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Nandini Bhattacharjee
Pathikrit Banerjee
Anisur Khuda Bukhsh
Cytogenetics and molecular biology laboratory, Department of zoology, University of Kalyani, Kalyani, India

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Abstract

The study was undertaken to examine whether Carcinosin-200 (Car-200) could provide additional ameliorative effect, if used intermittently with Natrum sulphuricum-30 (Nat sulph-30) against hepatocarcinogenesis induced by chronic feeding of p-dimethylaminoazobenzene (p-DAB) and phenobarbital (PB) in mice (Mus musculus). Mice were randomly divided into seven sub-groups: (i) normal untreated; (ii) normal+succussed alcohol; (iii) p-DAB (0.06%) + PB (0.05%); (iv) p-DAB + PB +succussed alcohol (v) p-DAB+PB+Nat sulph-30, (vi) p-DAB+PB+Car-200, and (vii) p-DAB+PB+ Nat sulph-30+Car-200. They were sacrificed at 30, 60, 90 and 120 days for assessment of genotoxicity through cytogenetical end-points like chromosome aberrations, micronuclei, mitotic Index and sperm head anomaly and cytotoxicity through assay of widely accepted biomarkers and pathophysiological parameters. Additionally, electron microscopic studies and gelatin zymography for matrix metalloproteinases (MMPs) were conducted in liver at 90 and 120 days. Results shower that administration of Nat sulph-30 alone and in combination with Car-200 reduced the liver tumors with positive ultra- structural changes and in MMPs expression, genotoxic parameters, lipid peroxidation, y-glutamyl transferase, lactate dehydrogenase, blood glucose, bilirubin, creatinine, urea and increased GSH, glucose-6-phosphate dehydrogenase, superoxide dismutase, catalase, glutathione reductase activities and hemoglobin, cholesterol, and albumin levels. Thus intermittent use of Car-200 along with Nat sulph-30 yielded additional benefit against genotoxicity, cytotoxicity hepatotoxicity and oxidative stress induced by the carcinogens during hepatocarcinogenesis.

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FUNDAMENTAL RESEARCH

Homeopathic drugs Natrum sulphuricum and Carcinosin prevent azo dye-induced hepatocarcinogenesis in mice*

Nandini Bhattacharjee, Pathikrit Banerjee and Anisur Rahman Khuda-Bukhshh**

The study was undertaken to examine whether Carcinosin-200 (Car-200) could provide additional ameliorative effect, if used intermittently with Natrum sulphuricum-30 (Nat sulph-30) against hepatocarcinogenesis induced by chronic feeding of p-dimethylaminoazobenzene (*p*-DAB) and phenobarbital (PB) in mice (Mus musculus). Mice were randomly divided into seven sub-groups: (i) normal untreated; (ii) normal+succussed alcohol; (iii) *p*-DAB (0.06%) + PB (0.05%); (iv) *p*-DAB + PB +succussed alcohol (v) *p*-DAB + PB + Nat sulph-30, (vi) *p*-DAB + PB + Car-200, and (vii) *p*-DAB + PB + Nat sulph-30+Car-200. They were sacrificed at 30, 60, 90 and 120 days for assessment of genotoxicity through cytogenetical end-points like chromosome aberrations, micronuclei, mitotic index and sperm head anomaly and cytotoxicity through assay of widely accepted biomarkers and pathophysiological parameters. Additionally, electron microscopic studies and gelatin zymography for matrix metalloproteinases (MMPs) were conducted in liver at 90 and 120 days. Results showed that administration of Nat sulph-30 alone and in combination with Car-200 reduced the liver tumors with positive ultra- structural changes and in MMPs expression, genotoxic parameters, lipid peroxidation, γ-glutamyl transferase, lactate dehydrogenase, blood glucose, bilirubin, creatinine, urea and increased GSH, glucose-6-phosphate dehydrogenase, superoxide dismutase, catalase, glutathione reductase activities and hemoglobin, cholesterol, and albumin levels. Thus intermittent use of Car-200 along with Nat sulph-30 yielded additional benefit against genotoxicity, cytotoxicity hepatotoxicity and oxidative stress induced by the carcinogens during hepatocarcinogenesis.

Keywords : biomarkers, genotoxicity, homoeopathy, hepatocarcinogenesis, mice, carcinosin-200, natrum sulphuricum-30

INTRODUCTION

Carcinosin-200 (Car-200), a nosode is occasionally used in treating stubborn cases of liver disorder as an intermittent remedy. Nosodes are homeopathic remedies prepared from disease agents or with other substances from an affected organ and are claimed in homeopathic literature to have good modifying effects when a well-selected remedy fails or stops acting any further after initial action.

An azo dye, p-dimethylaminoazobenzene (p-DAB), a group-2B carcinogen[1] on chronic feeding produces liver tumors with anaplastic characteristics[2,3] whereas phenobarbital (PB) is a promoter with positive carcinogenic effect[4], when chronically fed for a long time. Therefore, the hepato-toxicity and tumors formed due to chronic feeding of these carcinogens have been suitably utilized as a model for understanding sequential changes in the process of hepatocarcinogenesis[5], as well as for testing efficacy of drugs in combating carcinogenesis[6-10].

In our earlier study, we reported protective effects of Natrum sulphuricum-200, which is routinely used in treatment of various liver disorders[11,12] against induced hepatocarcinogenesis[10]. The efficacy of intermittent

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**Address for correspondence:
Dr. Anisur Rahman Khuda-Bukhsh
Cytogenetics and Molecular Biology Laboratory, Department of Zoology, University of Kalyani, Kalyani 741 235, India
E-mail: prof_arkb@yahoo.co.in;
khuda-bukhsh_48@rediffmail.com

Abbreviations:

Alc, alcohol; BUN, blood urea nitrogen, CA, chromosomal aberration; CAM, complementary and alternative medicine; Car-200, carcinosin-200; CAT, catalase; G6PD, glucose-6-phosphate dehydrogenase; GGT, gamma glutamyl transferase; GR, glutathione reductase; GSH, reduced glutathione content; Hb, hemoglobin; LDH, lactate dehydrogenase; LPO, lipid peroxidation; MDA, malondialdehyde; MI, mitotic index; MN, micronucleus; Nat sulph-30, natrum sulphuricum-30; PB, phenobarbital; *p*-DAB *p*-dimethylaminoazoben-zene; SHA, sperm head anomaly; SEM, scanning electron microscopy; SOD, superoxide dismutase; TEM, transmission electron microscopy.

use of Car-200 along with Chelidonium-200 is also reported to give better results as compared to when only Chelidonium-200 is used against induced hepatocarcinogenesis[6]. Therefore, in the present study, we investigated whether intermittent use of Carcinosin administered along with Natrum sulphuricum-30 (Nat suplh-30) could also produce additional beneficial effects against genotoxicity, hepatotoxicity, oxidative stress and liver tumors induced by chronic feeding of known carcinogens in mice.

The *p*-DAB after entry into body is metabolized to mono-amino azobenzene (MAB) by N-dimethylation and subsequently to amino azobenzene (AAB). The azo-dye produces reactive electrophiles[13] and free radicals, which lead to formation of reactive oxygen species (ROS). Cancer is a dreadful disease, which after a certain stage of development can hardly be contained and cured. In some cases, ROS generation is the primary cause of occurrence of most types of cancer. Therefore, for giving the patients a better quality of life, administration of various complementary and alternative medicines (CAM), including homeopathy is increasingly becoming popular[14,15].

Further, potentized homeopathic remedies are extremely diluted, often beyond Avogadro's limit, [16,18] and used in micro doses and have no known side-effects, unlike many of their highly toxic orthodox counterparts often used as chemotherapy (as for example, cisplatin[19,20] and its derivatives, or adriamycin[21] although liver cancer is known to resist most chemotherapeutic orthodox drugs[22,23]). In homeopathic centesimal (C) potentization process, 1 ml of original drug (mother tincture) is diluted in 99 ml of ethanol and given 10 jerks to produce potency 1, and then 1 ml of potency 1 is diluted again with 99 ml of ethanol and given 10 jerks to make it potency 2 and so on [16,17]. Therefore, at 30 or 200 potencies of the drugs used in this study, there is no original drug molecule(s) expected to be physically present. Therefore, it warrants demonstration of efficacy of such highly diluted remedies by analyzing results of tests conducted by involving multiple parameters against suitable controls to put them beyond any figment of doubt about their scientific validity.

In the present study, the effect of potentized homeopathic remedy Nat sulph-30 fed alone and in combination with Car-200 has been studied against *p*-DAB-induced hepatocarcinogenesis in mice.

Materials and Methods

Animals

Healthy inbred adults (from the same source and batch) of Swiss strain albino mice (*Mus musculus*) were used in the study. All animals were acclimatized for 7 days, prior to the commencement of the treatment and allowed free access to food (50% wheat, 40% gram and 10% powdered milk without any animal protein supplementation) and water *ad libitum* and kept in hygienic condition. Experiments were performed with clearance from the Animal Ethics Committee, University of Kalyani, and conducted under overall supervision of Animal Welfare Committee, University of Kalyani.

Experimental groups

Forty-two healthy mice of both sexes, weighing between 25-30 g were used for each of four fixation intervals – namely, 30, 60, 90 and 120 days, making a total of 168 animals for the entire study. For every fixation interval, 6 mice each were fed 7 different diets. The first group of 6 mice (Group I, control 1) was maintained on normal diet. The second group (Group II, control 2) was fed 0.06 ml of stock solution of succussed alcohol (90%) in addition to normal diet. The succussed 90% alcohol was prepared as per homeopathic procedure of potentization. 1 ml of 90% succussed alcohol was then diluted in 20 ml of double distilled water, in the same manner the drugs were diluted, to make it the stock solution of 90% alcohol (herein called placebo). Mice were fed through gavage in the same manner as the drug. Another group of mice (Group-III, carcinogen intoxicated) was kept on a diet mixed with 0.06% *p*-dimethyl-aminoazobenzene (*p*-DAB) (Sigma, D-6760) and provided 0.05% aqueous solution of Phenobarbital (PB) instead of water.

The fourth group of mice (Group IV, carcinogen intoxicated positive control) was chronically fed 0.06% *p*-DAB along with 0.05% aqueous solution of PB + succussed alcohol (as the "vehicle" of the drug was ethyl alcohol). The fifth group of mice (Group V, intoxicated drug-fed 1) was chronically fed *p*-DAB + PB + Nat sulph-30. The sixth group of mice (Group VI, intoxicated drug-fed 2) was fed *p*-DAB + PB + Car-200. A group of mice (Group VII, intoxicated drug-fed 3) was fed *p*-DAB + PB + Nat sulph-30 + Car-200. From the visual observation of food left over in cage in control and experimental sets (the quantity of food generally consumed by one mouse in a day was

measured in earlier range-finding trials), before they were cleaned everyday, it appeared that all mice consumed more or less equal amount of food.

All the experiments were carried out concurrently and in similar environmental set up. After sacrifice, blood was collected from jugular veins, since that proved to yield adequate amount of blood necessary for all the tests. Serum was separated and used for the assessment of renal function tests. Liver tissues were quickly processed and stored at -20°C till further biochemical estimations.

Source of the homeopathic drugs

Natrum sulphuricum-30 (Nat sulph-30) and Carcinosin-200 (Car-200) were procured from HAPCO, 165 BB Ganguly Street, Kolkata. The drugs were prepared and potentized as per procedures recommended in Homeopathic Pharmacopoeia of India[24]. While the base substance of Nat sulph-30 is sodium sulfate, Car-200 is a nosode, derived generally from cancerous tissue of liver[25]. For saving lives of mice and cost, only one set of data obtained by using succussed alcohol-200 [(Alc) homeopathically prepared from same batch of 90% alcohol which had been used for the preparation of the drugs also] was adopted as common positive control against drug-fed series. In our earlier experiments, when both alcohol 30 (Alc-30) and Alc-200 were used as controls, no significant difference was observed between the two treatments.

Feeding procedure and dose

With the aid of a fine pipette, each mouse was fed 0.06 ml (1 drop) of stock solution of either Nat sulph 30 or Car-200 or placebo, as the case may be, that conformed a single dose. Nat sulph-30 was fed twice a day and Car-200 was fed once a week to Nat sulph- 30 fed mice.

Methodology

Multiple parameters were used to understand whether and how the drugs could act in bringing about changes, if any.

Cytogenetic assay

The standard cytogenetic protocols like assays of chromosome aberrations (CA), micronuclei (MN), mitotic index (MI) from bone marrow cells and sperm head anomaly (SHA) from epididymis of testis were studied for testing genotoxicity[8,9,10].

Assessment of liver and renal functions

y-Glutamyl transferase (GGT) (E.C 2.32.2)[26] and serum total bilirubin[27] were estimated according to the manufacturer's protocol (Reckon Diagnostics, India). For renal function, serum samples were assayed for creatinine, urea and blood urea nitrogen (BUN) by using standard diagnostic kits (Span Diagnostics, Gujarat, India).

Assessment of pathophysiological parameters

For blood glucose determination, standard glucose test kit (enzymatic, GOD-POD method, Span Diagnostics Limited, Baroda India) was used. Hemoglobin content was determined by Sahli's method with the help of a hemometer (Marienfield, Germany). The blood cholesterol was measured as described previously[28]. Serum albumin level was assayed by the reagent kit (BCG method) supplied by Recon Diagnostics Pvt. Ltd., Gorwa, Baroda, India. The assay method was based on modified Doumas method with an extended linearity.

Biochemical assays

From whole blood and serum

Glucose-6-phosphate dehydrogenase (G6PD) (E. C 1.1.1.49) was assayed from whole blood by the reagent kit (UV-Kinetic method) according to the manufacturer's protocol (Reckon Diagnostics, India). Lactate dehydrogenase (LDH) (EC 1.1.1.27)[29] was assayed from serum according to the manufacturer's protocol (Reckon Diagnostics, India).

Preparation of tissue homogenates

The entire liver tissue was washed twice with ice cold 0.1 *M* PBS or phosphate- buffered saline (1:[illegible], pH 7.4, blotted, dried and weighted. The liver tissue was stored at -20°C for not more than 12 h before analysis. A 10% tissue homogenate was prepared in PBS by homogenizing the tissue in a glass homogenizer. The homogenate was centrifuged at 2000 × g for 15 min at 4°C to remove the cell debris and then the supernatant was centrifuged at 12,000 × g for 1 h at 4°C. The supernatant obtained was used for the determination of lipid peroxidation (LPO)[30], reduced glutathione (GSH) content[31], superoxide dismutase (SOD, E.C 1.15.1.1)[32], catalase (CAT, E.C 1.11.1.6)[33] and glutathione reductase (GR)[34,35] activities and estimation of protein[36].

Gelatin zymography

Liver extracts were thawed on ice and mixed 3:1 with substrate gel sample buffer (10% SDS, 4% sucrose, 0.25 M Tris-HCl, *p*H 6.8, 0.1% bromophenol blue). Each sample (20 µg) was loaded under non-reducing conditions on to electrophoretic mini-gels (SDS-PAGE) containing 1 mg/mL of type-1 gelatin (Sigma, USA). The gels were run at a running buffer temperature of 4°C. After SDS-PAGE, the gels were washed twice in 2.5% Triton X-100 for 30 min each, rinsed in water and incubated overnight in a substrate buffer at 37°C (Tris-HCl 50 m*M*, $CaCl_2$ 5m*M*, NaN_3 0.02%, *p*H 8). The gels were stained with Coomassie brilliant blue R250 and gelatinolytic activity of matrix metalloproteinases was detected as clear bands on a blue background[9,37].

Electron microscopic study

For electron microscopic study of liver at day 90 and 120, the standard gold coating technique using critical point-drier (CPD-Biorad, Microscience Division, Warford, England), and sputter-coater (model 198, Agar Sputter Coater, Stansted, United Kingdom) was adopted in case of scanning electron microscopy (LEO, 435 VP, United Kingdom). For transmission electron microscopy (TEM; CM-10, Philips Electron Optics, Eindhoven, The Netherlands), the ultra-thin sections (60-90 nm, cut by Reichert E Jung, England) were stained with uranyl acetate and lead citrate. Four serial liver sections obtained from each of four different mice at each fixation interval were analyzed[6,7].

Blinding

A laboratory scholar provided food and remedies or placebos to the experimental and control mice, but the actual observers were not permitted to know if the mice were from the treated or control lots, because the mice were given different codes, only to be deciphered after the various parameters of study were performed. Therefore, for all practical purposes, it was a "blinded" study. Uniformity in scoring data of the "control" and the "treated" series was all along maintained.

Statistical analysis

The significance test between different series of the data was conducted by students' t-test. Since we were more concerned with the differences between the drug-fed series and p-DAB + PB + Alc fed control (positive), these were highlighted. Difference between groups was assessed by Two-way-ANOVA using the SPSS software package for Windows (Table 1). Post-hoc testing was performed for inter-group comparisons using the least significance difference (L.S.D.) test. A value of $p<0.05$ was considered to indicate a significance difference between groups.

Table 1 — Two-way-ANOVA analyses

		df	Mean square	F	Sig
For cytogenetical parameters					
CA	Series	6	1148.095	994.487	0.000***
	Days	3	55.576	48.140	0.000***
	Interaction	18	28.780	24.930	0.000***
MN	Series	6	2.962	787.689	0.000***
	Days	3	8.577E-02	22.810	0.000***
	Interaction	18	3.196E-02	8.499	0.000***
MI	Series	6	123.718	7157.119	0.000***
	Days	3	25.514	1475.987	0.000***
	Interaction	18	5.035	291.261	0.000***
SHA	Series	6	27.404	1874.335	0.000***
	Days	3	3.509	240.036	0.000***
	Interaction	18	0.867	59.269	0.000***
For LPO and GSH content in liver					
LPO	Series	6	7.034E-02	163.515	0.000***
	Days	3	0.104	241.486	0.000***
	Interaction	18	7.442E-03	17.298	0.000***
GSH	Series	6	4.894E-04	97.070	0.000***
	Days	3	1.459E-05	2.895	0.37*
	Interaction	18	9.945E-06	1.973	0.015*
CAT activity	Series	3	121.296	366.137	0.000**
	Days	3	0.664	2.003	0.121n
	Interaction	7	3.803	11.481	0.000***
GR activity	Series	6	443.357	63.413	0.000***
	Days	1	6.926	0.991	0.323n
	Interaction	6	9.210	1.317	0.261n
For different blood parameters					
Glucose	Series	6	12732.778	161.044	0.000***
	Days	3	3720.917	47.062	0.000***
	Interaction	18	501.254	6.340	0.000***
Hemoglobin	Series	6	41.786	65.625	0.000***
	Days	3	0.280 0.	0.440	0.725n
	Interaction	18	45.210	71.002	0.000***
Cholesterol	Series	6	3506.260	288.444	0.000***
	Days	1	154.714	12.728	0.001**
	Interaction	6	212.199	17.457	0.000***
For serum total bilirubin and albumin					
Bilirubin	Series	6	13.750	110.274	0.000***
	Days	3	1.314	10.542	0.000***
	Interaction	18	0.615	4.930	0.000***

Cont. ....

For serum LDH and serum GGT and G6PD in whole blood sample					Albumin	Series	6	7.482	32.149	0.000***
Serum LDH activity						Days	3	0.341	1.464	0.227^{n}
Series	6	719363.042	1086.462	0.000***		Interaction	18	11.936	51.288	0.000***
Days	3	8205.295	12.393	0.000***						
Interaction	18	10703.366	16.165	0.000***	**For different renal function tests**					
Serum GGT activity					Serum creatinine	Series	6	0.405	60.626	0.000***
Series	6	12362.935	688.199	0.000***		Days	3	1.947E-02	2.916	0.036*
Days	3	587.434	32.700	0.000***		Interaction	18	0.545	81.535	0.000***
Interaction	18	272.602	15.175	0.000***						
G6PD activity					Serum urea	Series	6	3991.574	123.170	0.000***
Series	6	26.594	336.927	0.000***		Days	3	195.127	6.021	0.001**
Days	3	0.265	3.360	0.021*		Interaction	18	1775.160	54.777	0.000***
Interaction	18	0.510	6.458	0.000***						
For activities of different antioxidant enzymes in liver					BUN	Series	6	870.664	118.200	0.000***
SOD activity						Days	3	42.578	5.780	0.001**
Series	3	8.492E-03	34.196	0.000***		Interaction	18	386.985	52.536	0.000***
Days	3	1.134E-04	0.457	0.714^{n}						
Interaction	7	8.340E-05	0.336	0.935^{n}						

*p<0.05; **p<0.01; ***p<0.001; ^{n}insignificant

Table 2 — Number of mice with tumor incidence at different fixation intervals and in different groups [Six mice were used in each group for fixation intervals at 30, 60, 90 and 120 days]

Groups	No. of specimens	Tumor incidence and intensity			
		30 Days	60 Days	90 Days	120 Days
Normal	24	0/6	0/6	0/6	0/6
Normal+Alc	24	0/6	0/6	0/6	0/6
p-DAB+PB	24	0/6	6/6 (3^{+++}, 3^{++})	6/6 (5^{+++}, 1^{++})	6/6 (6^{+++})
p-DAB+PB+Alc	24	0/6	6/6 (4^{+++}, 2^{++})	6/6 (5^{+++}, 1^{++})	6/6 (6^{+++})
p-DAB+PB+Nat Sulph-30	24	0/6	2/6 (1^{++}, 1^{+})	3/6 (2^{++},1^{+})	3/6 (2^{++}, 1^{+})
p-DAB+PB+Car-200	24	0/6	3/6 (2^{++}, 1^{+})	3/6 (3^{++})	4/6 (3^{++}, 1^{+})
p-DAB+PB+Nat Sulph-30+Car-200	24	0/6	2/6 (2^{++})	2/6 (1^{++}, 1^{+})	1/6(1^{+})
TOTAL	168	0/42	19/42	20/42	20/42

$^{+}$Low intensity tumor; $^{++}$Moderate intensity tumor; $^{+++}$High intensity tumor

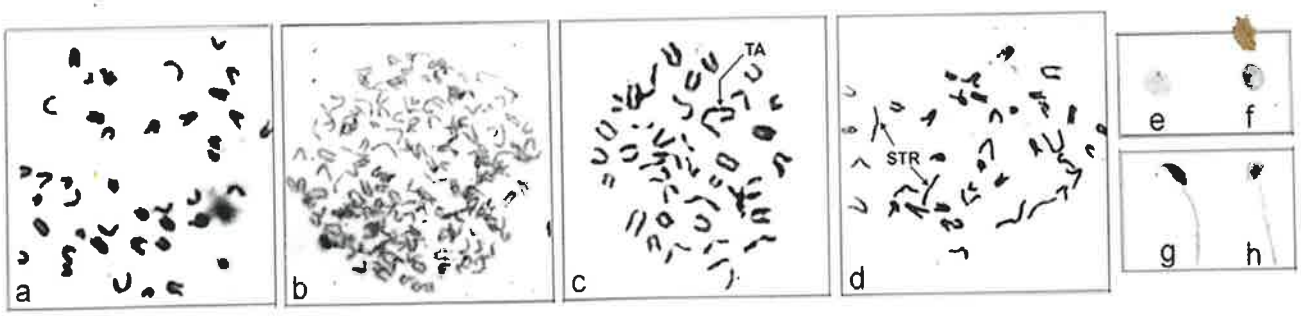

Fig. 1 — Representative photomicrographs showing (a): normal metaphase; (b): complement polyploidy; (c): terminal association (TA) and (d): stretching (STR); (e-f): erythrocyte showing micronucleus; (g): sperm with normal head morphology; and (h): sperm with abnormal head morphology

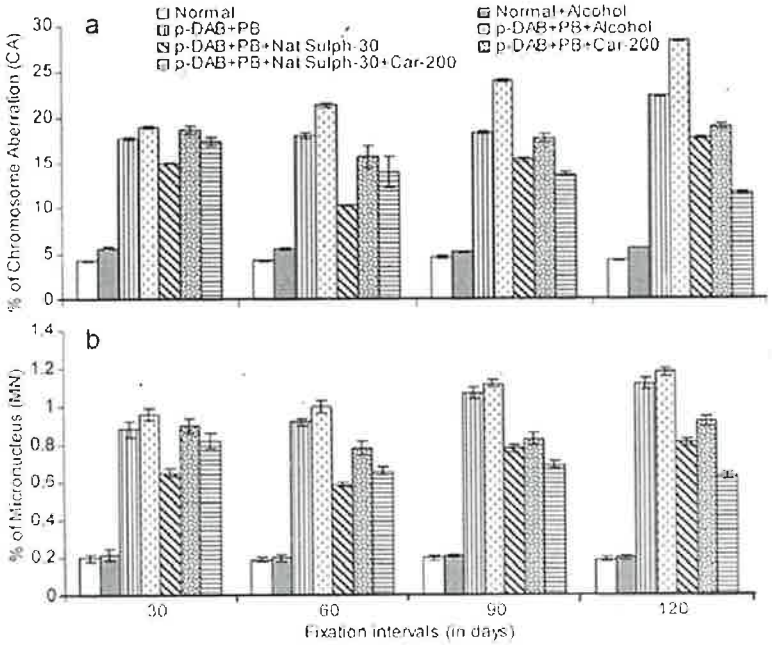

Fig. 2 — Percent of (a): chromosome aberrations (CA); and (b): micronucleus (MN) in different series of mice at different fixation intervals [Data presented as mean ± S.E.]

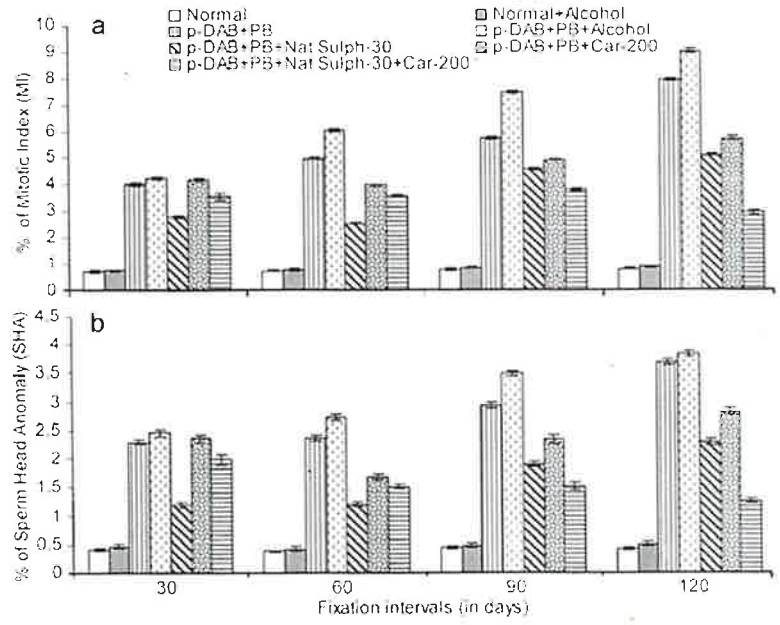

Fig. 3 — Percent of (a): mitotic index (MI)M; and (b): sperm head anomaly (SHA) in different series of mice at different fixation intervals [Data presented as mean ± S.E.]

Results

Incidence of liver tumors

Tumors were found on autopsy in the carcinogen-treated groups as well as in positive controls (Table 2). But, the incidence was much less in the drug-fed group, particularly in Nat sulph-30 + Car-200 fed group.

Effect on cytogenetical parameters

A few representative photomicrographs of normal and abnormal metaphase spreads are shown in Fig. 1a-d, MN in Fig. 1e-f, and normal and abnormal sperm (Fig. 1g and 1h, respectively). Chronic feeding of p-DAB + PB + Alc produced a significant increase of CA (Fig. 2a), MN (Fig. 2b), MI (Fig. 3a), and SHA (Fig. 3b). Administration of Nat sulph-30 or Car-200 alone brought about a significant restoration of CA, MN, MI and SHA and the values were comparable to those of normal controls. Application of the combination therapy of Nat sulph-30 and Car-200 produced better results than those with either Nat sulph-30 or Car-200 alone.

Effect on LPO

LPO was significantly decreased in all the drug-fed groups of mice (Fig. 4a), when compared with p-DAB + PB + Alc fed series at all fixation intervals. The combinational therapy showed better results, particularly at longer fixation intervals, while Nat sulph-30 showed better efficacy at shorter fixation intervals.

Effect on GSH content

A significant reduction in hepatic GSH content (Fig. 4b) was recorded at all fixation intervals in p-DAB + PB + Alc fed series of mice. Administration of Nat sulph-30 and Car-200 produced considerable repletion of hepatic GSH content, the combination therapy again showing better results.

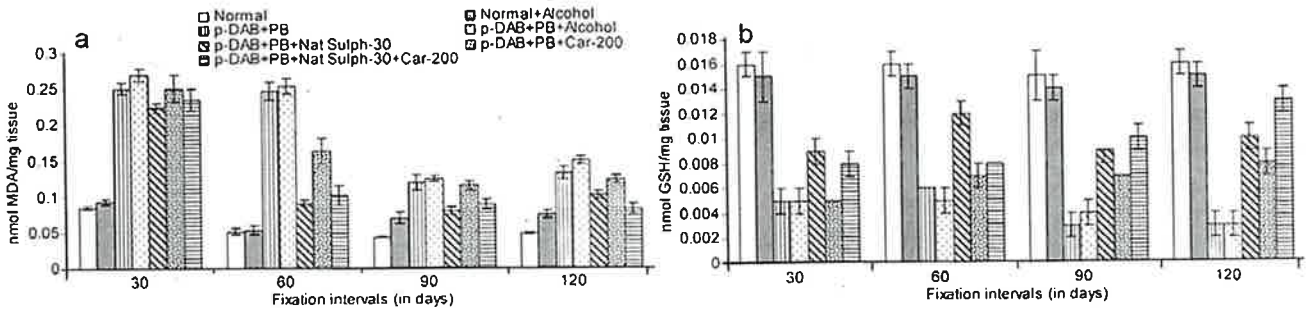

Fig. 4 — (a): Lipid peroxidation (nmol MDA/mg wet tissue); and (b): reduced glutathione (nmol GSH/mg tissue) content in different series of mice at different fixation intervals [Data presented as mean ± S.E.]

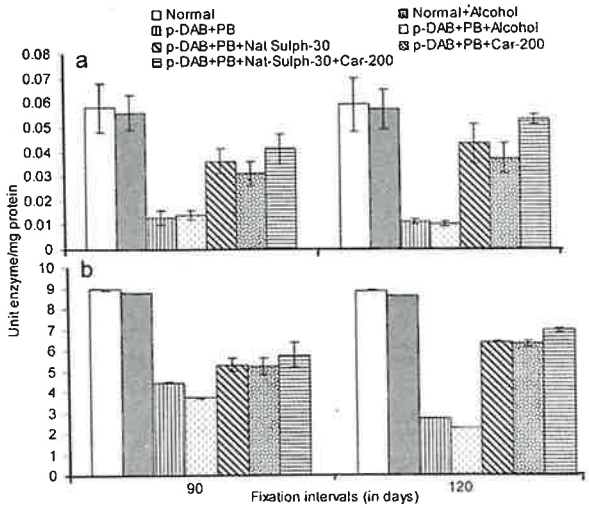

Fig. 5 — Activities of (a): SOD (Unit enzyme/mg protein); and (b): CAT (Unit enzyme/mg protein) in different series of mice at 90 and 120 days fixation interval [Data presented as mean ± S.E.]

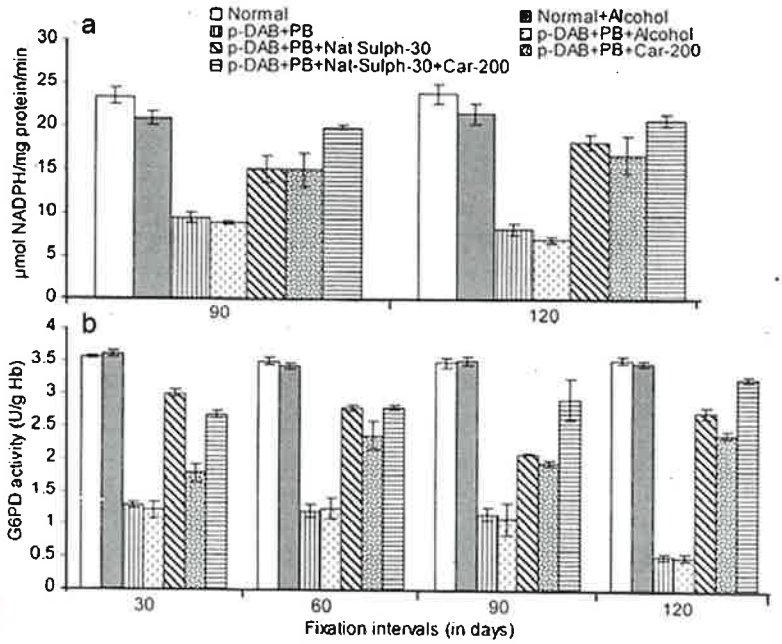

Fig. 6 — (a): GR activity (µmol NADPH/mg protein/min) in different series of mice at 90 and 120 days fixation interval; and (b): G6PD activity (U/g Hb) in different series of mice at different fixation intervals [Data presented as mean ± S.E.]

Effect on enzymes indicative of hepatic oxidative stress

Chronic feeding of *p-DAB* + PB induced considerable oxidative stress, which was evidenced by a significant decrease in the activities of SOD (Fig. 5a), CAT (Fig. 5b), GR (Fig. 6a) and G6PD (Fig. 6b). Treatment with Nat sulph-30 or Car-200 alone or as a combination therapy in carcinogen- intoxicated mice produced appreciable restoration of the said enzyme activities towards normal control levels, with Nat sulph-30 + Car-200 series showing the best results.

Effect on serum GGT and LDH

A significant increase in serum GGT (Fig. 7a) and LDH (Fig. 7b) was recorded in *p*-DAB + PB fed series of mice with minor deviations. Among the drug-fed series, the application of combinational therapy showed better results, particularly at longer fixation intervals, while Nat sulph 30 showed better efficacy at shorter fixation intervals with minor deviations.

Pathophysiological parameters

Effect on blood glucose, hemoglobin and cholesterol level

Blood glucose level (Fig. 8a) was increased in *p-DAB*+ PB and *p-DAB* + PB + Alc series and decreased in the drug-fed series. A significant decrease in blood hemoglobin (Hb) (Fig. 8b) and cholesterol levels (Fig. 9a) was observed in *p*-DAB + PB + Alc fed series of mice. Administration of the potentized homeopathic remedies produced appreciable restoration in the said levels, the combination therapy producing better results than the other two series.

Effect on serum bilirubin, serum albumin and renal functions

An increase in serum bilirubin level (Fig. 9b) was the characteristic of *p*-DAB + PB + Alc fed group of mice. Administration of the different potencies of homeopathic remedy brought down the values almost to levels comparable with those of normal controls.

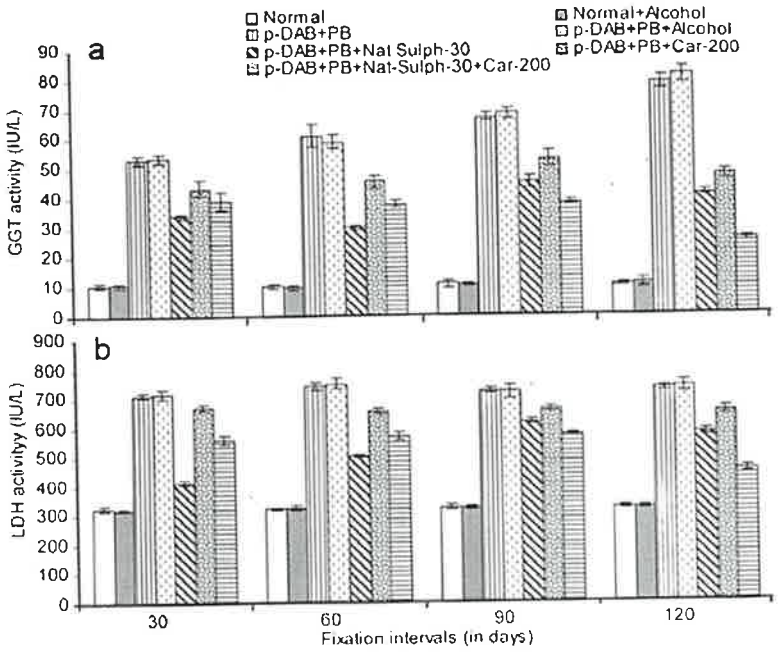

Fig. 7 — Activities of serum (a): γ-GGT (IU/L); and (b): LDH (IU/L) in different series of mice at different fixation intervals [Data presented as mean ± S.E.]

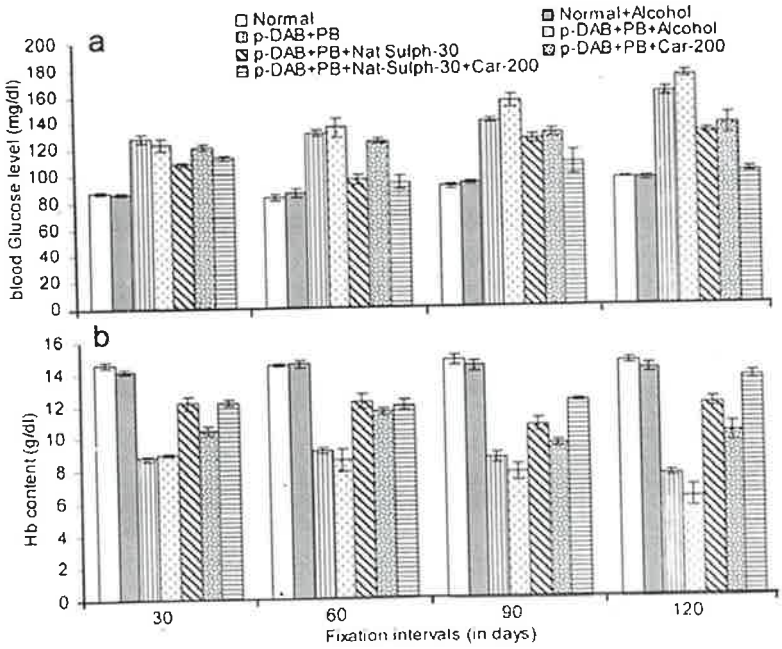

Fig. 8 — (a): Blood glucose level (mg/dl); and (b): haemoglobin content (g/dl) in different series of mice at different fixation intervals [Data presented as mean ± S.E.]

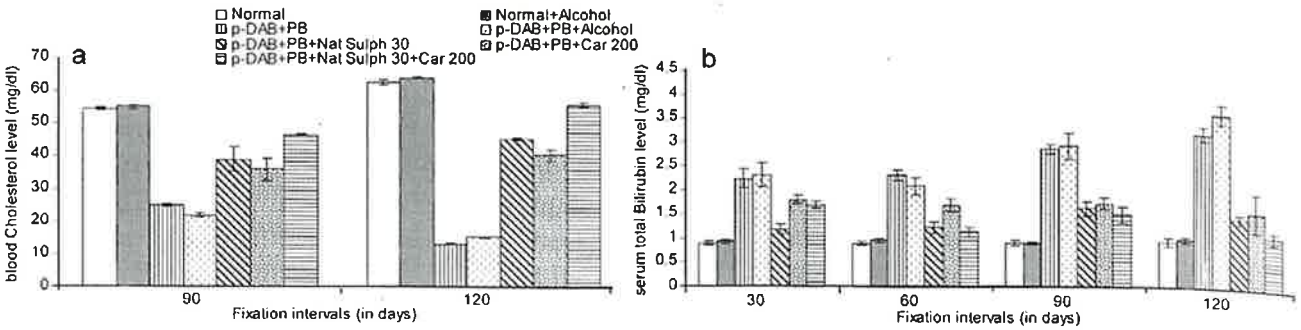

Fig. 9 — (a): Blood cholesterol level (mg/dl) in different series of mice at 90 and 120 days fixation interval; and (b): serum total bilirubin level (mg/dl) in different series of mice at different fixation intervals [Data presented as mean ± S.E.]

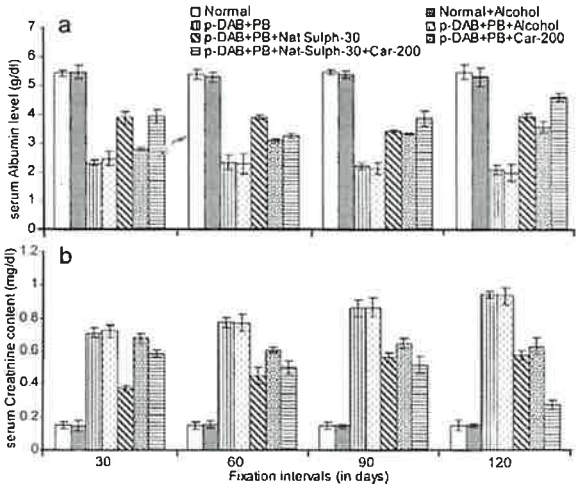

Fig. 10 — (a): Serum albumin level (g/dl); and (b): serum creatinine content (mg/dl) in different series of mice at different fixation intervals [Data presented as mean ± S.E.]

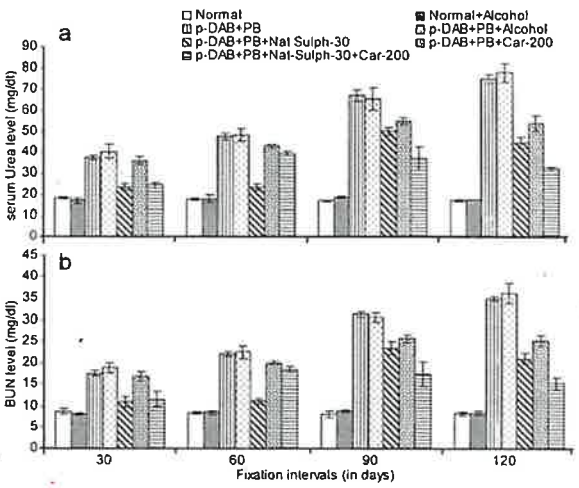

Fig. 11—(a): Serum urea level (mg/dl); and (b): BUN level (mg/dl) in different series of mice at different fixation intervals [Data presented as mean ± S.E.]

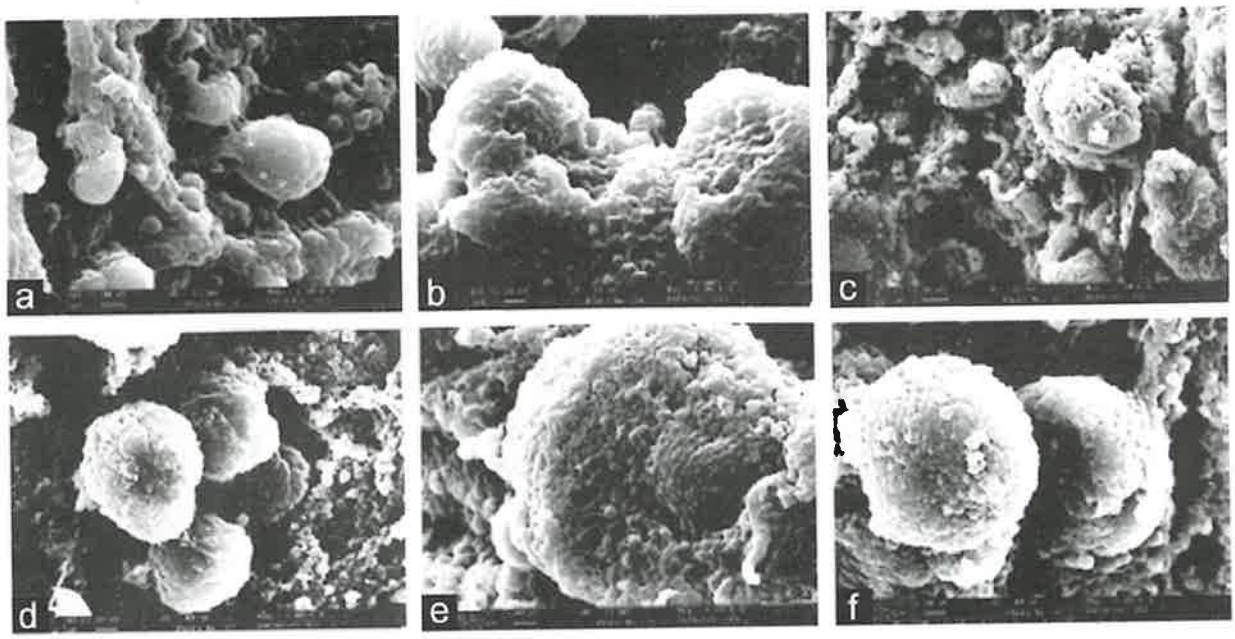

Fig. 12 — Representative photomicrographs of liver sections under SEM showing features of (a): normal; (b-c): *p*-DAB + PB + Alc; (d): *p*-DAB + PB + Nat sulph-30; (e) *p*-DAB + PB + Car-200; and (f): *p*-DAB + PB + Nat sulph-30 + Car-200

A decrease in serum albumin level (Fig. 10a) was the characteristic of *p*-DAB + PB + Alc fed group of mice. Administration of the different potencies of homeopathic remedy increased the values close to levels of normal controls. Nat sulph-30 + Car-200 fed mice showed the best results. A significant increase in serum creatinine (Fig. 10b), serum urea (Fig. 11a) and blood urea nitrogen (BUN) (Fig. 11b) was observed in mice chronically fed *p*-DAB + PB indicating possibility of renal failure. Administration of Nat sulph 30 or Car 200 separately along with *p-DAB* + PB brought about considerable positive modulation in the levels of serum creatinine, urea and BUN. In this regard, the combined treatment with Nat sulph-30 + Car-200 produced better results when compared to that of Nat sulph-30 or Car-200 fed alone group.

Electron microscopic studies

SEM study

The hepatocytes in normal controls are shown in Fig. 12a. Damaged hepatocytic cells were found in the *p*-DAB + PB and *p*-DAB + PB + Alc fed series (Fig. 12b-c), whereas relatively less damaged hepatocytes were found in the drug-fed groups (Fig. 12d-f); Nat sulph-30 + Car-200 (Fig. 12f) showed better results.

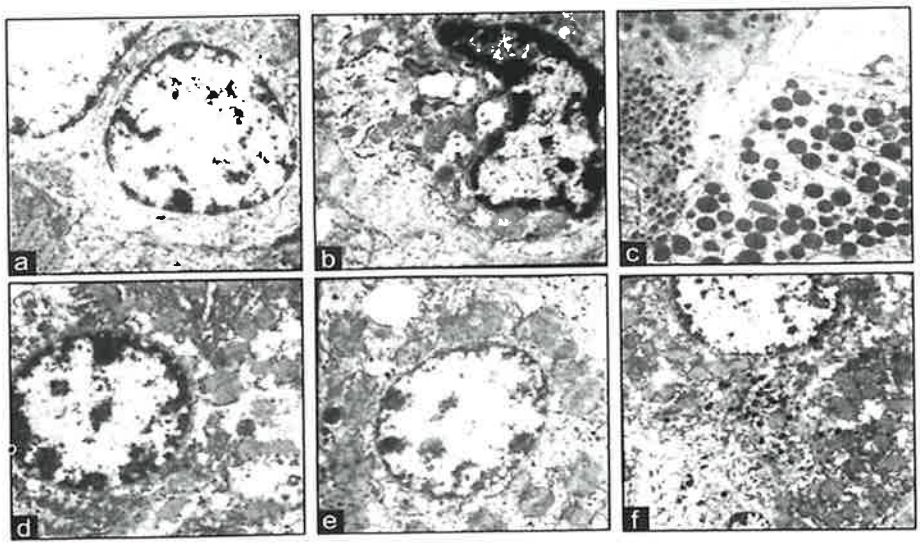

Fig. 13 — Representative photomicrographs of liver sections under TEM showing features of (a): normal; (b-c): *p*-DAB + PB + Alc; (d): *p*-DAB + PB + Nat sulph-30 fed; (e): *p*-DAB + PB + Car-200and; (f): *p*-DAB + PB + Nat sulph-30 + Car-200

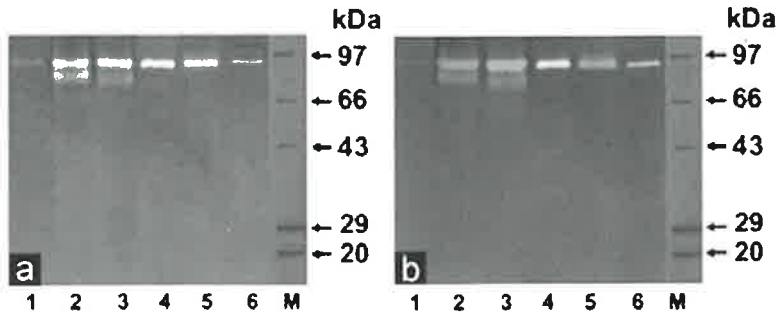

Fig. 14—Gelatin zymogram of liver samples showing the expression of MMP in experimental mice sacrificed at (a): day 90; and (b): day 120 [lane 1, normal; lane 2, *p*-DAB + PB; lane 3, *p*-DAB + PB + Alc; lane 4, *p*-DAB + PB + Nat Sulph-30; lane 5, *p*-DAB + PB + Car-200; lane 6, *p*-DAB + PB + Nat sulph-30 + Car-200; and M, molecular weight marker]

TEM study

Contrary to the intact nuclear membrane observed in liver cells of normal mice (Fig. 13a), it was found to be broken in liver cells of *p*-DAB + PB and *p*-DAB + PB + Alc (Fig. 13b-c) fed mice, and black lipid droplets were quite prominent (Fig. 13c). Necrotic changes were found in the cristae of *p*-DAB + PB or *p*-DAB + PB + Alc fed mice. Further, the number of vacuoles was higher in these carcinogen intoxicated groups, while cisternae of the Golgi bodies were absent in them. On the other hand, in mice administered either Nat sulph-30 (Fig. 13d) or Car-200 (Fig. 13e), or Nat sulph-30+Car-200 (Fig. 13f), these damaging features were less conspicuous. In regard to ameliorative changes, the combination therapy of Nat sulph-30 + Car-200 showed better results.

Gelatin zymogram profile for protease activity (matrix metalloproteinase or MMP activity)

At 90 day fixation interval, two bands were expressed near 92kDa (Fig. 14a) in *p*-DAB + PB and *p*-DAB + PB + Alc fed mice, which belonged to MMP family (from analysis of substrate specificity and molecular mass of 92 kDa, it appeared to be MMP-9). In the drug-fed series, single band was observed and the expression of MMP appeared to be somewhat less than that of carcinogen-fed series of mice. Similar results were obtained for 120 days also (Fig. 14b). This suggested that the expression of MMP was slightly less in Nat sulph-30 + Car-200 than either Nat sulph-30 or Car-200 (as depicted from width of the bands).

Discussion

One of the primary objectives of the present study was to examine, if positive results were obtained in an experimental mammalian model, mice, which could be extrapolated in a more meaningful and convincing manner for their possible human use. Results of the present study indicated that the individual treatment with Nat sulph-30 or Car-200 produced some ameliorative effects, but when in combination, they brought forth more striking changes in the various parameters. Administration of Car-200 in combination with Nat sulph-30 appeared to antagonize tumor formation to a considerable extent. These drugs effectively reduced the elevated chromosomal, nuclear and sperm head anomalies and also the various toxicity indices, suggesting their ability to combat carcinogenesis at the chromosomal and genomic levels.

Enhanced oxidative stress leads to a notable rise of cell proliferation and DNA synthesis, which result in promotion of tumors[38]. In most malignant tumors, neoplastic cells undergo chromosomal alterations, usually both structural and numerical aberrations[39] Chronic feeding of *p*-DAB + PB or *p*-DAB + PB + Alc increased the frequency of MN and MI of the bone marrow cells. A significant decrease in MN and MI observed in the Nat sulph-30, Car-200 and Nat sulph-30 + Car-200 fed mice also pointed to their anti-carcinogenic role. Considerable amount of repair or protection rendered to sperm head by the application of the combination therapy of Nat sulph-30 and Car-200 was indicative of its anti- spermatotoxic effect.

The reaction products of LPO e.g. malon-dialdehyde (MDA) are related to mutagenicity and carcinogenicity[40,41]. MDA has the capacity to bind to macromolecules like DNA and it appears to be mutagenic and carcinogenic[42]. GSH is a prominent cellular reductant, which is associated with defense against free radicals, peroxides and other toxic compounds[43]. Incidentally, the increased level of LPO observed in mice chronically fed *p*-DAB and PB could be due to the oxidative stress. In the present study, a

decrease in GSH content was accompanied by an increase in LPO in carcinogen-treated mice.The reversal of the levels of LPO and GSH towards normalcy in the drug-fed mice was in line with the activities of the other biomarkers. Furthermore, the cytoprotective process and antioxidant property of GSH possibly acted co-operatively to inhibit LPO, and thus had significant role in hepatic detoxification.

Chronic feeding of the carcinogens *p*-DAB and PB triggers generation of ROS over the lapse of time. SOD and CAT have the ability to remove ROS. Oxidation of glutathione (GSH) produces oxidized glutathione (GSSG) which is reduced to GSH by glutathione reductase utilizing NADPH as a reducing equivalent. It plays a significant role in maintaining adequate amounts of GSH. Accordingly, the reduction of GR results in decreasing GSH[44]. The rate-limiting step of the pentose phosphate pathway in which ribose synthesis occurs for nucleic acid generation is catalyzed by glucose-6-phosphate dehydrogenase (G6PD)[45].

GGT is a membrane bound enzyme that catalyzes the degradation of GSH and other *y*-glutamyl compounds by hydrolysis of the *y*-glutamyl moiety or by its transfer to a suitable acceptor[46]. Enhanced activity of GGT may occur due to oxidative stress, which has the capability to increase the transport of glutathione precursors into cells[47]. LDH, a glycolytic enzyme plays an important role in the reversible conversion of pyruvate to lactate[48]. The increase in the activities of the enzyme LDH and GGT brought about by the administration of the combination therapy of Nat sulph-30 and Car-200 further supported to the anti-toxic and anti-tumor potentials of the drugs.

Serum creatinine measurement is mostly considered as an indicator of renal function[49]. The amount of nitrogen in the blood resulting from urea is measured by blood urea nitrogen (BUN) test[48]. Although, serum creatinine level is more specific measure of renal function, elevated level of BUN is also an indicator of poor kidney function[48]. The positive modulation of creatinine and BUN levels due to the administration of Nat sulph-30 and Car-200 highlighted the protective ability of the drug on renal functions as well.

The occurrence of several types of cancer is related to elevated blood glucose level[50]. Also, decreased level of cholesterol in hepatocellular carcinoma (HCC) of human is reported[51]. Normal heme catabolism leads to formation of bilirubin (a yellow breakdown product) and severe liver failure with cirrhosis may lead to very high levels of bilirubin[48] The serum albumin level is of utmost significance and has been accepted as a test for liver function[52,53]. Treatment with Nat sulph-30 alone and in combination with Car-200 significantly reduced the bilirubin level and increased the albumin level, which is an indication of the hepatoprotective ability of the drugs.

An increase in the number of mitochondria, distorted nuclei and large black lipid droplets was observed in the TEM studies of liver tissues of carcinogen-fed mice. Similarly, SEM studies showed damaged hepatocytes and hepatic chords in the carcinogen-fed mice. In the drug-fed series, these features were less conspicuous, suggesting the positive effect of the drugs in the carcinogen-induced damage at the ultra-structural level.

MMPs are key enzymes that play a significant role in tumor invasion and metastasis[54,55]. The expression of MMP-9 is reported to increase in malignant tissues[56]. In the present study, MMP (presumably MMP-9) was overexpressed (depicted from number and intensity of the bands) at the longer fixation intervals in mice fed either *p-DAB* + PB or *p*-DAB + PB + Alc, but no such overexpression (only a single band) of these MMPs was noticed in the drug-fed mice. Thus, lack of expression in drug-fed group rendered strong evidence in favor of their anti-tumorigenic effects at the gene expression level. How the micro doses of the potentized homeopathic remedies can elicit response on receptors is still unresolved. Are there any specialized types of receptors capable of recognizing "homeopathic signal"? Similarly, the question of ligand binding in the conventional manner is an area that needs further research[57]. Earlier, Khuda-Bukhsh[16,17] advocated a working hypothesis that suggests that the homeopathic remedy has the ability to trigger relevant gene(s) into action by acting as a "molecular switch" and thereby initiating a cascade of chain reactions in downstream genes that can regulate the expression of right kind of protein necessary for recovery from the disease state or disorderly state of gene functioning. However, further studies at molecular level of gene expression through micro-array chips and also of signal transduction pathways could provide more light to understand the molecular mechanism of action of these ultrahigh diluted remedies.

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