± 0.81</td>
<td>2.20</td>
</tr>
<tr>
<td></td>
<td>Sonicated + alcohol 30</td>
<td>19.60 ± 0.93</td>
<td>1.78</td>
</tr>
<tr>
<td>8 hr</td>
<td>AMD</td>
<td>19.40 ± 1.60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sonicated + Arnica-30</td>
<td>21.20 ± 0.97</td>
<td>19.00</td>
</tr>
<tr>
<td></td>
<td>Sonicated + alcohol 30</td>
<td>13.40 ± 0.98</td>
<td>1.80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18.80 ± 0.80</td>
<td>4.27&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Chromosome aberrations include stickiness, precocious centromere separation, erosion, condensed, crumpled, c-mitotic effect, polydomy, aneuploidy etc.

<sup>c</sup> Standard Error, <sup>b</sup>P < 0.001, <sup>P</sup> < 0.01% level of significance at t-test

Due to apparent additive action of AMD on sonication-induced genotoxicity. The oral administration of Arnica 30 to these AMD-treated sonicated mice marginally reduced the frequency of aberrations, while the alcohol 30 in sonicated mice tended to increase the damage in some cases.

The frequencies of various chromosome aberrations differed across intervals encountered in unsonicated mice (S<sub>1</sub>), unsonicated fed with Arnica 30 (S<sub>2</sub>), sonicated mice (S<sub>3</sub>), sonicated mice fed with a homeopathic drug Arnica 30 (S<sub>4</sub>) and sonicated mice fed with alcohol 30 (S<sub>5</sub>, positive control) have been summarized in Tables 2 and 3 for single exposure and Tables 4 and 5 for multiple exposures to sonication. Representative photomicrographs of various types of chromosome abnormalities (Figs 1-9), micronucleated erythrocytes (Figs 10 and 11) and sperm head anomalies (Figs 12-14) have been provided.

The chromosome complements in both the experimental and control sets of mice were critically studied for possible abnormalities. In the normal healthy unsonicated mice (negative control), out of some 500 bone marrow cells examined, normal complements (e.g., 1) were obtained in all but two cells, one of which showed an achromatic lesion and another contained a chromosome with a constriction, which made the spontaneous aberration baseline as only 0.04%. Similarly, the baseline for micronucleated erythrocytes examined from 2000 cells and sperm head anomaly examined from some 2000 sperm in normal healthy unsonicated controls were extremely low, the mean being 0.001% for MN and 0.002% for SHA. In unsonicated healthy mice fed with Arnica 30 alone, no statistically appreciable difference in any of the protocols used was noticed. Therefore, the oral administration of Arnica 30 did not itself bring any apparent clastogenic/genotoxic effects in mice. On the other hand, in the sonicated mice the percentages of chromosome aberrations, mostly of the physiological and numerical types (Figs 2-6), were 30.00 at 2 hr, 24.20 at 24 hr and 20.00 at 48 hr which were all statistically significant at various levels (Table 1). Similarly, the incidence of micronuclei induction was 0.44% in the sonicated mice at 24 and 48 hr.

The same kind of increase in the frequencies of sperm with abnormal head shape was noticed in the sonicated mice, being 1.84 and 1.48% at 24 and 48 hr, respectively.

There was also some increase in the mitotic index in the sonicated mice, being 2.70% at 24 hr and 2.06% at 48 hr as compared to about 0.69% in unsonicated normal mice. Therefore, there was a positive change in these cytogenetical parameters even for the single exposure to ultrasonication. However, no break type or other more serious type of aberrations
Photomicrographs of normal (1) and aberrated (2-9) metaphase complements of mice (crumpled (2), pulvored (3), C-otic effect (4), stickiness and polyploidy (5), stickiness and ring (6), chromatid break and constriction (7), terminal association (8) and atirc fragment (9)), micronucleated polychromatic (10) and normochromatic (11) erythrocytes, sperm with normal (12) and abnormal id shape (13-14).

= Ring, BS = Break, TA = Terminal Association, F = Fragment; Bar = 10 μm.
major type) were encountered in this group of sonicated mice. In mice receiving multiple exposures, not only the percentages of CA (Table 4) were increased appreciably, but also the "major type" (Figs 7-9) aberrations appeared at all the four longer intervals, though not necessarily in a strictly cumulative manner. The percentages of MN, however, increased along with time sonicated mice (Table 5); the same was true for the incidence of SHA, thereby showing a somewhat time-dependent" and "cumulative" effect of sonication. The frequency of chromosome aberrations, which was at its peak at 24 hr, however, apparently lined appreciably at 48 hr, presumably because part of the aberrations were either restituted or else heavily damaged ones were eliminated after the cell cycle.

Interestingly enough, in the majority of cases, wherever Arnica 30 was fed to sonicated mice, there was a favourable alteration in the damaging effect, practically for all the parameters used (Tables 2-5); and the results were statistically significant at various levels (Tables 2-5).

Discussion

Even a single exposure to ultrasound irradiation could produce quantifiable genotoxic effects in mice as compared to normal unirradiated controls. Repeated

<table>
<thead>
<tr>
<th>Intervals</th>
<th>Series</th>
<th>Chromosome aberration</th>
<th>% ± SE</th>
<th>Mitotic index</th>
<th>% ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>S1 vs S3</td>
<td>S3 vs S4</td>
<td>S4 vs S3</td>
</tr>
<tr>
<td>2hr.</td>
<td>S1</td>
<td>0.04 ± 0.21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S2</td>
<td>0.03 ± 0.18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S3</td>
<td>0.00 ± 0.84</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S4</td>
<td>0.19 ± 0.50</td>
<td>29.96%</td>
<td>11.00%</td>
<td>10.80%</td>
</tr>
<tr>
<td></td>
<td>S5</td>
<td>0.29 ± 1.80</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24hr</td>
<td>S4</td>
<td>0.12 ± 0.65</td>
<td>24.16%</td>
<td>11.40%</td>
<td>6.20%</td>
</tr>
<tr>
<td></td>
<td>S5</td>
<td>0.20 ± 0.71</td>
<td>19.00%</td>
<td>19.96%</td>
<td>19.00%</td>
</tr>
<tr>
<td>48hr</td>
<td>S4</td>
<td>0.12 ± 0.57</td>
<td>19.96%</td>
<td>8.00%</td>
<td>6.00%</td>
</tr>
<tr>
<td></td>
<td>S5</td>
<td>18.00 ± 0.61</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2:** Frequency distribution of chromosome aberrations in 500 bone marrow cells examined (100 cells from each of 5 individuals) and mitotic indices at different fixation intervals in single exposure series S1—unsonicated, S2—unsonicated plus Arnica-30 fed S3—sonicated, S4—sonicated plus Arnica-30 fed and S5—sonicated plus alcohol-30 fed

<table>
<thead>
<tr>
<th>Intervals</th>
<th>Series</th>
<th>Micronuclei in PCE and NCE</th>
<th>% ± SE</th>
<th>Sperm head anomaly</th>
<th>% ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>P/N</td>
<td>S1 vs S3</td>
<td>S3 vs S4</td>
</tr>
<tr>
<td>2hr.</td>
<td>S1</td>
<td>0.001±0.03</td>
<td>0.40</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S2</td>
<td>0.003±0.02</td>
<td>0.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S3</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>S4</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>S5</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>24hr</td>
<td>S4</td>
<td>0.44±0.03</td>
<td>2.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S5</td>
<td>0.32±0.06</td>
<td>1.87</td>
<td>0.44%</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>S6</td>
<td>0.42±0.12</td>
<td>2.10</td>
<td></td>
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<tr>
<td></td>
<td>S7</td>
<td>0.44±0.06</td>
<td>0.93</td>
<td></td>
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</tr>
<tr>
<td>48hr</td>
<td>S4</td>
<td>0.30±0.03</td>
<td>0.88</td>
<td>0.44%</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>S5</td>
<td>0.43±0.11</td>
<td>0.92</td>
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</tr>
</tbody>
</table>

**Table 3:** Frequency distribution of micronuclei in normochromatic erythrocytes (NCE) and polychromatic erythrocytes (PCE) (1000 erythrocytes in each individual) and sperm with abnormal head shape (1000 sperm from each individual) at different fixation intervals in single exposure series S1—unsonicated, S2—unsonicated plus Arnica-30 fed S3—sonicated, S4—sonicated plus Arnica-30 fed and S5—sonicated plus alcohol-30 fed

- Standard Error, aP < 0.05, bP < 0.001, cP < 0.001 % level of significance at t-test

- Standard Error, aP < 0.05, bP < 0.001, cP < 0.001 % level of significance at t-test
exposures to ultrasonic waves further increased the
tent of cytogenetic damage. Earlier workers\(^3\), \(^24\)-\(^28\)
not get elevated frequencies of SCE in cultured
phocytes of human subjects exposed to ultrasonic
waves. On the other hand, several workers\(^7\), \(^29\)-\(^30\)
ported positive effects of ultrasonic sound waves in
phocytes of human beings and in egg lecithin
sitive genotoxic effects of ultrasonic sound waves
are also observed in fish genetic system\(^8\). Chatterjee
and his co-workers\(^4\),\(^5\),\(^7\) also documented positive
anges in enzymes related to lipid peroxidation and
grily held the view that ultrasonic irradiation
used cytoxicity. Therefore, ample evidence has
omulated now which would suggest that ultrasonic
will really cause some genomic damage to
exposed organisms.

The biophysical effects of ultrasound in aqueous
olutions can be categorized as thermal effects, cavi-
dation and direct effects\(^31\). The mechanism of action of
trasound is quite complex; in aqueous media the
on-thermal effects of ultrasound is mainly due to
vation. The degradation of the cavitation bubbles
uces free radicals\(^32\) and induces temporary local
ck waves. On the other hand the “to and fro” mo-
on of the cavitation bubbles produces hydrodynamic
ering stress\(^31\),\(^33\). This results in degradation of
DNA in aqueous solution and even destruction of
cells\(^34\),\(^35\). Therefore, present observations of the differ-
ent forms of cytogenetic damage caused by ultrasoni-
cation can be explained in the light of the above
findings, as also for the mechanical and psychological
stress caused due to exposure of sonication.

Further, the present findings suggest that AMD,
which is also a transcription-blocker, had genotoxic
effect of its own. It possibly bound itself to DNA by
ntercalating between bases and thereby changed the
ormal milieu of the DNA and interfered with normal
roofreading activities that might in turn be responsible
for the different aberrations encountered in AMD
treated mice. Further, it tended to increase the damage
already produced in sonicated mice. Thus, the use of
AMD may not be advisable as a protective measure
against sonication. On the other hand, the homeo-
pathic drug Arnica 30 was found to modulate
avourably the cytogenetic ill-effects of ultrasonica-
tion in mice while the administration of alcohol 30
appeared to increase the damaging effect of sonica-
tion. Although Arnica is claimed to have profound
regulatory action on various systems like blood
ascular, CNS, skin etc., on which sonication might
have produced some stressful effects, the exact
mechanism of action of this drug could not be known.

<table>
<thead>
<tr>
<th>Fix. Intervals</th>
<th>Series</th>
<th>Chromosome aberration</th>
<th>% ± SE</th>
<th>% of protection</th>
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<tbody>
<tr>
<td></td>
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<td></td>
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<td>S(_1) vs S(_3)</td>
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<tr>
<td>30 days</td>
<td>S(_1)</td>
<td>0.40 ± 0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S(_2)</td>
<td>0.38 ± 0.27</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S(_3)</td>
<td>35.90 ± 0.23</td>
<td></td>
<td>35 50(^a)</td>
</tr>
<tr>
<td></td>
<td>S(_4)</td>
<td>20.00 ± 0.86</td>
<td></td>
<td>15 90</td>
</tr>
<tr>
<td></td>
<td>S(_5)</td>
<td>21.60 ± 0.83</td>
<td></td>
<td>1 60</td>
</tr>
<tr>
<td>60 days</td>
<td>S(_3)</td>
<td>23.00 ± 0.77</td>
<td></td>
<td>22 60(^c)</td>
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<tr>
<td></td>
<td>S(_4)</td>
<td>19.80 ± 0.68</td>
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<td>3.20</td>
</tr>
<tr>
<td></td>
<td>S(_5)</td>
<td>26.80 ± 1.77</td>
<td></td>
<td>4 40</td>
</tr>
<tr>
<td>75 days</td>
<td>S(_3)</td>
<td>25.60 ± 0.50</td>
<td></td>
<td>25 20(^d)</td>
</tr>
<tr>
<td></td>
<td>S(_4)</td>
<td>8.40 ± 0.50</td>
<td></td>
<td>17 20(^b)</td>
</tr>
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<td></td>
<td>S(_5)</td>
<td>19.80 ± 1.80</td>
<td></td>
<td>11 40(^b)</td>
</tr>
<tr>
<td>90 days</td>
<td>S(_3)</td>
<td>29.40 ± 0.50</td>
<td></td>
<td>29 00(^c)</td>
</tr>
<tr>
<td></td>
<td>S(_4)</td>
<td>24.40 ± 0.40</td>
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<td>5 00</td>
</tr>
<tr>
<td></td>
<td>S(_5)</td>
<td>42.40 ± 3.29</td>
<td></td>
<td>18 00</td>
</tr>
</tbody>
</table>

*Chromosome aberrations include gap, break, centric fusion, translocation, fragment, pulverisation, ring, terminal association, polyplody, aneuploidy, precocious centromeric separation, centromeric stretching, stickiness, c-mitotic effect, etc.

SE = Standard Error, \(^a\) \(P<0.05\), \(^b\) \(P<0.001\), \(^c\) \(P<0.001\)% level of significance at t-test
Table 5—Frequency distribution of micronuclei in normochromatic erythrocytes (NCE) and polychromatic erythrocytes (PCE) (1000 erythrocytes in each individual) and sperm with abnormal head shape (1000 sperm from each individual) at different fixation intervals in single exposure series: S1-unsonicated, S2- unsonicated plus Arnica-30 fed S3-sonicated, S4-sonicated plus Arnica-30 fed and S5-sonicated plus alcohol-30 fed

<table>
<thead>
<tr>
<th>x Intervals</th>
<th>Series</th>
<th>Micronuclei in PCE and NCE</th>
<th>Sperm head anomaly</th>
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<tr>
<td></td>
<td></td>
<td>% ± SE</td>
<td>P/N</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>30 days</td>
<td>S1</td>
<td>0.06±0.03</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>S2</td>
<td>0.04±0.05</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>S3</td>
<td>0.40±0.09</td>
<td>1.14</td>
</tr>
<tr>
<td></td>
<td>S4</td>
<td>0.26±0.05</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>S5</td>
<td>0.43±0.04</td>
<td>0.54</td>
</tr>
<tr>
<td>60 days</td>
<td>S1</td>
<td>0.42±0.10</td>
<td>0.61</td>
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<tr>
<td></td>
<td>S2</td>
<td>0.16±0.02</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>S3</td>
<td>1.32±0.25</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>S4</td>
<td>0.49±0.11</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td>S5</td>
<td>0.38±0.05</td>
<td>0.60</td>
</tr>
<tr>
<td>75 days</td>
<td>S1</td>
<td>1.33±0.66</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>S2</td>
<td>0.60±0.09</td>
<td>0.37</td>
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<tr>
<td></td>
<td>S3</td>
<td>0.24±0.04</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>S4</td>
<td>0.30±0.14</td>
<td>0.83</td>
</tr>
<tr>
<td>90 days</td>
<td>S1</td>
<td>0.38±0.10</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td>S2</td>
<td>0.16±0.02</td>
<td>0.69</td>
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<td></td>
<td>S3</td>
<td>0.60±0.09</td>
<td>0.37</td>
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<tr>
<td></td>
<td>S4</td>
<td>0.24±0.04</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>S5</td>
<td>0.30±0.14</td>
<td>0.83</td>
</tr>
</tbody>
</table>

: Standard Error, *=P<0.05, b=P<0.001, c=P<0.001% level of significance at t-test.

Some relevant data about the drugs: Actinomycin D and Arnica Montana

**Actinomycin-D**
- Polypeptide containing antibiotic; inhibits transcription by binding tightly to DNA, preventing it from acting as template for RNA synthesis, binding enhanced by the presence of guanine residue
- Source/derived from: Specific strain of *Streptomyces*
- Working principle: The phenoxazone ring of AMD slips in between neighbouring base pair of DNA, mainly G-C

**Arnica Montana**
- Potentized form in alcohol vehicle can only be differentiated from alcohol by NMR studies, otherwise no chemical nature other than alcohol can be substantiated
- Source/derived from: Prepared from fresh roots of *Arnica montana*, (Compositae) that grows all over the world, tincture contains some alkaloids
- Working principle: Not precisely known, but claimed to act through CNS on skin, venous system, muscular system, digestive organs, serous membranes and circulation

It could not also be understood why and how the tiny doses of alcohol had accentuated the effect in sonicated mice, but it was at least a pointer that intake of alcohol alongside sonication should be discouraged in man subjects as well. It's difficult to perceive at the present state of knowledge how this ultra-low doses of the homeopathic drug could bring such spectacular modulating effect in sonicated mice since the precise mechanism of action of the homeopathic drugs has yet been completely understood. However, from different scientific evidences, Khuda-Bukhsh has posed a hypothesis to explain the mechanism of ion by attributing the major pathway through regulation of expression of certain genes by the homeopathic drugs in an unknown manner. The present findings of Arnica in reducing genotoxic effects of sonication in mice may have an application in human subjects (patients) as well, where repeated ultrasonic tests are absolutely necessary for diagnostic or therapeutic purposes, to minimize the possible ill-effects of ultrasonication. The use of the homeopathic drug can be considered safe because the administration of repeated doses of Arnica 30 alone in normal healthy mice did not reveal any clastogenic ill-effects by itself.

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References


