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Genotoxic and Carcinogenic Risks Associated with the Consumption of Repeatedly Boiled Sunflower Oil

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Repeated boiling of vegetable oils at high temperature in cooking and frying is a very common practice and leads to the formation of a class of toxic substances. Among them, polycyclic aromatic hydrocarbons (PAHs) are well-documented for their mutagenic/carcinogenic potential. The objectives of the present study were to evaluate the genotoxic and carcinogenic risks associated with the consumption of repeatedly boiled sunflower oil, which is one of the commonly consumed vegetable oils in southeast Asian countries. The presence of PAHs was analyzed using high-performance liquid chromatography (HPLC) methods in fresh, single-boiled, and repeatedly-boiled sunflower oil (FSO, SBSO, and RBSO) samples. A higher amount of known carcinogenic/mutagenic PAHs in RBSO samples were shown, as compared to FSO and SBSO. Oral administration of RBSO in Wistar rats resulted in significant induction of aberrant cells ($p < 0.05$) and micronuclei ($p < 0.05$) incidence in a dose-dependent manner. Oxidative stress analysis also showed a significant decrease in levels of antioxidant enzymes, such as superoxide dismutase and catalase, with a concurrent increase in reactive oxygen species and lipid peroxidation in animals following RBSO consumption, as compared to FSO or SBSO ($p < 0.05$). Additionally, RBSO administration alone and along with diethylnitrosamine for 12 weeks induced altered hepatic foci, as noticed by the alteration in positive (γ-glutamyl transpeptidase and glutathione-*S*-transferase) and negative (adenosine-triphosphatase, alkaline phosphatase, and glucose-6-phosphatase) liver biomarkers. A significant decrease in the relative and absolute hepatic weight in RBSO-supplemented rats was also noted ($p < 0.05$).

KEYWORDS: Sunflower oil; polycyclic aromatic hydrocarbons; altered hepatic foci; micronuclei; chromosomal aberration

INTRODUCTION

An increased risk of cancer is associated with the lifestyle-related factors of an individual, with diet being the major one. Diet is considered as a double-edged sword, which plays a pivotal role in the causation or prevention of cancer. High-temperature processing of foods can lead to the reduction of nutrients and formation of various toxic substances (*1*). Nevertheless, many south Asian countries, including India, use vegetable oil as an essential cooking medium. The use of repeated boiling of cooking oils is associated with the formation of genotoxic moieties (*2*). This may be attributed to the conversion of oil constituents to potentially toxic substances, such as volatile chain-scission products, non-volatile oxidized derivatives, dimeric, polymeric, or cyclic substances, etc. (*3*). Hageman et al. (*4*) reported that repeatedly frying fats possess mutagenic activity. Frying of foods in repeatedly heated oils causes the generation of various poly-aromatic hydrocarbons (PAHs) in food products and oil fumes (*5*). Rape seed oil vapors were shown to contain, mutagenic PAHs, benzo(*a*)pyrene [B(*a*)P], and dibenz(*a*,*h*)anthracene (*1*). Moreover, epidemiological studies confirmed that individuals exposed to mixtures containing PAHs have substantial susceptibility toward cancer development (*6*).

Next to tobacco usage, diet is the major non-occupational source of PAHs to humans, including meat and meat products, cereals, and oils and allied products as the major sources (*7*). Principle candidates among the risk-related foods are edible oils, predominantly in south Asian countries, because of their cooking and eating practices. Sunflower oil (SO), extracted from the seeds of sunflower (*Helianthus annuus*, family: Compositeae), is a combination of monounsaturated and polyunsaturated fats, predominantly linoleic acid. Linoleic acid is known to be essential for the maintenance of immune responses, but an excess of it promotes tumor growth in animals (*8*). It has been shown that oxidized SO consumption can be hepatotoxic (*9*) and may induce cancer (*10*). During deep frying when the fat is used repeatedly, oxidative and thermal effects result in the formation of many volatile and non-volatile products, some of which are potentially toxic (*3*). Consumption of the decomposition products formed by thermal abuse and oxidation of frying oils is reported to induce a number of pathological conditions (*11*). Although increased awareness about obesity has led to a dietary shift toward vegetable oils containing ω-6 polyunsaturated fatty acids

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(PUFAs), yet studies report PUFA to be pro-inflammatory, prooxidative, and atherogenic (*12*).

In view of the above information, the present study has been designed to investigate the presence of the PAH mixture in repeatedly boiled SO by high-performance liquid chromatography (HPLC) analysis and to evaluate the potential of thermally degraded oil to cause genotoxicity, oxidative stress, and carcinogenic risk in Wistar rats using a medium term carcinogenicity assay with the induction of altered hepatic foci (AHF) as the end point.

MATERIALS AND METHODS

Chemicals. Dimethyl sulfoxide (DMSO), *n*-hexane, cyclohexane, anhydrous sodium sulfate, dichloromethane (DCM), acetonitrile were purchased from Merck Chemical Company (Mumbai, India) and were of analytical grade and purity. The PAH standard mix was purchased from Supelco, Bellefonte, PA. Diethylnitrosamine (DEN), 2-acetylaminofluorene (2-AAF), γ-glutamyl-4-methoxy-β-napthylamide, glycylglycine, fast blue BB salt, adenosine triphosphate disodium salt, sodium-β-glycerophosphate, sodium diethylbarbiturate, glucose-6-phosphate monosodium salt, B(*a*)P, colchicines, dichlorodihydroflourescien diacetate dye (DCFH-DA), phenazine methosulfate, 2-thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane (TMP), nitroblue tetrazolium, nicotinamide adenine dinucleotide phosphate reduced, and nicotinamide adenine dinucleotide reduced were purchased from Sigma Chemical Company (St. Louis, MO). Anti-glutathione *S*-transferase-placental form (GST-P) and goat anti-rabbit immunoglobulin G (IgG) peroxidase secondary antibodies were procured from Calbiochem (Darmstadt, Germany). SO (refined) used in the experiment was purchased from the local market. The rest of the chemicals used in the study were of analytical grade and purity and procured locally.

Boiling Procedure for SO Sample Preparation. Three types of SO samples, viz., fresh (FSO), single-boiled (SBSO), and repeatedly boiled (RBSO), were used in this study. In brief, the SO was boiled (at its smoke point of 280 °C for 30 min) once, allowed to cool to room temperature, and labeled as SBSO. For RBSO samples, this process (boiling and cooling) was performed 6 times. FSO was given no heat treatment and used as such.

Extraction of PAHs. The FSO, SBSO, and RBSO samples were subjected to extraction and clean up procedure for PAHs according to Pandey et al. (*5*), with slight modification. Briefly, for extraction of PAHs, 20 mL of *n*-hexane was added to 10 g of oil, extracted 3 times with DMSO, following the addition of cold distilled water (4 °C) slowly, and re-extracted 3 times with cyclohexane (solvent was changed for specific extraction of PAHs).

Purification of PAHs. After washing, it was concentrated and passed through a glass column (20 × 2.2 cm) containing silica gel and anhydrous sodium sulfate. The elution was carried out with cyclohexane and DCM and evaporated to dryness. The final residue was dissolved in 1 mL of acetonitrile and passed through a 0.22 μm filter.

Animals and Treatment. Male Wistar rats at 125−150 g of body weight were obtained from the Indian Institute of Toxicology Research (IITR) animal colony. After acclimatization, rats were housed in stainless-steel cages in an air-conditioned room and fed a synthetic pellet basal diet (Ashirwad, Chandigarh, India) and water *ad libitum* under standard conditions with a 12/12 h light/dark period. The ethical approval for the experiments was obtained from the institutional animal ethics committee.

Feeding Procedures. *Chromosomal Aberration and Micronuclei Induction Assay*. The animals were divided into 2 sets of 8 groups, each comprising 6 animals. In each set, group I comprised of untreated rats and served as the negative control group, while group II (positive controls) was treated with B(*a*)P [100 mg/kg of body weight, intraperitoneally (ip)] 24 h prior to sacrifice. Animals of group III were administered FSO (0.5 mL) by gastric intubation. Animals of group IV-a (0.25 mL) and IV-b (0.5 mL) were administered SBSO by gastric intubation. Animals of group V-a (0.1 mL), V-b (0.25 mL), and V-c (0.5 mL) were administered RBSO by gastric intubation. After 1 week of administration, animals from all groups were sacrificed by cervical dislocation, and bone marrow was collected from their femurs, for chromosomal aberration and micronuclei induction assay.

Oxidative Stress Studies. The animals were divided equally into 5 groups of 10 animals each, where groups I, II, and III were treated in cytogenetic assays. Other groups, i.e., groups IV and V, were given SBSO and RBSO (0.5 mL/animal), respectively. After the animals were sacrificed at the end of 1 week, liver from each animal was excised and homogenized in ice-cold phosphate-buffered saline (pH 7.4) for biochemical estimations.

Induction of AHF. The animals were divided into 6 groups of 12 rats each. Animals of group I were maintained on a normal basal diet for the entire treatment period (i.e., 12 weeks) and served as controls. Groups II, III, IV, and V animals were given a single dose of DEN (200 mg/kg of body weight, ip). After 1 week, animals of group II were fed with a diet containing 0.05% 2-AAF and animals of groups III, IV, and V were given 0.5 mL of FSO, SBSO and RBSO, respectively, by gastric intubation for the entire study period. Simultaneously, animals of group VI were given 0.5 mL of RBSO only. Animals from all groups (groups I−VI) were subjected to two-third partial hepatectomy (PH) after 3 weeks (**Figure 1**). After completion of the study period, all of the animals were sacrificed humanly and their livers were excised, cleaned, weighed, and stored for further analysis.

Methodology. *HPLC Analysis of SO*. Quantification of extracted PAHs was performed with HPLC (Water Associates, Inc., Milford, MA) equipped with a dual pump and Rheo dyne injector with a 20 μL loop. The reverse-phase column used for the analysis was C-18 (E. Merck, Darmstadt, Germany) with a precolumn of the same type. The column was eluted at ambient temperature (27 °C) with 70:30 acetonitrile/water as the solvent, at a flow rate of 1.5 mL/min, and monitored on an ultraviolet (UV) detector at λ of 254 nm. The chromatogram was recorded and processed by Waters Millenium Software. The 16 PAH selection criteria were based on (i) the number of benzene rings in PAHs, (ii) PAHs generally found in oils following the literature search (*5*), and (iii) resolution of PAHs on HPLC. The peaks of the mixture of PAHs in oils were identified by comparing the retention time to that of the standard by an UV detector for the respective fractions. Quantification of PAHs was performed by comparing the integrated peak area to that of the standards.

Chromosomal Aberration Assay. Cytogenetic analysis was performed as described earlier (*13*). To arrest the metaphase stage, colchicine [4 mg (kg of body weight)$^{-1}$ animal^{-1}] was given 2 h prior to sacrificing the animals. Briefly, the bone marrow was flushed out from both femurs using Hanks buffered salt solution (HBSS, at pH 7.2) and centrifuged, and the pellet was redispersed in a hypotonic solution (0.56%; w/v) of KCl at 37 °C to permit osmotic swelling of cells. Swollen cells were fixed in ice-cold Carnoy's fluid, dropped onto slides, and stained with phosphate-buffered 5% Giemsa solution. A total of 75 well-spread metaphase plates per animal in each group were analyzed for chromosomal aberrations, and the mitotic index (MI) was calculated from a scan of 1000 cells per animal and classified as breaks, fragments, and exchanges. The incidence of aberrant cells was expressed as the percentage of damaged cells (aberrant metaphases).

Micronuclei Induction Assay. The frequency of micronucleated polychromatic erythrocytes (MNPCEs) was studied by the method as described by us (*13*). The bone marrow was flushed from femurs using HBSS, 1% (w/v) bovine serum albumin (BSA), and 0.15% (w/v) ethylenediaminetetraacetic acid (EDTA) (pH 7.2). Evenly spread bone marrow smears were stained using the May-Grunwald and Giemsa solutions. A minimum of 1000 polychromatic erythrocytes (PCEs) were scored for all treated and control groups.

Biochemical Estimations of Antioxidant Enzymes. The protein content of the tissue was determined by the method of Lowry et al. (*14*) using BSA as the standard. Cu,Zn−superoxide dismutase (SOD) activity was analyzed as per the protocol in ref *15* and expressed as specific activity in units min^{-1} (mg of protein)$^{-1}$. The activity of catalase (CAT) was analyzed by the method proposed in ref *16* using hydrogen peroxide (H_2O_2) as the substrate. The enzyme activity was measured following the disappearance of H_2O_2 at 570 nm and expressed as micromoles of H_2O_2 consumed per minute per milligram of protein. Lipid peroxidation (LPO) was analyzed by the method proposed in ref *17*. The peroxides were expressed as nanomoles of thiobarbutric acid reactive substance (TBARS) per hour per milligram of tissue protein, using TBA as the standard.

Flow Cytometric Analysis of Reactive Oxygen Species (*ROS*). ROS production was monitored by flow cytometry (BD-LSR II, San Jose, CA) using DCFH-DA, as described by Esposti and McLennan (*18*). The fluorescence increased because of the hydrolysis of DCFH-DA to

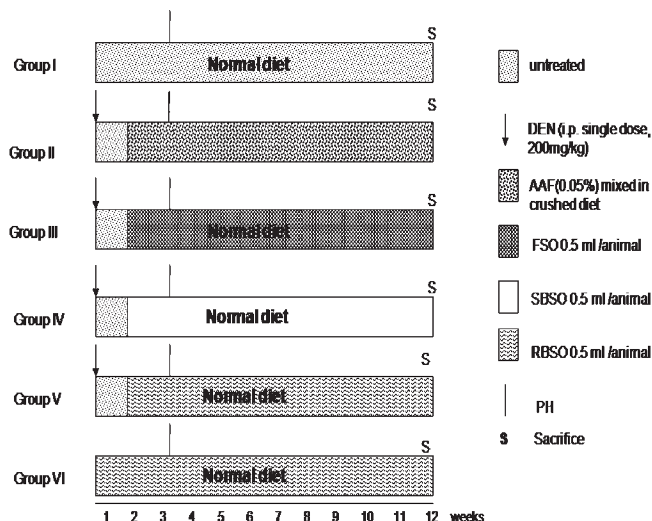

Figure 1. Treatment schedule for the induction of AHF in Wistar rats.

dichlorodihydrofluoroscein (DCFH) by some non-specific cellular esterases, and its subsequent oxidation by peroxides was measured. Values were given in terms of mean fluorescence intensity (MFI) using software "CellQuest".

Cryotomy and Immunohistochemistry for AHF. Tissue sections (10 μm) were cut from the frozen liver tissues using cryostat microtome (Slee Menz, Germany) and were stained for histochemical localization of GST-P, γ-glutamyl transpeptidase (GGT), glucose-6-phosphatase (G6-Pase), adenosine triphosphatase (ATPase), and alkaline phosphatase (AlkPase). Immunohistochemical detection of GST-P was performed as per the described protocol in ref *19*. In brief, exogenous peroxidase activity in fresh liver sections was quenched with methanol/H_2O_2 solution, and nonspecific binding was blocked by normal goat serum. The sections were incubated with anti-GST-P antibody (1:300) and then with horseradish-peroxidase-conjugated anti-rabbit IgG (1:20) at 4 °C in a humid chamber. The color was developed in 3-amino-9-ethylcarbazole in the dark and counter-stained with hematoxylin. The activities of GGT, G6-Pase, ATPase, and AlkPase enzymes in liver were detected (*20*). Briefly, for GGT, the sections were incubated in a medium containing γ-glumatyl-4-methoxy-β-napthlylamide dissolved in DMSO, glycylglycine, and fast blue BB salt, transferred in copper sulfate solution, washed, and mounted. For the staining of G6-Pase, the sections were incubated in a medium containing lead nitrate and glucose-6-phosphate at 37 °C for 30 min and developed in yellow ammonium sulfide. The brownish black deposits of lead sulfide indicated the active site of G6-Pase. The activity of ATPase was detected by incubating the sections in medium containing lead nitrate, magnesium chloride, and adenosine triphosphate for 60 min at room temperature and developed by yellow ammonium sulfide. Brown precipitates indicated the site of enzyme activity. For the localization of AlkPase activity, the sections were incubated in a freshly prepared medium containing sodium β-glycerophosphate, sodium diethyl barbiturate, calcium chloride, and magnesium sulfate. After incubation, sections were transferred to cobalt nitrate solution and then developed in yellow ammonium sulfide followed by in between rinses with distilled water. The black deposits of cobalt sulfide indicated the site of activity.

The enzyme activity of each biomarker was observed with reference to area and counts of AHF in each group using Leica Q Win500 image analysis software, and the negative and positive AHF were captured with a microscope attached to a charge-coupled device (CCD) camera (JVC). The image analysis was performed for each slide in triplicate, with at least 10 fields in each slide. The foci were counted only if their diameter was > 0.20 mm.

Statistical Analysis. The statistical analysis for different parameters, between different groups, was performed using Student's *t* test. $p < 0.05$ was considered significant for all of the assays.

RESULTS

PAHs in RBSO. PAHs have been categorized on the basis of their molecular weight and ring sizes into 2 classes: "light PAHs" (naphthalene, acenaphthylene, fluorene + acenapthene, phenanthrene, anthracene, fluoranthene, and pyrene) and "heavy PAHs" [benzo(*a*)anthracene + chrysene, benzo(*k*)fluoranthene, benzo(*b*)fluoranthene, B(*a*)P, dibenzo(*a*,*h*)anthracene, indino(1,2,3-*cd*)pyrene, and benzo(*g*,*h*,*i*)perylene]. Upon quantitative analysis, the content of PAHs in the RBSO extract (235.98 μg/kg) was found higher in comparison to FSO (64.56 μg/kg) and SBSO (134.56 μg/kg) samples (**Table 1** and **Figure 2**). Among the analyzed PAHs, heavy PAHs were present in higher amounts in RBSO (108.17 μg/kg), as compared to FSO (14.79 μg/kg). In RBSO, an increase in the amount of heavy PAHs of about 7-fold as compared to FSO and 6-fold as compared to SBSO was recorded (**Table 1**). The PAHs in RBSO detected in the highest content were naphthalene followed by fluoranthene, dibenzo(*a*,*h*)anthracene, B(*a*)P, benzo(*b*)fluoranthene, benzo(*k*)fluoranthene, and indino(1,2,3-*cd*)pyrene + benzo(*g*,*h*,*i*)-perylene, showing an increase of about 2-, 23-, 5-, 15-, 14-, 8-, and 5-fold, respectively, over FSO.

Table 1. HPLC Analysis for FSO, SBSO, and RBSO

PAHs	FSO (μg/kg)	SBSO (μg/kg)	RBSO (μg/kg)
naphthalene	37.4 ± 4.2	39.2 ± 4.9	78.96 ± 8.6
acenaphthylene	7.64 ± 1.1	6.64 ± 0.8	8.45 ± 1.8
fluorene + acenapthene	4.16 ± 0.6	16.8 ± 1.7	2.20 ± 0.8
phenanthrene	0.87 ± 0.04	11.9 ± 1.5	1.52 ± 0.2
anthracene	0.17 ± 0.02	29.06 ± 3.8	0.61 ± 0.03
fluoranthene	1.48 ± 0.07	9.75 ± 1.9	34.15 ± 3.8
pyrene	1.35 ± 0.06	3.45 ± 0.8	1.92 ± 0.05
benzo(*a*)anthracene + chrysene	1.89 ± 0.04	1.70 ± 0.4	5.90 ± 1.8
benzo(*k*)fluoranthene	2.44 ± 0.4	3.68 ± 0.9	18.88 ± 2.9
benzo(*b*)fluoranthene	1.42 ± 0.06	0.75 ± 0.05	20.22 ± 3.2
benzo(*a*)pyrene	1.40 ± 0.07	1.65 ± 0.09	21.55 ± 2.6
dibenzo(*a*,*h*)anthracene	4.64 ± 0.9	6.76 ± 1.8	24.96 ± 4.1
indino(1,2,3-*cd*)pyrene + benzo(*g*,*h*,*i*)perylene	3.00 ± 0.6	3.22 ± 1.6	16.66 ± 2.9
total	64.56	134.56	235.98

Clastogenic Effects of RBSO. The incidence of all types of aberrations, including breaks, fragments, exchanges, and multiple damages, along with micronuclei, was observed to increase in B(*a*)P and RBSO-treated groups (**Figures 3** and **4** and **Table 2**). The percentage of aberrant cells in the B(*a*)P-treated group increased up to 531%, and MI was decreased by 66% with respect to the untreated control. In RBSO-fed groups, V-a, V-b, and V-c, a dose-dependent increase in the percentage of aberrant cells was recorded, i.e., 100, 215, and 285% (**Table 2**), respectively, significantly higher than the control. RBSO-fed animals also showed a significant dose-dependent decrease in MI, which was 22% in group V-a, 36% in group V-b, and 42% in group V-c ($p < 0.05$) (**Table 2**). Induction in MNPCEs/1000 PCEs was 200% in the B(*a*)P-treated group and 66−133% in RBSO-fed animals (V-a, V-b, and V-c) over the control ($p < 0.05$). In this study, no significant change in FSO- and SBSO-fed animals was noted on any of the above-studied parameters for genotoxicity in comparison to the control (**Table 2**).

Effects of RBSO on Antioxidant Enzymes and Oxidative Stress. The levels of antioxidant enzymes CAT and SOD were significantly lowered ($p < 0.05$) in B(*a*)P-exposed animals (group II) by 52 and 75%, respectively, over group I. The levels of CAT and SOD activity also declined significantly ($p < 0.05$) in RBSO-fed animals by 33 and 50%, respectively. A significant increase (171%) in LPO was recorded following B(*a*)P administration, and RBSO exposure induced LPO by 106% over controls ($p < 0.05$). In animals of SBSO- and FSO-administered groups, no significant change in any of the above parameters for oxidative damage was noted ($p > 0.05$; **Table 3**).

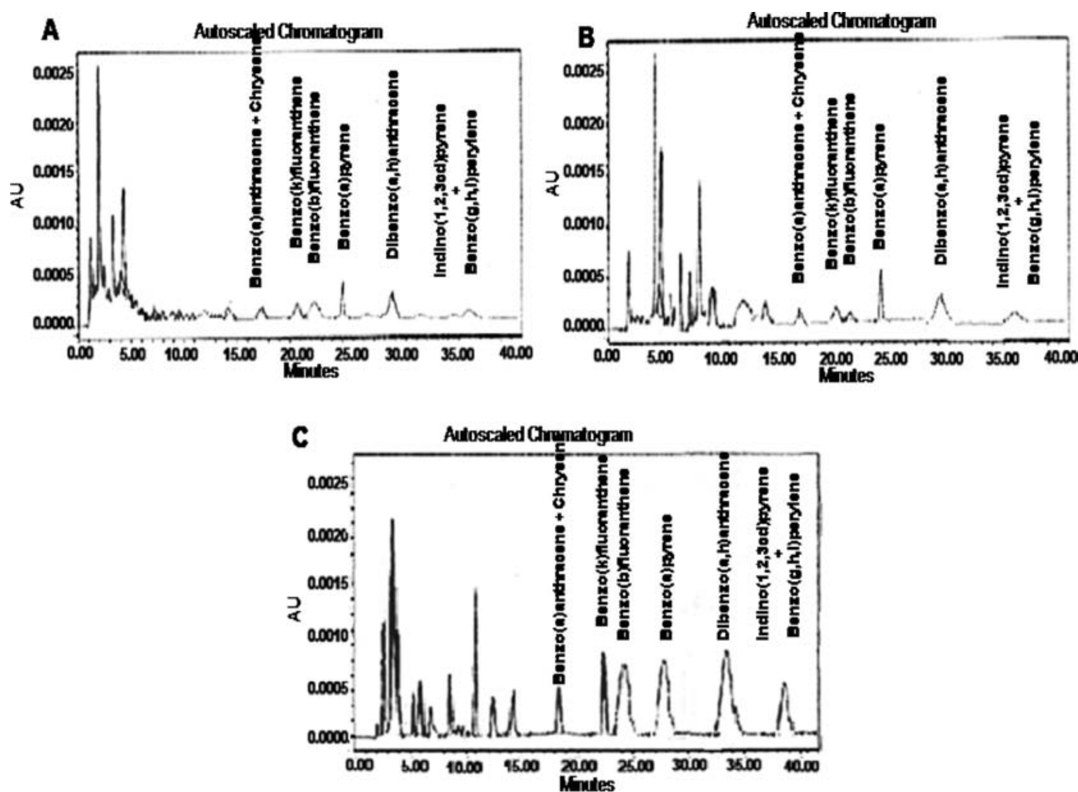

Figure 2. HPLC chromatograms showing the presence of PAHs in SO: (A) FSO, (B) SBSO, and (C) RBSO.

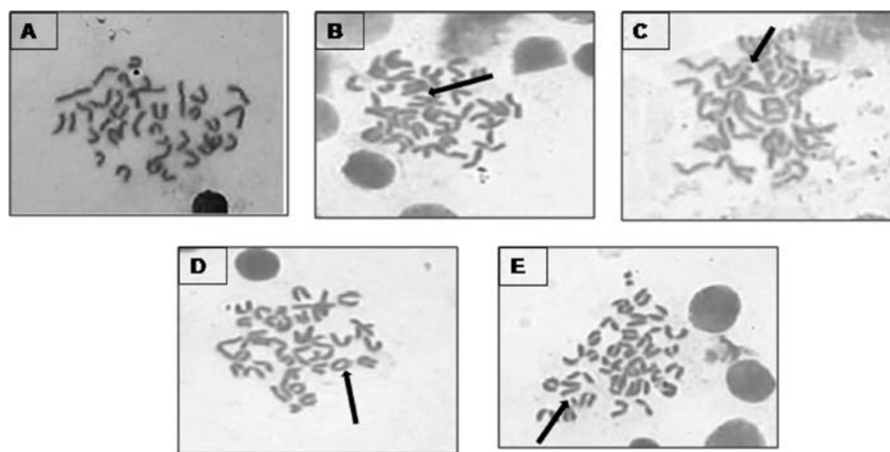

Figure 3. Representative pictures (100×) showing chromosomal aberration induced by RBSO in bone marrow of Wistar rats: (A) no aberration, (B) exchange, (C) break, (D) ring, and (E) fragment.

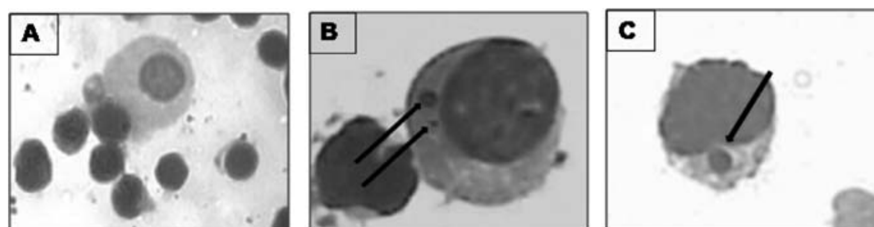

Figure 4. Representative pictures (100×) showing micronuclei in (A) untreated, (B) B(a)P-treated, and (C) RBSO-treated (0.5 mL/animal) groups.

The intracellular ROS levels were determined in terms of the fluorescence intensity of DCHF-DA by flow cytometry (**Figure 5**). The results showed that MFI in the control group was 89.8 ± 12.4, which increased to 553.9 ± 59.2 in B(*a*)P-treated animals. RBSO administration also induced the ROS levels (MFI of 499.6 ± 20.2) significantly ($p < 0.05$). However, ROS levels were 267.5 ± 24.5 in SBSO-administrated animals and 231.3 ± 17.1 in FSO-administrated animals ($p > 0.05$; **Figure 5**).

Table 2. Effects of RBSO on MI, Chromosomal Aberration, and Micronuclei Induction in Wistar Rats[a]

groups	MI	number of aberrant cells (%)					micronuclei induction (MNPCEs/1000 PCEs)
		breaks	fragments	exchanges	multiple damage	total number of aberrant cells	
I	13.43 ± 1.4	0.46	0.95	0.32	1.32	3.25 ± 0.4	4.50 ± 0.3
II	4.55 ± 0.6^{b}	5.55	3.48	2.35	8.62	20.50 ± 1.9^{c}	13.50 ± 1.7^{c}
III	13.22 ± 1.2	0.71	0. 87	0.18	1.63	3.39 ± 0.4	4.55 ± 0.6
IV-a	13.13 ± 1.6	0.64	0.77	0.41	1.79	3.61 ± 0.5	4.70 ± 0.4
IV-b	13.03 ± 1.5	0.60	1.02	0.45	1.67	3.74 ± 0.4	4.82 ± 0.3
V-a	10.36 ± 1.2^{b}	1.82	1.62	1.08	1.98	6.50 ± 0.7^{c}	7.50 ± 0.9^{c}
V-b	8.65 ± 0.8^{b}	2.86	2.98	1.22	3.19	10.25 ± 0.9^{c}	8.55 ± 1.2^{c}
V-c	7.80 ± 0.6^{b}	3.14	3.22	1.46	4.68	12.50 ± 1.3^{c}	10.50 ± 1.2^{c}

[a] All data are the mean ± standard error (SE) of 6 animals. MNPCEs, micronucleated polychromatic erythrocytes; PCEs, polychromatic erythrocytes. For doses and treatment, see the Materials and Methods. [b] Significant ($p < 0.05$) suppression over untreated group I. [c] Significant ($p < 0.05$) induction over untreated group I.

Table 3. Effects of RBSO on Levels of Antioxidant Enzymes and Lipid Peroxidation in Wistar Rats[a]

groups	SOD [unit min^{-1} (mg of protein)$^{-1}$]	CAT [μmol min^{-1} mg of protein)$^{-1}$]	lipid peroxidation (nm of TBARS/mg of protein)
I	0.08 ± 0.004	163.00 ± 8.2	1.43 ± 0.08
II	0.02 ± 0.001^{b} (75.0%)	78.71 ± 4.1^{b} (51.7%)	3.87 ± 0.20^{c} (170.6%)
III	0.08 ± 0.005 (0%)	156.50 ± 7.7 (4.6%)	1.47 ± 0.07 (2.8%)
IV	0.06 ± 0.005 (9.2%)	141.27 ± 6.2 (13.9%)	1.59 ± 0.08 (11.7%)
V	0.04 ± 0.004^{b} (50.0%)	109.54 ± 5.9^{b} (32.8%)	2.94 ± 0.11^{c} (105.6%)

[a] All data are the mean ± SE of 10 animals. For doses and treatment, see the Materials and Methods. Values in parentheses represent the percent change as compared to group I. [b] Significant ($p < 0.05$) decrease in the level of antioxidant enzymes over untreated group I. [c] Significant ($p < 0.05$) increase in lipid peroxidation of animals over untreated group I.

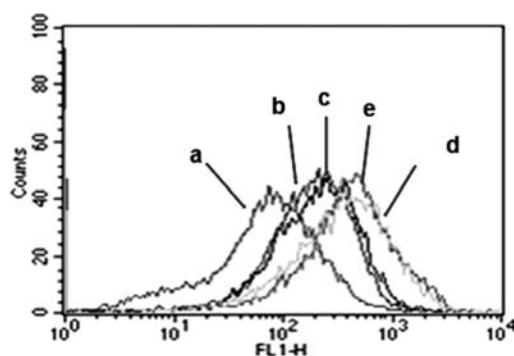

Figure 5. Representative overlay of flow cytometric analysis showing ROS generation in the liver of rats induced by RBSO: (a) untreated, (b) FSO, (c) SBSO, (d) RBSO, and (e) B(a)P administration. The horizontal axis represents DCHF-DA fluorescence verses count.

Effect of RBSO on the Induction of AHF. Quantification of stained liver sections in terms of area and count of foci showed a significant increase in the activity of GST-P and GGT and a decrease in the activity of ATPase, G6-Pase, and AlkPase (negative biomarkers) following the administration of DEN + 2AAF (group II) with respect to control animals (**Tables 4** and **5**). RBSO administration caused depleted expression levels of ATPase, G6-Pase, and AlkPase and induced expressions of GST-P and GGT activity (**Tables 4** and **5** and panels **A**−**C** of **Figure 6**). However, the administration of FSO (group III) and SBSO (group IV) in animals failed to modulate the activity of these hepato-specific biomarkers (**Tables 4** and **5**). Although a change in the expression of these selected biomarker enzymes was also noticed in only the RBSO-administered group (group VI), changes were far less as compared to groups II and V (**Tables 4** and **5** and panels **A**−**C** of **Figure 6**).

DISCUSSION

The association between dietary fat intake and mutagenic/cancer risk has been reported for a long time; however, studies quantifying such risks and the mechanism of pathogenesis are obscure. The present study was conducted to find out the carcinogenic risk associated with the consumption of thermally degraded SO. PAHs are suggested as one of the toxic compounds formed in oil during thermal degradation and are well-known for their carcinogenic and mutagenic properties. Therefore, taking them as representative of toxic compounds, we explored the effect of repeated boiling on the presence and amount PAHs and investigated the mechanism for mutagenicity/carcinogenicity of RBSO on dietary consumption, if any. Analysis of PAHs in fresh or boiled SO (single/repeatedly) carried out with liquid−liquid phase extraction followed by reverse-phase HPLC and spectrometric detection showed the presence of a higher amount of PAHs in RBSO samples (**Figure 2** and **Table 1**). These results are in accordance with an earlier study, where the repeated boiling of vegetable oils led to the generation of PAHs and their quantity was dependent upon the number of cycles used for boiling (*5*). Heavy PAHs are well-documented as potential mutagens/carcinogens (*21*). Our study also showed that RBSO contained higher amounts of heavy PAHs over FSO and SBSO samples (**Table 1**).

The presence of PAHs in substantially high amounts (**Table 1**) and the speculation that RBSO consumption may pose a risk toward genotoxicity/carcinogenicity impelled us further to assess the associated health hazards. RBSO treatment resulted in an increased incidence of aberrant cells and micronuclei, in a dose-dependent manner (**Figures 3** and **4** and **Table 2**). B(a)P is well-documented for chromosomal aberrations and micronuclei induction (*13*), and the European Community has fixed its concentration to be below the 2 parts per billion (ppb) limit; however, here, we detected that levels of B(a)P in RBSO were much above the permissible limits (**Table 1**).

RBSO administration led to significant depletion of antioxidant enzymes and increased levels of TBARS and ROS as compared to FSO or SBSO (**Table 3** and **Figure 5**; $p < 0.05$). Dung et al. (*22*) also showed cooking oil fumes induced DNA damage via ROS production. Altered levels of antioxidant enzymes are reported to cause cytotoxicity, mutagenicity, and carcinogenicity (*21*, *22*), and PAHs are linked to imbalance ROS generation

Table 4. AHF-Inducing Effects of RBSO in Wistar Rats in Terms of Area (mm^2/cm^2)[a]

groups	GST-P	GGT	ATPase	G6-Pase	AlkPase
I	1.66 ± 0.20	0.92 ± 0.07	1.92 ± 0.09	2.71 ± 0.30	1.78 ± 0.08
II	4.01 ± 0.09^b (141%)	2.48 ± 0.50^b (169%)	0.91 ± 0.10^c (53%)	1.42 ± 0.20^c (48%)	0.59 ± 0.06^c (67%)
III	1.73 ± 0.02 (4%)	0.85 ± 0.03 (5%)	1.98 ± 0.03 (3%)	2.47 ± 0.20 (8%)	1.71 ± 0.04 (5%)
IV	1.83 ± 0.02 (10%)	1.00 ± 0.04 (9%)	1.75 ± 0.02 (9%)	2.39 ± 0.10 (12%)	1.62 ± 0.05 (9%)
V	3.45 ± 0.05^b (108%)	1.81 ± 0.07^b (97%)	1.24 ± 0.10^c (35%)	1.89 ± 0.07^c (30%)	1.17 ± 0.05^c (34%)
VI	2.98 ± 0.10^b (79%)	1.53 ± 0.04^b (66%)	1.44 ± 0.08^c (25%)	2.17 ± 0.02^c (20%)	1.46 ± 0.04^c (18%)

[a] All data are the mean ± SE of 12 animals. For doses and treatment, see the Materials and Methods. Values in parentheses represent the percent change as compared to group I. [b] Significant ($p < 0.05$) induction over group I. [c] Significant ($p < 0.05$) suppression in the level of enzymes over group I.

Table 5. AHF-Inducing Effects of RBSO in Wistar Rats in Terms of Count of Foci/mm^2 [a]

groups	GST-P	GGT	ATPase	G6-Pase	AlkPase
I	1452.2 ± 67	1992.2 ± 122	1756.6 ± 82	914.2 ± 81	1224.4 ± 108
II	1993.4 ± 101^b (37%)	2856.3 ± 136^b (43%)	1268.3 ± 91^c (28%)	529.1 ± 68^c (42%)	845.3 ± 72^c (31%)
III	1471.7 ± 41 (1%)	2059.2 ± 152 (3%)	1717.9 ± 76 (2%)	853.1 ± 79 (7%)	1166.8 ± 112 (5%)
IV	1532.4 ± 52 (5%)	2141.6 ± 126 (7%)	1647.7 ± 88 (6%)	864.8 ± 71 (5%)	1136.2 ± 92 (7%)
V	1884.4 ± 68^b (30%)	2733.4 ± 121^b (37%)	1392.0 ± 156^c (21%)	611.6 ± 76^c (33%)	926.0 ± 81^c (24%)
VI	1620.6 ± 75^b (12%)	2342.1 ± 98^b (17%)	1580.9 ± 68^c (10%)	804.5 ± 51^c (12%)	1077.5 ± 80^c (12%)

[a] All data are the mean ± SE of 12 animals. For doses and treatment, see the Materials and Methods. Values in parentheses represent the percent change as compared to group I. [b] Significant ($p < 0.05$) induction over group I. [c] Significant ($p < 0.05$) suppression in the count of foci over group I.

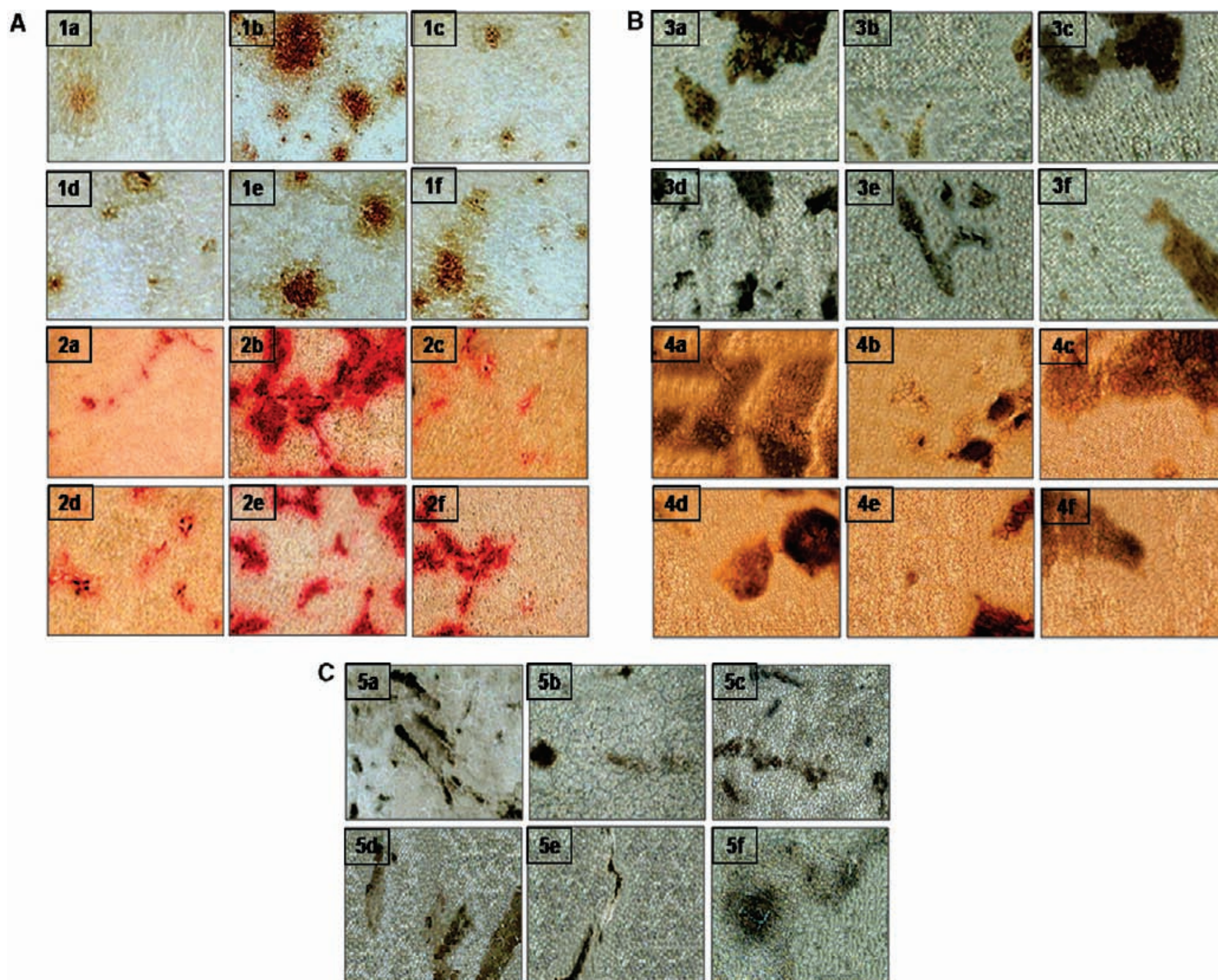

Figure 6. Representative pictures (10×) for cellular localization of the expression of liver-specific enzyme markers showing (A) (1) GST-P and (2) GGT, (B) (3) G6-Pase and (4) ATPase, and (C) (5) AlkPase. 1a−5a represent the untreated group. 1b−5b represent the DEN + 2AAF-treated group (200 mg/kg of body weight + 0.05% in crushed diet). 1c−5c represent DEN + FSO (0.5 mL/animal). 1d−5d represent DEN + SBSO (0.5 mL/animal). 1e−5e represent DEN + RBSO (0.5 mL/animal). 1f−5f represent RBSO (0.5 mL/animal).

and LPO and antioxidant enzyme levels (*23*, *24*). Similarly, in our study, thermally degraded SO administration, which revealed to have high PAHs, caused imbalance in antioxidant enzyme levels and ROS production may increase susceptibility toward the neoplastic transformation (**Tables 1** and **3** and **Figure 5**).

The quantitative analysis of enzymes altered foci as an end point in rodent hepatocarcinogenesis using marker enzymes is a useful tool for the rapid detection of the carcinogenic potential of compounds. Here, we have evaluated the AHF-developing potential of RBSO using biomarker enzymes (GST-P, GGT, AlkPase, ATPase, and G6-Pase) in Wistar rats. Our study demonstrated alteration in positive (GGT and GST-P) and negative (AlkPase, ATPase, and G6-Pase) hepatic foci following RBSO administration with and without DEN, showing that thermally challenged SO has hepatocarcinogenic potential (**Tables 4** and **5**). The deficiency in canlicular ATPase and G6-Pase is considered as reliable markers for the larger foci and nodules and, thus, identifies preneoplastic alterations in liver, and GST-P is employed as a reliable diagnostic marker for hepatocellular carcinoma (*19*, *25*). The RBSO sample contains higher amounts of known carcinogenic PAHs, which have been shown to be responsible for ROS production and, in turn, causes lipid peroxidation, resulting in the formation of high-molecular-mass protein aggregates within the membrane (**Table 3** and **Figure 5**). This subsequently inhibits the activity of enzymes, such as G6-Pase and ATPase, within the cells (*25*). ROS production is also related to epigenetic toxicity and accounts for GST-P induction in hepatocytes (*26*). In a study by Aruna et al. (*27*), initiation of hepatocarcinogenesis has been shown to be influenced by thermally oxidized SO. Repeated use of the vegetable oils when administered to rats has been shown to generate indications of cellular damage to liver and kidneys, increase urine mutagenicity, and enhance cell proliferation by oxidation products of linoleic acid (*28*). Because tumor initiation involves a genetic pathway and tumor promotion entails an epigenetic pathway, it is apparent by the present study that thermally degraded SO, having higher amounts of carcinogens and shown to induce AHF, therefore, poses a great health risk to humans (*29*).

In conclusion, consumption of thermally degraded RBSO afforded substantial genotoxicity, via induced oxidative stress, and led to preneoplastic changes in rat liver and, hence, may pose a health hazard to humans. Thus, use of the repeatedly boiled cooking and frying medium may be avoided in kitchen practices to minimize the risk from such sources.

ABBREVIATIONS USED

2-AAF, 2-acetylaminofluorene; ATPase, adenosine triphosphatase; AlkPase, alkaline phosphatase; AHF, altered hepatic foci; CAT, catalase; DCFH-DA, dichlorodihydroflourescien diacetate dye; DEN, *N*-diethylnitrosamine; GGT, γ-glutamyltranspeptidase; G6-Pase, glucose-6-phosphatase; GST-P, glutathione *S*-transferase-placental form; HPLC, high-performance liquid chromatography; 8- OHdG, 8-hydroxylguanine; LPO, lipid peroxidation; PUFA, ω-6 polyunsaturated fatty acid; PH, partial hepatectomy; PAH, polycyclic aromatic hydrocarbon; ROS, reactive oxygen species; SOD, superoxide dismutase; TBA, 2-thiobarbituric acid; TMP, 1,1,3,3-tetramethoxypropane.

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