Research Article

Evaluation of the Chemopreventive Impacts of Lactoferrin on the Initiation of Liver Cancer Induced by Diethylnitrosamine in Male Mice

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ABSTRACT

Lactoferrin (LF) was examined in this study for its protective influence on liver tissues using a mice carcinogenesis model because it is previously shown to be a strong chemopreventive of colon carcinoma development. Male albino mice were treated sequentially with diethylnitrosamine (DEN, i.p.), in DMSO solution during the first 8 weeks (DEN treatment), and then was administered in the basal diet, at a dose of 0.2 and 2% starting at 2 weeks after the completion of DEN treatment until the end of the experiment. Other groups were given DMSO or LF alone as negative controls. All surviving animals were sacrificed and major liver tissues were examined for expression analysis of tumor related genes (mdr1b, CYP1A2 and p53), DNA damage and reactive oxygen species (ROS) formation. The results revealed that treatment of male mice with DEN was associated with over expression of tumor related genes, DNA damage and ROS formation. However, a markedly reduction in the expression of tumor related genes, DNA damage and ROS formation in all doses (0.2 and 2%) of Lactoferrin was clearly found. The present results indicate that the situation of imbalance between the generation and the elimination of reactive oxygen species (ROS) named oxidative stress, which is characterized by oxidizing biological macromolecules including lipoid, cellular protein, and nucleic acid inducing tumor was inhibited by LF. The results suggest that that LF exerts chemopreventive effects in the liver tissues in addition to the colon tumor which reported in the literature.

Keywords: Lactoferrin, Diethylnitrosamine, Cancer related genes, ROS formation, DNA damage.

INTRODUCTION

Liver cancer is a major health care problem worldwide and is the fifth most common diagnosed cancer in men and the second most leading cause of cancer death[1]. Hepatocellular carcinoma (HCC) accounts for 70–85% of the primary malignant tumors of the liver[2] and its development is frequently related to chronic inflammation in the liver induced by persistent infection with hepatitis B virus and/or hepatitis C virus[3]. Recent epidemiological evidence indicates that the incidence of HCC is rising in developed countries.

This is attributed to an increasing prevalence of hepatitis C virus infection and conditions such as non-alcoholic fatty liver disease, which are associated with obesity[4,5]. Indeed, the incidence and mortality of HCC Arabian countries and also in the USA are rapidly increasing[6]. Since effective and established chemotherapeutic agents for HCC are currently unavailable and its recurrence rate is high, the prognosis of HCC is still poor.

Diethylnitrosoamine (DEN), also known as N-nitrosodiethylamine, is widely used as a carcinogen in experimental animal models. Upon administration of DEN to mice, either administered orally or by interperitoneal injection, various tumors including ones of the livers, the gastrointestinal tract, skin, the respiratory tract and hematopoietic cells are induced. Many investigators have employed DEN to induce liver tumors in mice by injecting DEN i. p. into weaning mice at 2 weeks after birth, giving rise to hepatic tumors 8 months later[7,8]. Since DEN does not itself exert carcinogenicity, it needs to be bioactivated by cytochrome P450 (CYP) enzymes in the liver, resulting in DNA-adducts that form through an alkylation mechanism[9].

These alkylation adducts can be removed by a DNA repair gene O6-methylguanine-DNA methyltransferase (MGMT), also known as O6-alkylguanine-DNA alkyltransferase[10]. Recently, Kang et al.[11] demonstrated that CYP2E1-deficient mice show lower tumor incidence and multiplicity compared with wild-type (WT) mice for DEN-induced hepatocarcinogenesis.

This result strongly suggests that CYP2E1 plays an essential role in the activation of DEN, although several other CYP enzymes are proposed to catalyze DEN bioactivation *in vivo*[9].

Lactoferrin (LF) is a multifunctional iron-binding glycoprotein which is particularly abundant in colostrums (approximately 10 mg/ml) and is also present in mammalian epithelial cell secretions such as tears, saliva and seminal fluid in various amounts (0.01–2 mg/ml)[12]. It is reported to have bacteriostatic properties[13] antiviral activity[14,15] and immunomodulatory functions, such as natural killer (NK) cell activation,[16] stimulation of lymphokine-activated killer (LAK) cell activity[17] and potentiation of macrophage cytotoxicity[18]. Additionally, it was reported that lactoferrin can directly activate NK cells and arrest cell proliferation of epithelial tumor cell lines[19]. Thus, its physiological importance may be related to host primary defense mechanisms.

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It has been recently shown that dietary supplementation with bovine lactoferrin (BLF), derived from bovine milk,[13] can inhibit the development of azoxymethane (AOM)-induced aberrant crypt foci (ACF) as precursor lesions of tumor development,[20] as well as carcinomas[21] in the rat colon, without any toxic effects in major organs. It was also found to reduce growth and metastasis of solid tumors[22]. Therefore, LF is considered to be a good candidate for a chemopreventive agent of human cancer development.

However, several studies of chemopreventive agents have demonstrated that preventive and occasionally promoting or carcinogenic effects of individual exogenous agents may markedly differ from organ to organ[23-27]. Therefore, research into chemoprevention should be based on a wide-spectrum organ analysis. To assess whether LF might influence tumorigenesis in liver tissues, we investigated the effects of low and high doses of LF supplementation using mice carcinogenesis model[28,29].

MATERIALS AND METHODS

Chemicals

Diethylnitrosamine (DEN) and lactoferrin (LF) were purchased from Sigma (St. Louis, MO, USA). Trizol was bought from Invitrogen (Carlsbad, CA, USA). The reverse transcription and PCR kits were obtained from Fermentas (Glen Burnie, MD, USA). SYBR Green Mix was purchased from Stratagene (La Jolla, CA, USA).

Experimental Animals

Seventy adult albino male mice (range: 20-25 g) from the Biology Department, College of Science, King Abdul Aziz University, Saudi Arabia, were used in this study. They were maintained on standard laboratory diet (protein, 16.04%; fat, 3.63%; fiber, 4.1%; and metabolic energy, 0.012 MJ) and water *ad libitum* at the Animal House Laboratory, Biology Department, College of Science, King Abdul Aziz University, Saudi Arabia. After an acclimation period of 1 week, animals were divided into groups (10 mice/ group) and housed individually in filter-top polycarbonate cages, housed in a temperature-controlled (23 ± 1°C) and artificially illuminated (12 h dark/light cycle) room free from any source of chemical contamination. All animals received humane care in compliance with the guidelines of the Animal Care and Use Committee of the College of Science, King Abdul Aziz University, Saudi Arabia.

Experimental Design

The male mice were randomly allocated in 7 groups (n = 10 per group) and treated for 8 weeks as follows: Group 1, control group: animals were treated intragastrically with solvent vehicle control (NaCl). Group 2: animals were administered with DMSO which is the solvent for DEN, Groups 3 and 4, animals were administered with LF in the basal diet, at a dose of 0.2 and 2% for 8 weeks[30]. Group 5, single dose of DEN given dissolved in DMSO at a dose of 25mg/kg body wt by i.p. injection[31]. Groups 6 and 7, animals were administered with LF in the basal diet, at a dose of 0.2 and 2% starting at 2 weeks after the completion of DEN treatment until the end of the experiment.

Tissue Collection

At 24 h after the last injection, mice in each group were sacrificed by decapitation after anesthetized. The liver tissues were collected on ice bath and separated in order to investigate the ROS, total RNA (for the determination of the hepatic related cancer_mRNAs), and DNA (for determination DNA damage).

Gene Expression Analysis

Total RNA Extraction

TRIzol® Reagent (Invitrogen, Germany) was used to extract total RNA from liver tissues of male mice according to the manufacturer's instructions with minor modifications. Tissue samples (50 mg) were homogenized in 1 ml of TRIzol® Reagent. Afterwards, the homogenized sample was incubated for 15 min at room temperature. A volume of 0.2 ml of chloroform per 1 ml of TRIzol® Reagent was added.

Then, the samples were vortexes vigorously for 20 seconds and incubated at room temperature for 5 min. The samples were centrifuged for 12 000 xg for 15 min at 4°C. Following centrifugation, the mixture was separated into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA was remained exclusively in the aqueous phase. Therefore, the upper aqueous phase was carefully transferred without disturbing the interphase into a fresh tube. The RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. A volume of 0.5 ml of isopropyl alcohol was added per 1 ml of TRIzol® Reagent used for the initial homogenization. Afterwards, the samples were incubated at 15 to 30°C for 10 min and centrifuged at 12,000 x g for 10 min at 4°C. The RNA was precipitated which was often invisible before centrifugation, formed a gel-like pellet on the side and bottom of the tube. The supernatant was removed completely. The RNA pellet was washed once with 1 ml of 75% ethanol. The samples were mixed by vortexing and centrifuged at 7500 x g for 5 min at 4°C. The supernatant was removed and RNA pellet was air-dried for 10 min. RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water by passing solution a few times through a pipette tip.

Isolated total RNA was treated with one unit of RQ1 RNAse-free DNAse (Invitrogen, Germany) to digest DNA residues, re-suspended in DEPC-treated water and quantified photospectrometrically at 260 nm. Additionally, integrity was assured with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis (data not shown). Aliquots were used immediately for reverse transcription (RT), otherwise they were stored at -80°C.

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Reverse Transcription (RT) Reaction

Complete Poly(A)$^+$ RNA isolated from liver tissues was reverse transcribed into cDNA in a total volume of 20 µl using RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas, Germany). An amount of total RNA (5 µg) was used with a master mix. The master mix was consisted of 50 mM $MgCl_2$, 10x RT buffer (50 mM KCl; 10 mM Tris-HCl; pH 8.3), 10 mM of each dNTP, 50 µM oligo-dT primer, 20 IU ribonuclease inhibitor (50 kDa recombinant enzyme to inhibit RNase activity) and 50 IU MuLV reverse transcriptase. The mixture of each sample was centrifuged for 30 sec at 1000 g and transferred to the thermocycler. The RT reaction was carried out at 25°C for 10 min, followed by 1 h at 42°C, and finished with a denaturation step at 99°C for 5 min. Afterwards the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for cDNA amplification through quantitative Real Time- polymerase chain reaction (qRT-PCR).

Real Time- PCR (qPCR)

QIAGEN's real-time PCR cycler (Rotor-Gene Q, USA) was used to determine the liver cDNA copy number. PCR reactions were set up in 25 µL reaction mixtures containing 12.5 µL 1× SYBR® Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd.), 0.5 µL 0.2 µM sense primer, 0.5 µL 0.2 µM antisense primer, 6.5 µL distilled water, and 5 µL of cDNA template. The reaction program was allocated to 3 steps. First step was at 95.0°C for 3 min. Second step consisted of 40 cycles in which each cycle divided to 3 steps: (a) at 95.0°C for 15 sec; (b) at 55.0°C for 30 sec; and (c) at 72.0°C for 30 sec. The third step consisted of 71 cycles which started at 60.0°C and then increased about 0.5°C every 10 sec up to 95.0°C. At the end of each sqRT-PCR a melting curve analysis was performed at 95.0°C to check the quality of the used primers. Each experiment included a distilled water control. The sequences of specific primer of the genes used are listed in Table 1.

Table 1: Primers used for qPCR

Genes	Primer sequence	Annealing Temp. (°C)	References
mdr1b	GAA ATA ATG CTT ATG AAT CCC AAA`	54	Zhang et al.[32]
	GGT TTC ATG GTC GTC GTC TCT TGA		
CYP1A2	GGA CCC TGG GGC TTG CCC TTC	60	Wang et al.[33]
	AGC CTC TTT GCT CAG CTC		
P53	GCG GTA CCC CAG GTC GGC GAG AAT CC	57	Qin et al.[34]
	GGG CTC GAG TCT AGA CTT TTG AGA AGC		
β-Action	GTG GGC CGC TCT AGG CAC CAA	65	Qiu et al.[35]
	CTC TTT GAT GTC ACG CAC GAT TT		

At the end of each qPCR a melting curve analysis was performed at 95.0°C to check the quality of the used primers.

Calculation of Gene Expression

First the amplification efficiency (Ef) was calculated from the slope of the standard curve using the following formula found in the manufacturer's instruction pamphlet:

$$Ef = 10^{-1/slope}$$

$$Efficiency\ (\%) = (Ef - 1) \times 100$$

The relative quantification of the target to the reference was determined by using the $2^{-\Delta\Delta CT}$ method if Ef for the target (GLAST, GLT-1, SNAT3 and ASCT2) and the reference primers (β-Actin) as follows:

$$\Delta C_{T(test)} = C_{T(target,\ test)} - C_{T(reference,\ test)},$$

$$\Delta CT(calibrator) = C_{T(target,\ calibrator)} - C_{T(reference,\ calibrator)},$$

$$\Delta\Delta CT = \Delta C_{T(Test)} - \Delta C_{T(calibrator)}.$$

The relative expression was calculated by $2^{-\Delta\Delta CT}$.

Comet Assay for DNA Strand Break Determination

Isolated hepatic cells of all groups of rats were subjected to the modified single-cell gel electrophoresis or comet assay[36]. To obtain the cells, a small piece of the liver was washed with an excess of ice-cold Hank's balanced salt solution (HBSS) and minced quickly into approximately 1 mm^3 pieces while immersed in HBSS, with a pair of stainless steel scissors. After several washings with cold phosphate-buffered saline (to remove red blood cells), the minced liver was dispersed into single cells using a pipette. In brief, the protocol for electrophoresis involved embedding of the isolated cells in agarose gel on microscopic slides and lysing them with detergent at high salt concentrations overnight (in the cold). The cells were treated with alkali for 20 min to denature the DNA and electrophoresis under alkaline conditions (30 min) at 300 mA, 25 V. The slides were stained with ethidium bromide and examined using a fluorescence microscope (Olympus BX60 F-3) with a green filter at × 40 magnification. For each experimental condition, about 100 cells (about 25 cells per fish) were examined to determine the percentage of cells with DNA damage that appear like comets.

The non-overlapping cells were randomly selected and were visually assigned a score on an arbitrary scale of 0–3 (i.e., class 0 = no detectable DNA damage and no tail; class 1 = tail with a length less than the diameter of the nucleus; class 2 = tail with length between 1× and 2× the nuclear diameter; and class 3 = tail longer than 2× the

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diameter of the nucleus) based on perceived comet tail length migration and relative proportion of DNA in the nucleus[36]. A total damage score for each slide was derived by multiplying the number of cells assigned to each class of damage by the numeric value of the class and summing up the values. Slides were analyzed by one observer to minimize the scoring variability.

Determination of ROS Formation

Intracellular ROS generation was measured in liver tissues by a flow cytometer with an oxidation-sensitive DCFH-DA fluorescent probe, after single-cell suspensions were made[37]. DCFH-DA is a non-fluorescent compound that is freely taken up into cells. DCFH is oxidized to fluorescent dichlorofluorescein (DCF) by the action of cellular oxidants. The suspension was loaded by DCFH-DA solution with a final concentration of 50 μM and was incubated for 30 min at 37°C. Then samples were centrifuged at 1000 rpm for 5 min (4°C), and cells were resuspended with phosphate buffered saline (PBS, pH 7.2–7.4). The fluorescence was detected by flow cytometer (with excitation 488 nm and emission 525 nm). For each treatment, 1×10^5 cells were counted, and the experiment was performed in triplicate.

RESULTS

Gene Expression Analysis

Expression of multidrug resistance (mdr1b), cytochrome-P 1A2 (CYP1A2) and tumor proteins (p53) genes in liver tissues of mice is summarized in Figures 1-3, respectively. The expression level of mdr1b gene was significantly higher ($P \leq 0.01$) in the liver tissues of mice treated with DEN in comparison to control and DMSO groups (Figure 1).

However, the expression level of mdr1b gene was significantly lower in mice supplemented with LF in basal diet, at a dose of 0.2 and 2% for 8 weeks ($P \leq 0.05$) compared to animals treated with DEN (Figure 1).

Moreover, treatment of male mice with low or high doses of LF decreased significantly the expression of mdr1b in DEN-treated animal compared with animals treated with DEN alone (Figure 1).

In the same line, the expression of CYP1A2 gene increased significantly ($P \leq 0.01$) in the liver tissues of mice treated with DEN compared to control and DMSO groups (Figure 2).

DEN increased the expression of CYP1A2 gene to 314% compared to control animals. While, the expression level of CYP1A2 gene decreased significantly in mice fed with LF in basal diet, at a dose of 0.2 and 2% for 8 weeks ($P \leq 0.05$) compared to animals treated with DEN (Figure 2).

Furthermore, treatment of male mice with low or high doses of LF decreased the expression of CYP1A2 gene in DEN-treated animal by 21 and 37%, respectively, compared with animals treated with DEN alone (Figure 2).

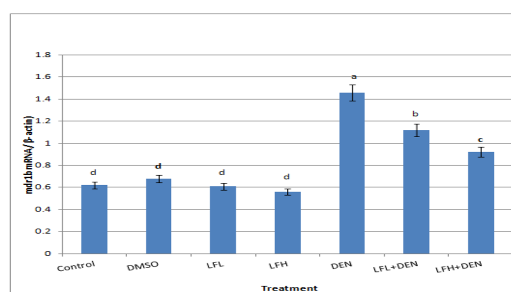

Figure 1: The relative expression of mdr1b gene in liver of male mice after exposure to Diethylnitrosamine (DEN) and/or lactoferrin (LF, L: low dose, H: high dose). Mean values in the same column with different superscript differ significantly (*P*< 0.05).

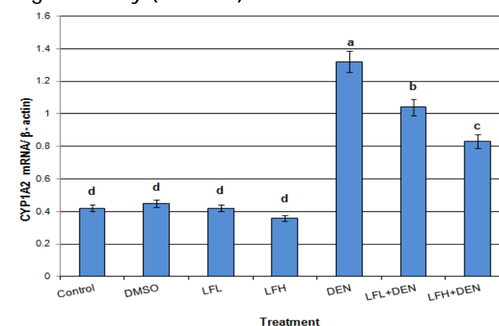

Figure 2: The relative expression of CYP1A2 gene in liver of male mice after exposure to Diethylnitrosamine (DEN) and/or lactoferrin (LF, L: low dose, H: high dose). Mean values in the same column with different superscript differ significantly ($P < 0.05$).

Regarding the expression of p53 gene, the results revealed that treatment of male mice with DEN increased significantly ($P \leq 0.01$) its level compared to control and DMSO groups (Figure 3). DEN increased the expression of p53 gene to 342% compared to control animals. In contrary, the expression level of p53 gene decreased significantly in mice fed with LF in basal diet, at a dose of 0.2 and 2% for 8 weeks ($P \leq 0.05$) compared to animals treated with DEN (Figure 3). Furthermore, treatment of male mice with low or high doses of LF decreased the expression of p53 gene in DEN-treated animal by 17 and 41%, respectively, compared with animals treated with DEN alone (Figure 2).

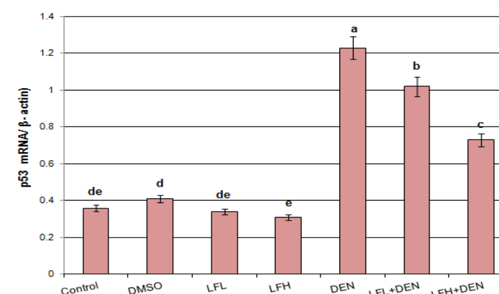

Figure 3: The relative expression of p53 gene in liver of male mice after exposure to Diethylnitrosamine (DEN) and/or lactoferrin (LF, L: low dose, H: high dose). Mean values in the same column with different superscript differ significantly ($P < 0.05$).

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DNA Damage Assessed by Comet Assay

In the present study, DNA damage determination as evident from (Table 2) and (Figure 4) corresponds to DNA from control, DMSO, LF, DEN and DEN+LF animals, respectively. It is observed that the treatment with DEN resulted in significantly ($P \leq 0.01$) higher DNA damage as compared to control, DMSO and the both doses of LF.

However, DNA damage was significantly ($P \leq 0.05$) reduced in mice fed diets contains low or high doses of LF. This reduction was ever more pronounced in the high dose of LF group than in mice fed low dose of LF diet.

In addition, treatment of male mice with low and high doses of LF decreased significantly the DNA damage induced by DEN treatment, however, the high dose of LF was more effective than low dose in preventing the DNA damage (Table 2).

Table 2: Visual score of DNA damage in male mice treated with Diethylnitrosamine (DEN) and/or lactoferrin (LF) using comet assay.

Treatment	Number of Animals	No. of cells		Class¥ of comet				DNA damaged cells (%)
		Analyzed(*)	Total Comets	0	1	2	3	
Control	5	500	26	474	21	5	0	5.2
DMSO	5	500	29	471	20	7	2	5.8
LFL	5	500	27	473	19	6	2	5.4
LFH	5	500	25	475	18	6	1	5
DEN	5	500	91	409	31	37	23	18.2
LFL+DEN	5	500	52	448	21	18	13	10.4
LFH+DEN	5	500	36	464	15	14	7	7.2

¥: Class 0= no tail; 1= tail length < diameter of nucleus; 2= tail length between 1X and 2X the diameter of nucleus; and 3= tail length > 2X the diameter of nucleus. (*): No of cells analyzed were 100 per an animal.

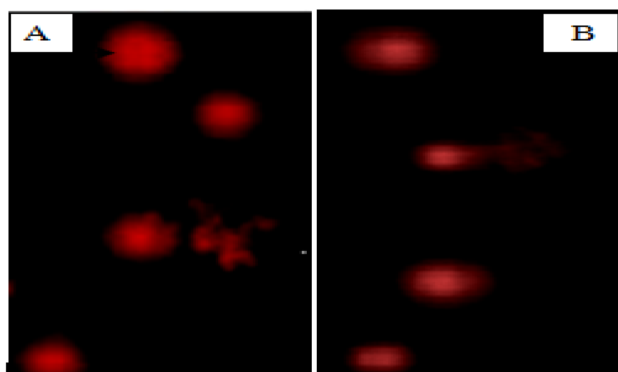

Figure 4: DNA damage in male mice treated with Diethylnitrosamine (DEN) and/or lactoferrin (LF).A) Class 0= no tail; 1= tail length < diameter of nucleus and 3= tail length > 2X the diameter of nucleus. B) 2= tail length between 1X and 2X the diameter of nucleus.

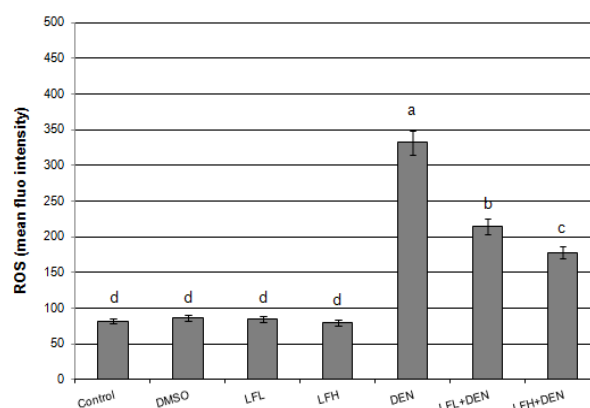

Figure 5: The changes of intracellular ROS levels in liver of male mice after exposure to Diethylnitrosamine (DEN) and/or lactoferrin (LF, L: low dose, H: high dose). Mean values in the same column with different superscript differ significantly ($P < 0.05$).

Effects of LF on Oxidative Stress after DEN Exposure

Effect of DEN on intracellular ROS changes and the protective effects of LF are summarized in Figure 4.

The results of the present study revealed that there were no increases in ROS levels as no significant differences of ROS levels were found between animals in control and DMSO groups. Moreover, treatment of male mice with LF at low or high doses did not increase the levels of ROS compared with control animals (Figure 5).

However, DEN increased intracellular ROS levels by 405% compared to the control group. On the other hand, low and high dose of LF reduced significantly the production of intracellular ROS induced by DEN compared to DEN exposure alone (Figure 5). It was found that high dose of LF was more effectively to reduce the intracellular ROS production than low dose of LF.

Low and high dose reduced intracellular ROS levels by 36 and 46%, respectively, compared to the DEN group (Figure 5).

DISCUSSION

Liver cancer is a major health care problem worldwide and is the fifth most common diagnosed cancer in men and the second most leading cause of cancer death[1]. Hepatocellular carcinoma (HCC) accounts for 70–85% of the primary malignant tumors of the liver[2] and its development is frequently related to chronic

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inflammation in the liver induced by persistent infection with hepatitis B virus and/or hepatitis C virus[3].

In current study, we have focused our attention on some genes related to liver diseases and cancer such as multidrug resistance (mdr1b), cytochrome p1A2 (CYP1A2) and p53 genes in liver tissues of mice. The expression level of mdr1b gene was significantly higher in the liver tissues of mice treated with DEN in comparison to negative control animals. These results were in same line with those reported by Brady et al.[38]. They reported that mdr1a and mdr1b mRNA levels increased in kidney and liver when some cancer chemotherapeutic drugs and efflux of xenobiotics were administered to rats. Yao and Hou[39] found that the administration of carcinogenic compounds to rats caused changes in the activity of the immunological system, the mechanism of action was related to modulation of gene expression that are responsible for mRNA synthesis in thymocythes. Also, over–expression of imprinted genes was observed in human placenta during exposure carcinogenic compounds[40]. Moreover, two genes, a putative pirin protein a putative inner membrane protein were up-regulated in ammonia-oxidizing bacterium *Nitrosomonas europaea* during exposure to carcinogens[41].

Multiple drug resistance is a condition enabling a disease-causing organism to resist distinct drugs or chemicals of a wide variety of structure and function targeted at eradicating the organism. Organisms that display multidrug resistance can be pathological cells, including bacterial and neoplastic (tumor) cells. Multidrug resistance (MDR)–associated genes are known to be inducible by heat and drugs. MDR induction was reported for a variety of drugs/chemicals and radiation *in vitro* systems, but exposure to increased temperatures (mostly approximately 42°C to 43°C) also led to enhanced MDR1 levels, whether they followed single or repeated heat treatments[42]. MDR over-expression was reported for human sarcoma and melanoma cell lines and for human tumors[43].

On the other hand, the cytochromes P450 constitute a multigene family of enzymes involved in the oxidation of many endogenous and xenobiotics substrates[44]. CYP450 is widely used as biomarker when assessing exposure to contaminants in environmental system[45]. Also in the present study, we have observed an over expression of its messenger in the analyzed liver tissues exposed to DEN compared to the control group. Similarly, Sun et al.[46] examined the effects of propiconazole (as a fungicide) on the expression of hepatic cytochrome P450 genes and on the activities of P450 enzymes in male rats and mice. Quantitative real time RT-PCR assays of rat hepatic RNA samples revealed significant mRNA over-expression of the followed genes as compared to control: CYP1A2, CYP2B1, CYP3A1/CYP3A23 and CYP3A2. In mouse livers, propiconazole produced mRNA over-expression of CYP2b10 and CYP3a11. Also, propiconazole significantly induced both pentoxyresorufin O-dealkylation (PROD) and methoxyresorufin O-dealkylation (MROD) activities in both rat and mouse livers.

Additionally, the present study revealed that DEN increased the tumor protein p53 gene in liver of male mice. In the same line, several findings revealed the exposure to carcinogenic and mutagenic compounds such as BaP was able to induce alteration in the gene expression of several liver cancer related genes (CYP1A1, CYP1A2, CYP3A4, CYP2B6, CD59, hTRET and P53) and increased the rate of DNA damage in male mice[47,48].

Concerning the DNA damage, the present study showed that the treatment with DEN resulted in significantly higher of DNA damage as compared to the control mice. Other investigations reported that carcinogenic compounds such as phenols or phenol compounds had inhibited synthesis and replication of DNA in each of Hela cells[49] and diploid human fibroblasts[50]. Additionally, carcinogens also inhibit the enzyme activity of rybonucleotide reductase that participates in DNA synthesis[51]. These phenol compounds are capable of interacting with genetic material causing damage to the DNA structure of mouse and human lymphocytes[52].

The current study revealed that DEN treatment increased significantly the intracellular ROS levels compared to the control group. These results confirm and extend previous data which have demonstrated that the carcinogenic compounds induce a significant increase in LPO under *in vivo* condition[53].

The accumulation of carcinogens in many animal organs lead to formation of lipid peroxidation that is responsible for damage and finally degradation of cell's membrane[53].

The current study showed that LF was able to suppress the expression alterations of cancer related genes, decreased the DNA damage and reduced the intracellular ROS induced by DEN treatment in liver of male mice.

In the same line, it has been shown that LF inhibits the development of azoxymethane (AOM)-induced aberrant crypt foci (ACF) as precursor lesions of tumor development[20] as well as carcinomas[21] in the rat colon when given as a dietary supplement, and also suppresses spontaneous intestinal polyposis in ApcMin mice as a model of familial adenomatous polyposis[30].

Findings have accumulated pointing to a wide array of functions of LF in the immune system; thus NK cell activation[16] stimulation of LAK cell activity[17] and potentiation of macrophage cytotoxicity[18] have been documented, among other effects. The chemopreventive potential of LF on carcinogenesis in animal models might therefore result from activation of immune responses.

In spite of no obvious dose-dependence was observed in the present carcinogenesis mice model, LF tended to inhibit liver carcinogenesis, DNA damage and ROS formation at the 0.2–2% doses, along with significant inhibition at the 0.2% dose in liver tissues.

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In conclusion, LF reveals a beneficial effect which is limited in terms of the target organ spectrum. However, no adverse effects were noted. The main point that whether LF stimulation of immune responses is responsible for its influence on carcinogenesis in animal models remains to be determined. Further investigations of its absorption, distribution and biological effects are warranted.

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