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Research article

AMELIORATIVE EFFECTS OF CURCUMIN AND GREEN TEA AGAINST GASOLIN-INHALATION HEMATOTOXICITY

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ABSTRACT: This study aimed to investigate the ameliorative role of curcumin or green tea on gasoline-induced hematotoxicity. Green tea extract and powdered curcumin were chosen as antioxidant and antihematoxicity natural products. CD1 mice were taken as experimental model.

Mice were exposed to gasoline vapor 2hours/day for 3 weeks in inhalation chamber. The concentration of gasoline is 9375 ppm and the concentration of benzene is 100 fold less than gasoline in equilibrium with pure benzene being 93.75 ppm. Hematological parameters in bone marrow and peripheral blood were measured and histopathological investigations were done.

The results of this study were concluded as follow:

1-Bone marrow depression was occurred by gasoline as reduction in bone marrow cellularity and slow rate of cells maturation. Apptosis appeared in bone marrow cells by histopathological examination for biopsies. All these were improved in the groups provided with green tea and curcumin in the diet.

2-Reduction in blood cell counts was occurred, in RBCs, WBCs, platelets, and hemoglobin. Lymphocytes percentages in blood were depressed and neutrophils percentages were elevated ingasoline inhalation group. All these changes returned to the normal levels by green tea extract and curcumin.

Key words: Curcumin, Green tea, Antioxidant, Hematotoxicity, Gasoline

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INTRODUCTION

A major development over the past two decades has been the realization that free radical mediated peroxidation of membrane lipids and oxidative damage of DNA are associated with a variety of chronic health problems, such as cancer, atherosclerosis, neurodegenerative diseases and aging (Finkel and Holbrook, 2000; Perwez Hussain et al., 2003; Barnham et al., 2004). Therefore, inhibition of oxidative damage by supplementation of antioxidants becomes an attractive therapeutic strategy to reduce the risk of these diseases (Rice-Evans and Diplock, 1993; Brash and Harve, 2002).

Curcumin is a powerful scavenger of many free radicals such as anion, hydroxyl radical and nitric oxide (Elizabeth and Rao, 1990; Sreejayan and Rao, 1997 and Barzegar *et al*., 2011). Jayaprakasha *et al*. (2006) demonstrated in vitro the antioxidant capacities and activities of curcumin, bisdemethoxycurcumin and demethoxycurcumin using the phosphomolybdenum method and linoleic acid peroxidation method. They reported that, by using phosphomolybdeum method curcumin, demethoxycurcumin and bisdemethoxycurcumin exhibited various degrees of antioxidant capacity. The antioxidant capacities of curcuminoids were found to decrease in the order: curcumin > demethoxycurcumin > bisdemethoxycurcumin. Also by using linoleic acid peroxidation method, they found the same orders of antioxidant activities of the three curcuminoid compounds.

Recent studies provide scientific evidence regarding the potential pharmacological, prophylactic or therapeutic use of Cur, as anti-inflammatory, anti-carcinogenic, anti-tumoral, anti-viral, antifungal, anti-parasitic, anti-mutagen, anti-infectious, anti-hepatotoxic and anti-oxidant compound (Chen et al., 2006; Aggarwal *et al*., 2007; Ciftci *et al*., 2010; 2011 and 2012; Shehzad
et al., 2011).

Epidemiological and laboratory studies have reported that green tea presents diverse beneficial health effects including antioxidant (Sung et al., 2000; Nakagawa and Yokozawa, 2002), hypocholesterolemic (Lin et al., 1998; Riemersma et al., 2001; Erba et al., 2005 and Lee et al., 2005) , anti-hyperglycemic (Tsuneki et al., 2004 and Li et al., 2006), hepatoprotective (Chung et al., 2003; Fujiki et al., 2005; Bun et al., 2006, Kaviarasan et al., 2007 and Inoue et al., 2013) , anticarcinogenic (Wang et al., 1992 ; Lou et al., 1999; Hayakawa et al., 2001 and Zaveri, 2006).

The tea leaves are distinguished by their content of methylxanthines, and polyphenols especially flavonols of the catechin type. The major green tea polyphenols are: (-)-epigallocatechin-3-gallate (EGCG), (-)-epicatechin-3-gallate (ECG), (+)-gallocatechin (GC), (-)-epicatechin (EC), gallocatechin gallate (GCG) and catechin (C) which together may constitute 30% of the dry leaf weight, in addition to caffeine, theobromine, theophylline and phenolic acid, such as gallic acid are also present as minor constituents of green tea (Gupta et al., 2002).

The percentages of catechines in green tea extract according to Sartippour et al.(2001) as follow: EGCG 46.8%, ECG 13.54%, GCG 7.24%, EC 8.07%, EGC 2.28%, GC 2.46%, CG 1.28%, C 2.22% and caffeine <0.3%. Tea also contain small amount of flavonols (kaempferol, quercetin and myricitin) in the form of glycosides. The flavonol content is less affected by
processing, and flavonols are present in comparable amount in green and black tea (Balentine et al., 1997).

Green tea polyphenols are good antioxidant against free radical initiated lipid peroxidation in solutions (Jia et al., 1998) in micelles (Zhou et al., 2000; 2004 and 2005) in human red blood cells (Ma et al., 2000; Dai et al., 2006 and Rizvi et al., 2006) in human low density lipoprotein (Liu et al., 2000) and in rat liver microsomes (Cai et al., 2002) , and that the antioxidant activities of these polyphenols depend significantly on the structure of the molecules, the initiation conditions and the microenvironment of the reaction medium (Cai et al., 2002). It was found that these green tea polyphenols could interact with α-tocopherol, synergistically to enhance their antioxidant activity (Zhou et al., 2005; Wei et al., 2006a). Dietary green tea catechins inhibit colonic mucosal lipid peroxidation in 1,2-dimethylhydrazine-induced colonic carcinogensis . Intake of green tea catechins in rats fed monounsaturated fatty acids suppressed iron-induced lipid peroxidation of intestinal mucosa homogenate. Age-dependent and ethanol induced lipid peroxidation in the study of Augustyniak et al. (2005) was decreased by 7 g/L green tea in liquid diet. The study of El-Beshbishy (2005) on the effect of tamoxifen on lipid peroxidation of liver homogenate , this showed that, increment of TBRS level significantly in comparing to normal , administration of green tea extract resulted in high improvement in lipid peroxidation . In the study of Coimbra et al.(2006) on 34 portuguese subjects drinking green tea for 4 weeks, the levels of malondialdehyde and malondialdehyde+4-hydrroxy-2(E)-nonenal and the oxidative stress in erythrocyte membrane, namely membrane bound hemoglobin, were reduced significantly.

MATERIALS AND METHODS

Experimental animals

Sixty male mice (*Mus musculus*) weighting 20 – 25 g were purchased from the Egyptian Organization for Serological and Vaccine Production, Egypt, were used as experimental animals throughout the present work. The animals were housed individually in plastic cages and acclimated for 1 week before gasoline-fume exposure. Food and water were offered *ad libitum*. Animals were maintained at 22± 2 °C at normal light/dark cycle.

Preparation of green tea extract:

Green tea (*Camellia sinensis*) was purchased from Shanghai tea import & export Corporation, China. The green tea extract was made according to Maity et al. (1998), by soaking 15 gm of instant green tea powder in 1L of boiling water for 5 minutes. The solution was filtered to obtain 1.5% green tea extract; this solution was provided to mice as their sole source of drinking water.

Preparation of curcumin in the diet:

The dried ground rhizomes of *Curcuma longa* were purchased from local market in Cairo, Egypt, grinded, powdered, and added to the diet of mice, 30gm to 1Kg of diet to form concentration of 3% (Conney et al., 1997).

Inhalation of gasoline:

A glass cubic box its length is 70cm, width is 70cm and high is 70cm, was manufactured to make as gasoline inhalation chamber, there are two orifices in both right and left sides of the box in the upper portion of the box to make aeration, each orifice 5cm in diameter covered with wire mesh to prevent mice escaping. At 10cm distance from the bottom of box, a wire mesh shelf 70x70 cm was fixed to put the mice on it. Under this shelf a 200 ml cans containing 150 ml of gasoline were placed in the exposure chamber and the animals were allowed to inhale the fumes

evaporating from the cans. The gasoline which evaporated during the time of inhalation was about 80 ml/2hours. The time of exposure was 10.00 to 12.00 am and the cans were withdrawn and the inhalation stopped. The experimental fume gasoline inhalation was exceeded for successive three weeks as 2hours/day/three weeks.

The gasoline

The Egyptian commercial unleaded gasoline (octane 90) was purchased from filling station. Gasoline is a petroleum-derived liquid mixture consisting mostly of more than 300 individual hydrocarbons primarily (in volume) of paraffins (30–90%), cycloparaffins (1–35%), olefins (0–20%), and aromatics (5–55%), distilling in the approximate range of 30°C–220°C. Composition of gasoline varies with source of the crude oil, refinery processes, conditions, and the blending of refinery streams in the gasoline boiling range to meet performance criteria as well as regulatory requirements (Roberts et al., 2001). Volatile organic compound emissions from gasoline storage showed that total organic compounds per cubic meter gasoline loaded is 35 g/m^3 saturated vapor at 25°C.

Gasoline Dose

Based on analysis reported by Johnson et al. (1990) the concentration in equilibrium with gasoline is 9375 ppm. Benzene is 100-fold less than in equilibrium with pure benzene being 93.75 ppm. This dose of benzene is in equilibrium with gasoline in the inhalant mice cages in the current study. However, gasoline fraction differs from whole gasoline by containing far less aromatics, longer chain and longer aliphatic hydrocarbons. Analysis of workplace exposure to gasoline vapors revealed that C4–C5 length hydrocarbons constitute from 67 to 74% by weight of the typical vapor (Halder et al., 1986).

Animal Groups

After an acclimation period for 1 week, animals were classified into six groups, each group consists of ten mice as follow:

1- ***Control group***, received only the ordinary mice diet and drinks water without any additions and kept two hours daily in the inhalation chamber without gasoline for three weeks.
2- ***Green tea group***, received ordinary diet, drink green tea extract (1.5%) as a sole source of drinking water and kept two hours daily in the inhalation chamber without gasoline for three weeks.
3- ***Curcumin group***, these animals received powdered dried ground rhizomes of *Curcuma longa* (turmeric) in the diet (3%) and kept two hours daily in the inhalation chamber without gasoline for three weeks.
4- ***Gasoline inhalation group***, this is the intoxicated group with gasoline inhalation, these mice were kept 2 hours daily in inhalation chamber with gasoline for three weeks. This group drinks water and eat the ordinary diet.
5- ***Gasoline and green tea group***, these animals exposed to gasoline 2 hours daily in inhalation chamber for three weeks and received green tea extract (1.5%) eat the ordinary diet.
6- ***Gasoline and curcumin group***, this group exposed to gasoline in the inhalation chamber, 2 hours daily for three weeks and received powdered dried ground rhizomes of *Curcuma longa* in their ordinary diet along the time of the experiment and drinks water.

METHODS

Blood collection

Twenty four hours after stopping gasoline inhalation, animals were anaesthetized by diethyl ether, dissected and blood was collected by heart puncture with syringe (3ml capacity). The required amount of blood was collected in three tubes, one of them contain EDTA anticoagulant for hematological studies, the second contain 0.1 ml sodium citrate solution (3.6%) and 0.9 ml blood was added in this tube for detection of prothrombin time, the last tube was empty where the blood allowed to coagulate in water bath at 37 °C for 30 minutes. Serum was separated by centrifugation in cooling centrifuge (Hettich, Germany) at 3000 xg for 15 minutes, transported into another dry and clean Eppendorf tubes and was kept in deep freezer at -20 °C for biochemical analysis.

Hematological Parameters in Bone Marrow Aspirate

Total bone marrow cell count

Total bone marrow cells were counted according to Lezama et al., (2001). As follows: 1- Remove one femur, clean it from muscles, and cut the epiphyses.

2- Inject 1 ml of isotonic saline solution into the medullary channel and receive the cell suspension in a glass tube.

3- Take 10µl of cell suspension and dilute it with 200µl of Turk's solution.

4- Fill the counting chamber of hematocytometer slide by one drop of diluted bone marrow cell suspension under the cover with smooth flow of fluid.

5- Count bone marrow cells in 4 corners of the large squares (64 large squares), and multiply the total count by 50 to get the bone marrow cell count per µl of cell suspension.

Differential count of bone marrow aspirate

Differential count for bone marrow aspirate was made as follows:

1- Remove the other femur, clean it from muscles, and cut the epiphyses.

2- Insert a needle into one side of the femur and receive bone marrow aspirate from the other side on a clean and dry glass slide.

3- Spread it by using another slide to make bone marrow aspirate film. 4- Dry the film in air.

5- Fix it with absolute methyl alcohol.

6- Place the bone marrow film in diluted Giemsa stain (1:10 with distilled water) (Atlas Medical Company, UK) for 45 minutes.

7- Wash with distilled water and allow to dry.

Observe under oil immersion lens and differentiate bone marrow cell types.

Histopathological Investigations

Tissues were removed from the mouse, placed into 10% formalin for 48 hours, and then transferred to 70% ethanol. Femur underwent decalcification in 7.5% formic acid for 5 days before further processing, and dehydrated in 80, 96, and 100% ethanol.

The tissues were then washed with a 1:1 mixture of 100% ethanol and xylene, washed twice in absolute xylene, and finally embedded in paraffin, and at least two sections per tissue were prepared, Serial 7µm sagital cuts were obtained and stained with hematoxylin and eosin. Bone marrow cellularity was assessed by evaluating five fields per femur (with magnification power 100 and 1000 times). Images were acquired from standardized regions of femur using a Spot RT digital camera (Diagnostic Instruments Inc., Sterling Heights, MI) on a Zeiss Axioscop 2 microscope (Carl Zeiss Inc., Thornwood, NY). All chemical reagents were purchased from El-Gomhoria Company, Egypt.

Determination of Hematological Parameters in Peripheral Blood

Hemoglobin in blood was determined according to method of Van Kmpen and Zijlstra (1961) using the kit of Randox Company, United Kingdome.

Red blood cells, white blood cells, platelets, and reticulocytes were counted using hematocytometer method according to Krupp et al. (1976).

PCV percentage was determined according to Turgeon (2005) using microhematocrit tubes coated with anticoagulant.

Leucocyte differential counted was preceded according to Turgeon (2005) by using Giemsa stain (Atlas Medical Company, UK).

Prothrombin time detection was preceded according to Turgeon (2005). This basic procedure involves adding plasma on an excess of extrinsic thrmboplastin-Ca substrate by using thrmboplastin-Ca kit (Biomeriux – France).

Statistical Analysis

Data are expressed as mean±SD. The level of statistical significance was taken at $P < 0.05$, using one way analysis of variance (ANOVA) test followed by Dunnett test to detect the significance of differences between each group and control. All analysis and graphics were performed by using, INSTAT and graphPad Prism software version 4.

RESULTS

Hematological parameters in bone marrow aspirate

The data obtained from bone marrow aspirate examination were illustrated in table (1), these to give us a complete picture about the bad effects of gasoline inhalation on bone marrow cellularity, maturation of bone marrow cells to give the blood cells and percentages of myeloid and erythroid precursor cells.

By making total count for bone marrow nucleated cells, observed sever bone marrow depression caused by gasoline alone reached to a half of the normal control group (-51.45%). This huge depression was recovered and returned near to normal level by oral taking of green tea extract 1.5% as a sole source of drinking water or by eating curcumin 3% with the diet simultaneously with gasoline inhalation to reach only -2.33% in gasoline+green tea group and -
4.53% in gasoline+curcumin group compared to control.

By studying the myeloid precursor cells, differential count for the stained bone marrow aspirate films, a slowing in a maturation rate of cells by gasoline inhalation was occured as illustrated in table (1), they showed a shift toward immature cells, in other mean, the immature cell percentages were elevated compared to control and mature cells percentages were decreased
by a remarkable degrees.

Myeloblasts and promyelocytes percentages did not affected in all intoxicated and treated groups with a remarkable degrees. On the other hand, by measuring the percentages of myelocytes in the six groups a remarkable elevation for this cell type in gasoline alone group (83.67%) was occurred compared to control ($P<0.01$), returned near to normal by green tea extract and curcumin (12.65%, 9.15%) compared to control . This also the case of metamyelocytes, when they affected by gasoline toxicity which caused elevation of them by 40.92% ($P<0.01$), returned to normal level by simultaneously consumption of green tea or
curcumin.

A significant depression of band granulocyte percentages occurred as a result of gasoline intoxication alone with about a third of control (-33.45%) ($P<0.01$) and recovered to their normal count by addition of green tea or curcumin to the animal's diet to reach only -7.89% in
gasoline+green tea group and -7.43% in gasoline+curcumin group compared to control.

Lymphocytes in bone marrow were affected by gasoline inhalation which caused their depression to reach -39.69% of control, but in gasoline+green tea group was not significant change in compared to control. In gasoline+curcumin group this percentage was approximately equal to control group, this reflect the protective role of green tea and curcumin on lymphocytes.

By comparison the percentages of monocytes in all animal's groups using ANOVA test statistical analysis, there was not any significant changes.

The rate of maturation of erythroid precursor cells also became slower by gasoline intoxication, the percentages of cells were shifted toward the immature cells to yield a case of hypocellularity. Proerythroblasts showed significant increase (44.23%) by gasoline inhalation compared to control ($P<0.05$) this was lowered by coadministration of green tea to reach only12.18% and by curcumin to reach 23.93% compared to control. These lowering effects of natural products is significant in case of green tea (-20.36%) and non-significant in case of curcumin (-12.01%) compared to gasoline alone group. This also the feature of basophilic erythroblasts and polychromatic erythroblasts when these immature cells were exposed to gasoline inhalation they behaved a similar behavior by increase their percentages with 50.74% and 46.83% respectively compared to control ($P<0.01$), these increases were returned near to normal by green tea extract to become only 16.94% and 6.4% respectively compared to control, also curcumin exerted its protective effect by lowering these percentages to reach only 25.59% and 22.07% compared to control.

On the other hand a significant depression occurred to orthochromatic erythroblasts percentage by gasoline inhalation (-23.92%) compared to control (P<0.05), this depression was reduced by co-administration of green tea or curcumin to reach - 9.93% and -0.13% compared to control.

Myeloid/Erythroid (M/E) ratio, megakaryoblasts and megakaryocytes by ANOVA test statistical analysis did not show any significant changes among all groups of animals as illustrated in table (1).

Green tea alone or curcumin alone did not show any significant changes in neither bone marrow cellularity nor bone marrow aspirate differential count.

Hematological parameters in peripheral blood

Hematological parameters were examined in blood samples obtained by heart puncture and mixed with anticoagulant (EDTA). These samples were analyzed for red blood cells (RBCs), white blood cells (WBCs), and platelet count also for determination of hemoglobin concentration (Hb), packed cell volume percentage (PCV%), reticulocyte percentage and differential count for WBCs.

The influences of gasoline inhalation (2 hours/day for 3 weeks) as illustrated in table (2) showed that the inhalation of gasoline alone resulted in a state of decline in hemoglobin concentration (-19.43%), RBCs count (-12.71%) and PCV% (-11.43%) significantly compared to control (P<0.05).

The addition of green tea extract or curcumin to the diet alone did not show any considerable changes in these parameters, on the other hand, co-administration of these natural products with gasoline inhalation caused a highly significant improvement of these declines to elevate them again around the normal levels. In other mean by comparing the groups of gasoline+green tea and gasoline+curcumin to gasoline alone group we found that in gasoline+green tea group the hemoglobin concentration elevated by 25.42% and in gasoline+curcumin group was elevated by 33.29% compared to gasoline alone group, also the cases of RBCs count depression were protected by green tea (23.02%) and curcumin (24.42%), thereby, PCV% were elevated by green tea and curcumin as a result of elevation of RBCs count by 15.94%, 16.45% respectively (P<0.05).

Blood indices (MCH, MCHC and MCV) were examined in this study and MCH was reduced significantly (P<0.01)by gasoline inhalation compared to control(-21.33%), but the two others (MCHC and MCV) did not affect by gasoline inhalation. Both green tea and curcumin failed to protect MCH falling down.

Reticulocytes are erythrocytes still possessing RNA. The enumeration of reticulocytes is important in assessing the status of erythrocyte production in the bone marrow. By observing table(2) was noticed that a huge depression in reticulocyte percentage by intoxication with gasoline inhalation alone (2 hours/day for 3 weeks) to reach about the third of control (-71.45% compared to control) (P<0.01), this bad effect of gasoline was protected and the decline in reticulocyte percentage among erythrocytes was compensated by adding green tea extract 1.5% as a sole source of drinking water and curcumin 3% to the animal's diet to reach the differences compared to control to -44.15% and 2.41% for gasoline+green tea group and gasoline+curcumin group respectively. Adding of green tea or curcumin alone did not show any significant changes in reticulocyte percentage when compared to control.

Green tea or curcumin addition to the diet alone did not cause any considerable changes in platelet count when compared to control but they could slightly reduce the bad effect of gasoline inhalation (-60.65%) to reach -43.52% and -40.33% respectively in compared to control, in the other meaning, they elevated the platelet's depression which caused by gasoline by 43.54% and 51.64% respectively on comparing to gasoline alone group.

A huge decline in total WBCs count as a marker of gasoline hematotoxicity as illustrated in table (2) to reach about the half of control, this decrease mainly in lymphocytes number, these markers declined by -51.04% and -36.39% for total WBCs count and lymphocyte percentage respectively, but these decreases were compensated and reached to the normal levels by using green tea extract and curcumin simultaneously with gasoline.

Neutrophils percentage behaved another behavior that it was elevated significantly (P<0.01) as a response to gasoline inhalation alone to reach 78.05 more than control but this elevation was returned to normal by co-administration of green tea or curcumin in the diet to reach only 22.05% for gasoline+green tea group and 10.29% for gasoline+curcumin group more than control .

Other leucocyte cell types (monocytes, eosinophils and basophils) did not show any significant changes neither in intoxicated group nor protected groups. Neither total WBCs count nor differential count affected by green tea extract or curcumin administration alone.

Prothrombin time is prolonged by gasoline inhalation which means that the activity of prothrombin was reduced. The time is increased by 14.01% compared to control and shorted by the simultaneously administration of green tea or curcumin to overlap this depression of prothrombin activity.

Histopathological studies

Plates (2) demonstrate the effects of gasoline toxicity on bone marrow of the femur from CD1 mice in case of inhalation gasoline alone. It shows marked hypocellularity, decreased the haematopoeitic cells and increased apoptosis, plasma cells and fibrous tissue amount also increased. There is marked decrease in the segmented leucocytes and slow cellular maturation which was found as accumulation of myelocytes and metamyelocytes.

Plates (3) demonstrate the effects of gasoline toxicity on bone marrow of the femur from CD1 mice in case of co-administration of green tea extract with gasoline inhalation. It shows
mild hypocellularity, mild decrease in the haematopoeitic cells. There are mild apoptosis and mild increase in fibrous tissue amount as well as mild increase in the metamyelocytes.

Plates(4) demonstrates the effects of gasoline toxicity on bone marrow of the femur from CD1 mice in case of co-administration of curcumin. It shows mild hypocellularity, mild decreased the haematopoeitic cells. There is mild apoptosis and mild increase in fat cells and fibrous tissue amount as well as mild increase in the metamyelocytes.

Table-1:Hematological parameters in bone marrow aspirate of CD1 mice exposed to gasoline inhalation and effect of green tea or curcumin

Animal groups	Control group Mean±SD	Green tea group	Curcumin group	Gasoline group	Gasoline and green tea group	Gasoline and curcumin group
Bone marrow count/µl of cell suspension	12360±1033	11260±1305	11620±1543	6000**±533.9	12071±1195	11800±873.7
Myeloblasts%	0.328±0.049	0.32±0.081	0.344±0.053	0.38±0.058	0.302±0.06	0.298±0.027
Promyelocytes%	0.72±0.13	0.632±0.163	0.68±0.113	0.762±0.119	0.666±0.122	0.712±0.146
Myelocytes%	9.125±2.307	8.767±3.179	9.86±1.866	16.76**±3.084	10.28±2.969	9.96±1.724
Metamyelocytes%	11.41±1.965	10.98±3.029	11.86±1.339	16.08*±1.628	11.95±1.936	11.54±1.555
Band granulocytes%	45.62±8.487	46.09±7.654	44.97±6.26	30.36**±2.978	42.02±5.746	42.23±5.185
Lymphocytes%	14.6±2.023	15.14±1.921	13.82±2.445	8.804**±1.422	11.16±4.426	14.2±3.098
Monocytes%	1.21±0.143	1.28±0.164	1.16±0.24	1.32±0.216	1.267±0.186	1.18±0.13
Proerythroblasts%	0.394±0.044	0.368±0.046	0.436±0.042	0.555*±0.057	0.442±0.043	0.488±0.072
Basophilic erythroblast%	1.340±0.1327	1.311±0.09403	1.438±0.1149	2.020**±0.1356	1.567±0.1476	1.683±0.1721
Polychromatic erythroblasts%	4.860±0.7393	4.700±0.6458	5.300±0.7362	7.136**±0.5952	5.178±0.2683	5.933±0.3853
Orthochromatic erythroblasts%	15.30±1.699	13.84±1.100	15.02±1.876	11.64*±1.453	13.78±2.911	15.28±1.469
M/E ratio	3.776±0.54	3.972±0.38	3.564±0.51	3.844±0.38	3.798±0.78	3.378±0.33
Megakaryblasts%	0.354±0.045	0.32±0.081	0.34±0.056	0.364±0.04	0.318±0.053	0.318±0.023
Megakaryocytes%	0.708±0.091	0.62±0.142	0.68±0.113	0.712±0.075	0.665±0.065	0.638±0.042

(*) significant difference compared to control group ($P < 0.05$).
(**) highly significant difference compared to control group ($P < 0.01$).

Table-2: Hematological parameters in peripheral blood of CD1 mice exposed to gasoline inhalation and effect of green tea or curcumin

Animal groups	Control group Mean ± SD	Green tea group	Curcumin group	Gasoline group	Gasoline and green tea group	Gasoline and Curcumin group
Hb gm/dl	12.31±1.360	12.64± 1.329	12.20± 1.548	9.918*± 0.8635	12.44± 1.029	13.22± 0.6691
RBCs mill/µl	8.02± 0.45	7.696± 0.17	8.03± 0.503	7*± 0.59	8.612± 0.33	8.71± 0.74
PCV%	44.6± 3.362	45.5± 7.688	45.2±2.049	39.5*± 1.73	45.8± 2.49	46± 3
MCH Pg/cell	17.48± 1.252	17.02± 1.442	17.56± 0.62	13.75**± 1.464	13.35**± 1.939	13.42**±1.598
MCHC%	26.66± 3.621	26.23± 2.966	25.19± 2.996	24.94± 1.777	26.34± 2.196	27.35 ± 1.541
MCV(fL)	64.69± 10.47	65.31± 4.387	65.45± 7.555	61.88± 11.98	55.47± 3.653	52.78± 1.984
Reticulocytes%	3.277± 1.085	2.7± 0.755	3.038± 0.427	0.935**± 0.236	1.83**± 0.4	3.198± 0.965
Platelets 1000/µl	1004± 240.6	895.6± 320.3	823.1± 121.7	395**± 116.5	567**± 138.1	599*± 141.9
WBCs/µl	9780± 1254	8960± 1352	9217± 1452	4788**± 559.2	8900± 624.5	8825± 2620
Neutrophils%	27.2± 2.168	25.6± 3.507	26.6± 4.827	48.43**± 5.74	33.2± 5.933	30± 1.87
Lymphocytes%	65.4± 4.159	66.8± 3.564	66.8± 5.263	41.6**± 4.393	59.2± 6.76	62.76± 4.763
Monocytes%	5.55± 1.236	6± 0.707	5.3± 1.204	6.11± 1.833	5.167± 0.752	5.717± 1.036
Eosinophils%	1.44± 0.384	1.72± 0.408	1.66± 0.522	1.68± 0.348	1.52± 0.277	1.44± 0.378
Basophils%	0.516± 0.172	0.6± 0.212	0.48± 0.204	0.475± 0.138	0.48± 0.083	0.44± 0.134
Prothrombin time with second	11.7± 0.833	11.78± 0.746	11.76± 0.714	13.34*± 1.062	12.48± 0.715	12.26± 0.929

(*) significant difference compared to control group ($P < 0.05$).
(**) highly significant difference compared to control group ($P < 0.01$).

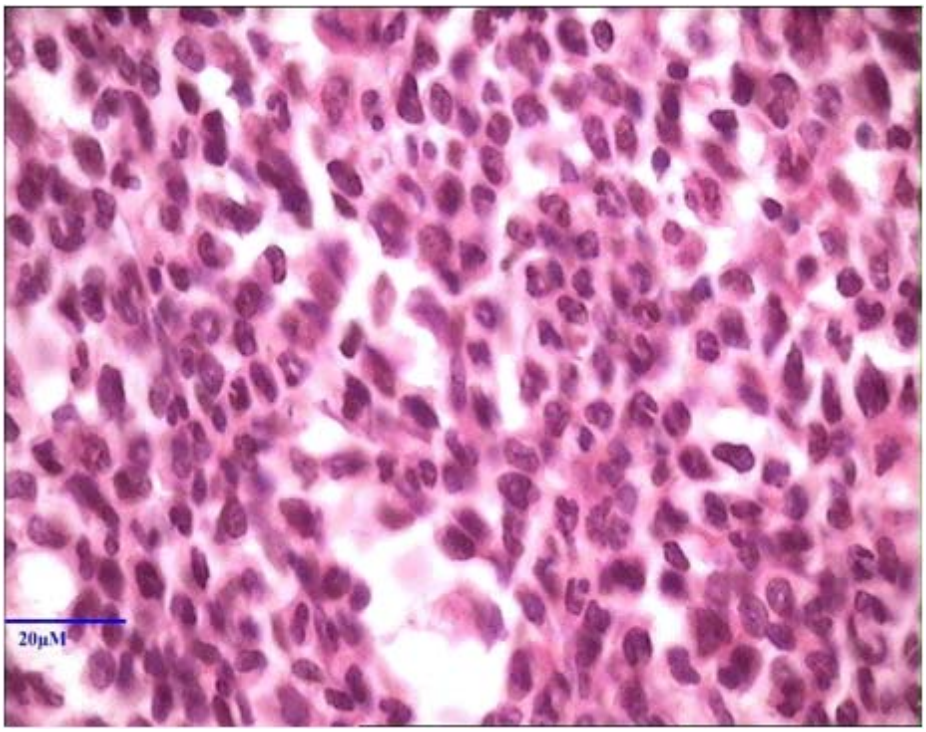

Plate-1: Section in femur of CD1 mice stained with hematoxylin and eosin stain illustrate the bone marrow of control group

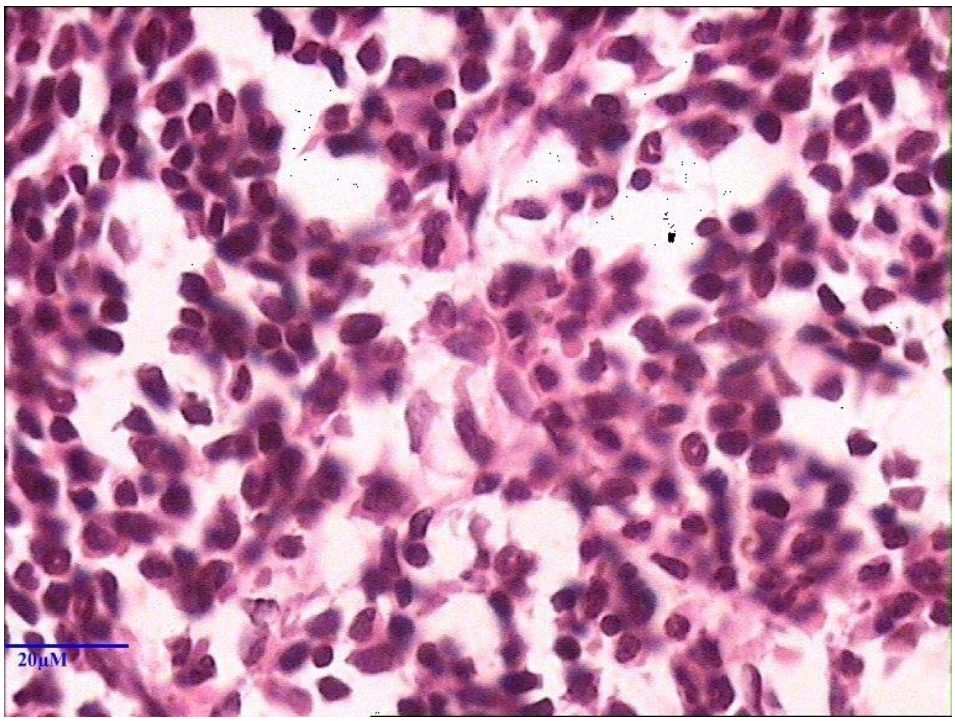

Plate-2: section in femur of CD1 mice stained with hematoxylin and eosin stain illustrate the bone marrow of gasoline group

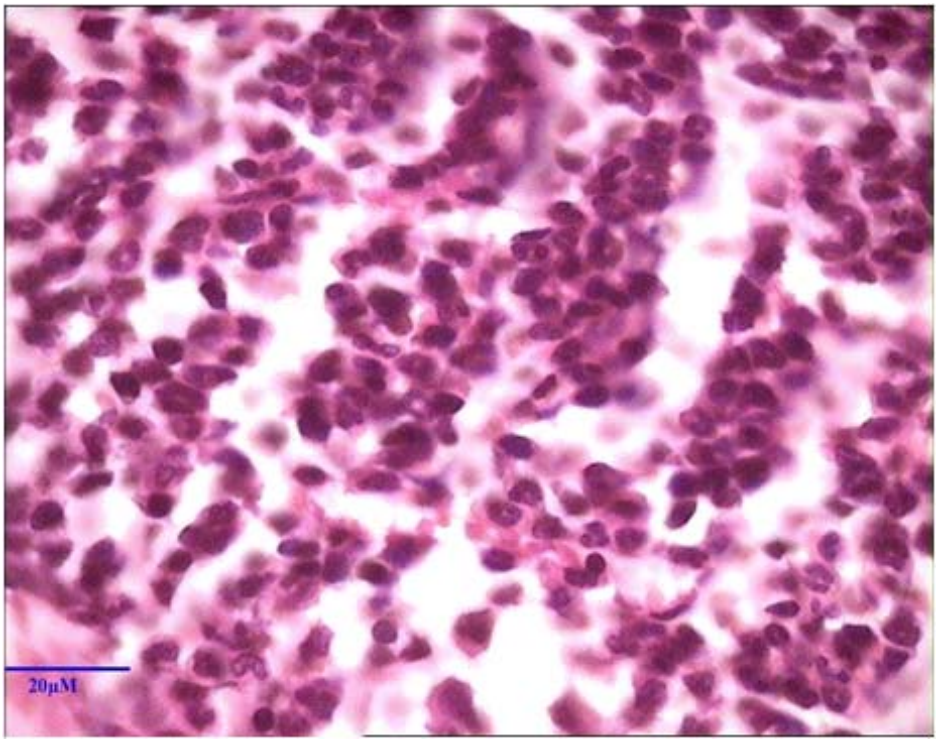

Plate-3: section in femur of CD1 mice stained with hematoxylin and eosin stain illustrate the bone marrow of gasoline+green tea group

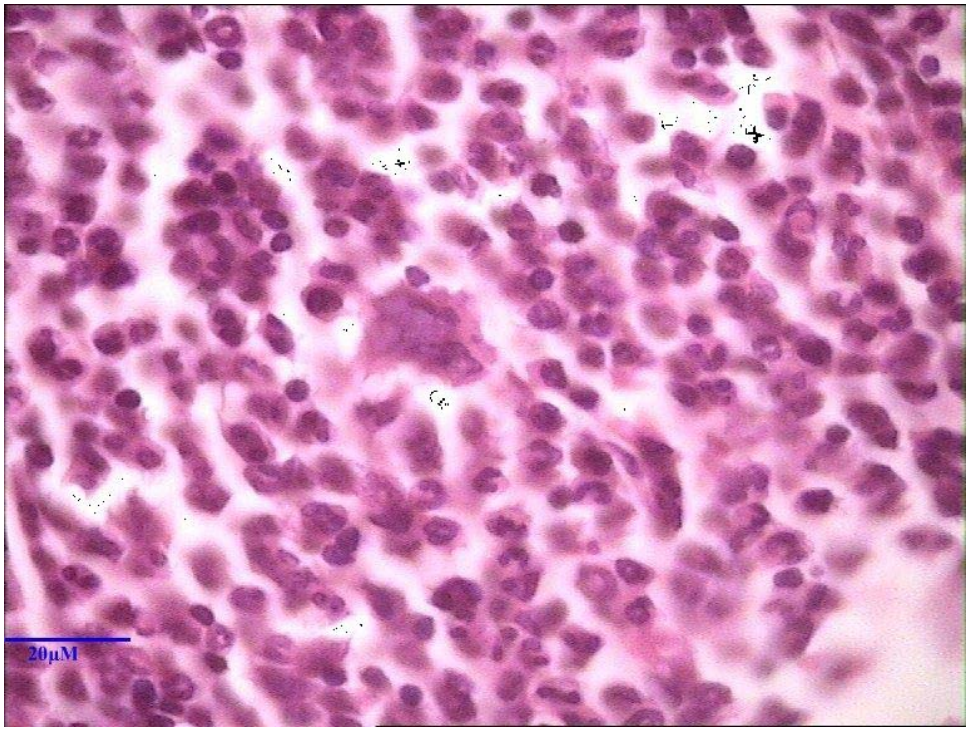

Plate (4) section in femur of CD1 mice stained with hematoxylin and eosin stain illustrate the bone marrow of gasoline+curcumin group

DISCUSSION

The present data showed that gasoline fume exposure induced neutrophilia in male CD1 mice. This probably caused due to intensive mobilization of segmented cells from the bone marrow compartment (Macedo et al., 2006). The relative increase in neutrophils may also have been due to a non-specific response, or to a response to the specific stimulus of benzene metabolites, such as hydroquinone, which stimulate the granulocyte/macrophage progenitor cells both in vivo and in vitro (Lezama et al., 2001; Macedo et al., 2006).

There is a characteristic signs of aplastic anemia as decreased bone marrow cell counts associated with decreased peripheral erythrocyte, platelet, and leukocyte counts in male CD1 mice. The present results are in accordance with other authors who have injected mice with benzene subcutaneously (Lezama et al., 2001) also the study of Irons et al. (2005) who discovered bone marrow displasia in 23 workers exposed to high concentrations of benzene, this benzene-induced displasia include marked dyserythropoeisis, eosinophilic dysplasia and abnormal cytoplasmic granulation of neutrophilic precursors. Hematophagocytosis, stromal degeneration and bone marrow hypoplasia also seen in the study of irons et al. (2005).Also the study of Liu et al. (2001) on mice inhaled benzene and developed a type of aplastic anemia.

The bone marrow is an ordered environment with the hemopoietic stem cell being in close proximity to protective stromal cells. Ordered maturation of myeloid progenitors can be seen in relation to the normal hemopoietic stem cells and one of the diagnostic features of myelodysplasia is abnormal location of these immature precursor cells. In addition to these myeloid components, mature B and T cells are present which may exert significant effects on the stem cell compartment. Thus, hemopoietic stem cells are found in a relatively protected environment within the bone marrow. Oxygen and toxins are delivered by the vascular system, and the differentiating myeloid precursors, which are rich in myeloperoxidase, provide an environment which easily generates oxidative stress (Morgan and Alvares, 2005).

Various benzene metabolites can cause oxidative DNA damage, lipid peroxidation in vivo, formation of hydroxylated deoxyguanosine residues in DNA, and DNA strand breaks, thus, implicating a role for reactive oxygen species (ROS) in benzene-induced toxicity (Faiola et al., 2004). Formation of DNA double-strand breaks by ROS and other mechanisms can lead to increased mitotic recombination, chromosomal translocations, and aneuploidy.

Such genetic consequences may result in protooncogene activation, tumor suppressor gene inactivation, gene fusions, and other deleterious changes in stem cells that can ultimately result in leukemic responses. Thus, DNA damage following benzene exposure must be properly repaired or the affected cells must be eliminated to prevent proliferation of mutated cells and subsequent transformation into malignancies (Faiola et al., 2004).

Various studies have suggested that hematopoietic stem cells (HSC) are the target cells for benzene-induced alterations. In the BM, HSC are a small population (<0.05% of BM cells) of self-renewing, pluripotent cells that give rise to all blood cells (Morrisson et al., 1994). Inhalation exposure to benzene significantly reduced the number of transplantable spleen colony-
forming units (CFU-S), granulocyte/monocyte colony-forming units (CFU-GM), and erythroid colony-forming units (CFU-E) in the bone marrow of male and female mice, indicating a decrease in the number of HSC following exposure to benzene. Benzene was found to affect cell cycle kinetics as the fraction of CFU-GM in S phase was suppressed in male mice exposed to 300 ppm benzene for 2 weeks compared to unexposed control mice (Yoon et al., 2001). In addition, persistent benzene-induced DNA damage was observed as an increased frequency of aneuploidy in the long-term self-renewing population of HSC (Lin−, c-kit+, Sca-1+) from male and female mice 8 months after gavage with benzene compared to the corn-oil-exposed control mice. Thus, benzene has short- and long-term deleterious effects on HSC (Giver et al., 2001).

Exposure to 100 ppm benzene for 2 weeks was sufficient to cause hematotoxicity in the blood and bone marrow as well as significant genotoxicity. At the time point examined, the percentage of dead cells and cells undergoing apoptosis in the bone marrow did not change in response to inhaled benzene. The benzene metabolite hydroquinone can inhibit apoptosis, and some have postulated that damaged cells that have not been repaired could then proliferate and thereby lead to leukemia (Snyder, 2000). Other investigators found that treatment of HL60 human promyelocytic leukemia cells and CD34+ human bone marrow progenitor cells with some benzene metabolites induced time- and concentration-dependent apoptosis (Moran et al., 1996).

Martınez-Velazquez et al. (2006) found that HepG2 cells exposed to 75 mM of benzene produced mainly muconic acid (an indicator of muconaldehyde formation). The benzene and metabolite combination was able to induce a more marked viability decrease than benzene alone. They explored the possible induction of apoptosis as the cause of this viability decrease. They also found morphological and biochemical changes typical of this type of cell death. These in
agreement with the present histopathological study on bone marrow biopsies of mice intoxicated with gasoline alone which showed increased apoptosis in bone marrow cells.

Exposure of mice to benzene severely reduces the number of hemopoietic and lymphoid cells in the peripheral blood by inhibiting DNA synthesis in progenitor cells in the bone marrow. Studies by Cronkite et al. (1982) and Farris et al. (1997) indicated that the cycling fraction of hemopoietic stem cells is elevated dramatically by benzene, whereas a study by Lee et al. (1988) indicated that benzene suppresses these parameters.

Lymphopenia, a decreased number of lymphocytes in peripheral blood is the most common disorder resulted from benzene exposure in many epidemiological studies (Irons et al.,2005; Tsai et al., 2006) and in animal studies (Lezama et al., 2001; Macedo et al., 2006). These results in accordance with the present results which showed a remarkable decrease in peripheral lymphocytes and also the percentage of lymphocytes in bone marrow aspirate.

The hemopoietic system dynamically but conditionally responds *in vitro* as well as *in vivo* to the various endogenous and exogenous stimuli that induce changes in blood and hemopoiesis in normal mice under highly controlled conditions (Yoon et al., 2001).

The sensitivity of persons to benzene toxicity elevates with possessing high levels of CYP2E1 and myeloperoxidase, and low levels of GSH transeferas and NQ01. On the other hand the resistance to benzene toxicity increases with low levels of CYP2E1 and myeloperoxidase, and high levels of GSH transferase and NQ01. Snyder (2000; 2002 and 2004) reported that progressive bone marrow depression ranging from leucopenia, anemia, or thrombocytopenia, through pancytopenia indicative of bone marrow aplasia. In addition recognizing that aplastic anemia was one end point in benzene toxicity and acute leukemia, primarily of myelocytic variety, is an alternative end point to benzene toxicity.

Benzene toxicity can be characterized with respect to the intensity and duration of exposure. Chronic exposure to low doses over a period of weeks or months may lead to decrease in circulating blood cells, and, to some extent in stem cells, but sufficient functional residual capacity of the bone marrow may remain to permit circulating cell levels to be restored to normal if exposure to benzene ceases. Exposure to high levels of benzene may cause sufficient loss of both stem cells and cells of bone marrow stroma that pancytopenia and aplastic anemia results. At intermediate exposure the bone marrow may appear dysplastic, a condition characterized by abnormal morphology, inadequate hematopoiesis, and chromosome damage (Snyder, 2000).

Myelodysplastic syndrome in considered to be preleukemic state and usually proceeds to give rise to full blown leukemia. It is significant, that damage to the immune system can lead to death in aplastic anemia, myelodysplastic syndrome or acute myelocytic leukemia (Snyder, 2000). This in agreement with the present results in that gasoline inhalation caused a high degree of hypocellularity in bone marrow appeared with bone marrow aspirate and bone marrow biopsy examinations, also a marked decrease in leukocyte, erythrocyte and platelet counts in peripheral blood.

Hazel and Kalf (1996), using 32D cells, reported that hydroquinone promoted cellular proliferation and differentiation of the myeloblast to the myelocyte stage, but inhibited further maturation to the neutrophil. Normally the size of the myelocyte pool is controlled by the rate of myelocyte formation and the rate of maturation to neutrophils. When the numbers of myelocytes exceed the need, some cells undergo apoptosis. In the present study was noticed shifting toward immature leukocytes especially myelocytes and metamyelocytes on the other hand decrease of the mature band granulocytes which means slow rate of maturation of leukocytes or inhibiting it by gasoline intoxication, also erythropoisis became slower by gasoline intoxication which
appeared by increasing the percentages of immature erythroblast (proerythroblasts, basophilic erythroblasts, and polychromatic erythroblasts) and decrease the more mature orthrochromatic erythroblasts, and reticulocytes percentage also decreased in peripheral blood, this inhibiting effect of gasoline for leukocytes and erythrocytes maturation discuss the low count of total leukocytes and erythrocytes in the present results, and these results in accordance with the previous discussion of Hazel and Kalf.

Hazel et al. (1996) demonstrated that hydroquinone can also inhibit apoptosis thereby resulting in expansion of the clone of myelocytes. Any mutated cells in this population, which have not undergone DNA repair, will now proliferate, and in effect, promote the development of the leukemia. It may be that the example of benzene-induced promotion was observed in a study of Spalding et al. (1999).

Among the hematological alterations reported to be associated with benzene exposure, peripheral lymphocytes of benzene-exposed workers have shown higher frequencies of somatic mutations and several types of chromosomal aberrations and aplastic anemia, which is characterized by pancytopenia, variable hypocellularity of the bone marrow in the absence of a malignant myeloproliferative disease. Aplastic anemia may be caused by anomalies at three different stem cell functional levels, (A) Bone marrow microenvironment abnormalities, which modify totipotential cell differentiation; (B) inadequate function of cellular hematopoietic regulators (T lymphocytes and their lymphokines); and (C) Immunological inhibition of hematopoiesis (Lezama et al., 2001).

Thrombocytopenia which appeared in the present study as a result of bone marrow depression reflected only in platelet count and increase in prothrombin time which means decrease in prothrombin concentration. On the other hand megakaryblast and megakaryocyte
percentages did not show any significant changes, this may be due to their low percentages, and thus the statistical analysis could not determine the significance of differences. These results are in accordance with Escorcia et al. (1997) study on rats administered benzene orally and bleeding from nasal and gastric mucosa occurred as a result of low platelets count and prothrombin concentration.

A viable alternative to stem cell transplantation is to design approaches that stimulate endogenous stem cells to promote healing and regenerative medicine. Many natural compounds have been shown to promote healing. Bickford et al. (2006) reported about the effects of several natural compounds on the proliferation of human bone marrow and human CD34(+) and CD133(+) cells. A dose-related effect of blueberry, green tea, catechin, carnosine, and vitamin D was observed on proliferation with human bone marrow as compared with human granulocyte-macrophage colony-stimulating factor (hGM-CSF). They further showed that combinations of nutrients produce a synergistic effect to promote proliferation of human hematopoietic progenitors. This demonstrates that nutrients can act to promote healing via an interaction with stem cell populations.

In the present study on the possible protective role of green tea and curcumin on bone marrow cellularity, the hematological and histopathological studies on bone marrow showed a remarkable degree of protection of green tea exerted on bone marrow by returning bone marrow cells count to the normal levels. The next studies judged the present results. In the study of Wang et al. (2003) the water soluble extractives of green tea have protective effects on mice with the irradiating damage in bone marrow induced by gamma-ray. Also Sadzuka et al. (2000) have examined the effect of theanine, a specific amino acid in green tea, on idarubicin-induced
hematotoxicity. The numbers of leukocyte and bone marrow cells decreased significantly on idarubicin injection. Theanine significantly reversed these changes.

In the study of Pal et al. (2005), they used Ehrlich's ascites carcinoma cells grown in peritoneal cavity of Swiss albino mice and curcumin was fed every alternative day. They reported that curcumin administration to tumor-bearing mice decreased tumor cell number significantly in a dose-dependent manner. Furthermore, tumor-induced depletion of immune cell number of the host, as was evidenced from the decrease in bone marrow progenitor as well as thymic and splenic mononuclear cell numbers was reintrated by curcumin. In fact, curcumin inhibited tumor-induced apoptosis of both thymocytes and splenocytes thereby restoring immune cell numbers to normal level in treated Ehrlich's ascites carcinoma-bearing mice. Moreover, curcumin was not toxic to the host; rather in tumor-bearing mice it inhibited hematopoietic toxicity, acted as a hepatoprotective agent and activated depressed anti-oxidant and detoxification systems.

Leukopenia, a decrease in white blood cells, is the most commonly cited hematological change associated with benzene exposure (Qu et al., 2002). In the present study green tea and curcumin protected leukocytes from depression caused by gasoline. This effect of green tea and curcumin on hematopoeisis my be due to its strong inhibiting effect on myeloperoxidase activity which is the corner stone enzyme in benzene hematotoxicity. Kato et al. (2003) estimated that ferulic acid, gallic acid, quercetin, caffeic acid (green tea constituents) and curcumin strongly inhibited myeloperoxidase activity in vitro.

The study of Katiyar and Mukhtar (2001) which shown that topical application of polyphenols from green tea or its major chemopreventive constituent (-)-epigallocatechin-3-gallate (EGCG) prevents ultra violet radiation-induced immunosuppression in mice. To define
the mechanism of prevention, they found that topical application of EGCG (3 mg/mouse/3 cm^2 of skin area) to C3H/HeN mice before a single dose of ultra violet radiation (90 mJ/ cm^2) exposure inhibited ultra violet radiation-induced infiltration of leukocytes, specifically the CD11b+ cell type, and myeloperoxidase activity, a marker of tissue infiltration of leukocytes. EGCG treatment was also found to prevent ultra violet radiation-induced depletion in the number of antigen-presenting cells when immunohistochemically detected as class II MHC+Ia+ cells. UV-B-induced infiltrating cell production of H_2O_2 and nitric oxide (NO) was determined as a marker of oxidative stress. They found that pretreatment of EGCG decreased the number of ultra violet radiation-induced increases in H_2O_2-producing cells and inducible nitric oxide synthase-expressing cells and the production of H_2O_2 and NO in both epidermis and dermis at a UV-B-irradiated site. Together, these data may discuss the mechanisms by which green tea could protect the immunity system from benzene-induced suppression.

In the present observations on WBCs count and its protection with green tea extract are in agreement with Sabu et al. (2002) who studied the effect of polyphenols on total WBCs in alloxan diabetic rats which reversed alloxan-induced WBCs damage to reach the normal level. The immunomodulatory functions of curcumin had appeared in the study of Antony et al.(1999), when WBCs count, circulatory antibody titer against sheep RBCs, the plaque forming cells in the spleen, significantly increased with curcumin administration to Balb/c mice.

Several studies have found associations between benzene exposure and lowered red blood cell counts at various exposure levels (Kipen et al., 1988; Georgieva et al., 1998; Khuder et al., 1999; Qu et al., 2002). Red blood cell decreases in these studies were found at benzene exposure levels ranging from 0.14–2.08 ppm (Khuder et al., 1999) to 75 ppm (Kipen et al., 1988).

Since the lipid peroxidation is a free radical chain reaction and one initiating radical could induce up to twenty propagation reactions (Liu et al., 2000), the RBC membrane is quickly damaged, leading to hemolysis. On the other hand, if antioxidants, such as flavonoids in green tea are present or added to RBCs they would react with the chain propagating peroxyl radicals to stop the peroxidation, hence inhibit hemolysis. All flavonols and their glycosides are effective antioxidants which can protect human red blood cells from free radical induced oxidative hemolysis (Dai et al., 2006).

Deng et al. (2006) concluded that, curcumin and its analogues are effective antioxidants which can protect human red blood cells from free radical- induced oxidative haemolysis and the H-atom abstraction from the phenolic group is responsible for the activity. The observations of Deng et al. (2006) that the compounds bearing ortho-diphenoxyl functionality exhibit markedly higher anti-haemolysis activities than those bearing no such functionality gives us useful information for antioxidant drug design.

Benzene intoxication significantly diminishes the antioxidant defense system and in consequence leads to changes in erythrocyte membrane lipid structure and properties what may disturb their functions. Green tea causes the improvement in the antioxidant system and partially prevents cell membrane disorganization. The results obtained in this study and taking into consideration that metabolism of benzene and catechins is the same in the animals as in humans suggest that green tea may protect human membrane erythrocyte cells against results of oxidative stress caused by different factors. These which prove the increase in RBCs count and hematocrit percentage in gasoline+green tea group compared to gasoline alone group.

The present findings are judged by the study of Varilek et al. (2001) on Interleukin-2-deficient mice, which were received green tea plyphenols in drinking water for 6 weeks appeared when hematocrit was measured at the beginning and the end of the experiment that the hematocrit was improved significantly. Kempaiah and Srinivasan (2005) concluded that curcumin has displayed a protective influence on the erythrocyte integrity in the high fat diet-induced hyperlipidemia.

Other blood count measures, such as platelet count, hemoglobin, and mean corpuscular volume (MCV) also show mixed evidence of an association with gasoline exposure. The strongest evidence is for hemoglobin; several studies show a decrease in hemoglobin in exposed populations (Georgieva et al., 1998; Khuder et al., 1999). These in line with the present findings that gasoline caused hemoglobin reduction compensated by green tea extract or curcumin addition to the diet.Several studies have found a positive relationship between benzene exposure and MCV (Bogadi-Sare et al., 1997). Khuder et al. (1999) have found an inverse association with platelet count. Only one study reported decreased MCV among workers exposed to low levels of benzene (Khuder et al., 1999). All these studies disagree with the present study on MCV and platelets count, in the present study MCV was not affected by gasoline alone nor by co-administration of green tea or curcumin with gasoline. On the other hand platelets count were reduced significantly with gasoline inhalation and improved by green tea extract drinking and addition of curcumin to the diet.

CONCLUSION

This study concluded that, addition of curcumin by 3% to diet or drinking of green tea extract 1.5 as a sole source of drinking water to CD-1 mice, ameliorated the hematotoxicity which induced by gasoline fumes inhalation.

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