The 5-HT$_{1A}$ Agonist Buspirone Decreases Liver Oxidative Stress and Exerts Protective Effect Against CCl$_4$– Toxicity

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Abstract

We aimed to study the effect of buspirone, an anxiolytic drug and 5-HT$_{1A}$ agonist on liver injury induced by carbon tetrachloride (CCl$_4$) in rats. Rats were orally treated with CCl$_4$ (2.8 mL/kg in olive oil) along with buspirone at 10, 20 or 30 mg/kg once daily starting with CCl$_4$ and for one week thereafter. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) as well as alkaline phosphatase (ALP) activities were determined in the serum. Markers of oxidative stress: lipid peroxidation (malondialdehyde; MDA), reduced glutathione (GSH), nitric oxide (nitrite/nitrate) levels were measured in the liver. Moreover, paraoxonase 1 activity was determined in the liver and serum. The administration of CCl$_4$ led to significant increases in serum ALT, AST, and ALP activities. Results showed that there were significantly increased hepatic MDA, nitrite and decreased GSH levels.   PON1 activity decreased both in the liver and serum, respectively. The immunohistochemical investigations using anti-caspase-3 antibody revealed that CCl$_4$ caused apoptosis to many hepatocytes. DNA studies showed that CCl$_4$ caused hypoploidy in hepatocytes. Rats treated with 20-30 mg/kg buspirone showed significant decrease in serum ALT and AST by 19.5-34.3% and 24.2-31.4%, respectively. Serum ALP decreased by 21.7% after 30 mg/kg buspirone. In the liver, the higher dose of the drug resulted in decreased MDA (by 15.8%), decreased nitric oxide (17.4%) and increased GSH (by 20.1%). Significantly increased serum PON1 activity by 43.9-53.5% was observed after treatment with 20-30 mg/kg buspirone. On histopathologic examination of liver sections, there was mild protective effect for the drug at 30 mg/kg. Sections stained with anti- caspase- 3 confirmed the results obtained from histopathological examination. Moreover, buspirone given at 30 mg/kg resulted in an increase in % of cells containing normal values of DNA. These results indicate that buspirone decreases liver oxidative stress and exerts protective effect against CCl$_4$- toxicity. The study thus indicates more beneficial effects of buspirone as an anxiolytic drug and that the drug could be used safely in patients with liver disease.

Introduction

Buspirone is a partial $5\text{-}HT_{1A}$ receptor agonist that is widely used in treating anxiety disorders and depression [1]. $5\text{-}HT_{1A}$ receptors are presynaptically located as somatodendritic receptors on the 5-HT neurons in the midbrain raphe nuclei and also postsynaptically in limbic and cortical regions. Stimulation of $5\text{-}HT_{1A}$ receptors decreases the firing of 5-HT neurons and 5-HT terminal release. This results in inhibition of serotonergic neurotransmission [2]. The drug in addition has a weak affinity for $5\text{-}HT_2$ receptors and act as an antagonist at dopamine D-2, D-3 and D-4 receptors [3-5]. In animal models of pain, buspirone exerted analgesic action increasing the threshold to thermal, electrical, chemogenic, and visceral pain. The drug inhibits gastric acid secretion and exerts gastric mucosal protective and anti-inflammatory effects [6].

Serotonin has wide distribution in the brain and gut. In brain, serotonin plays an important role in mood, behavior, aggression, and sexual function [7]. Serotonin is decreased in depression and thus drugs which increase brain serotonergic activity eg., the serotonin reuptake inhibitors (SSRIs) are the most common agents used nowadays in treating depressive disorders [8] including depression that occurs in the course of liver disease and/or results from antiviral therapy [9]. There is also evidence involving the brain serotonergic system in the development of depression in patients with chronic liver disease [10] and in hepatic encephalopathy [11] and it is likely that changes in brain serotonin could modulate liver injury.

In the body, serotonin exists mainly in the gut, being produced by the enterochromaffin cells, whilst a small amount is present in plasma stored in platelets [12]. Studies suggested an important role for serotonin derived from platelets in liver regeneration [13,14] and also in causing hepatic injury [15,16]. $5\text{-}HT_{2A}$ receptors are upregulated in activated hepatic stellate cells, the principal cells mediating liver fibrosis and $5\text{-}HT_{2A}$ antagonists inhibit activation of HSCs [17]. In hepatocytes, $5\text{-}HT_{1/2A}$ receptors stimulate, whereas $5\text{-}HT_{2B}$ receptors inhibit glycogen synthesis [18]. There is also a decrease in hepatic $5\text{-}HT_{1A}$ receptor function during hepatocyte regeneration and neoplasia while stimulation of $5\text{-}HT_{1A}$ receptor inhibited hepatocyte DNA synthesis [19]. The above data point to the important role of serotonin in modulating the integrity of the liver.

The administration of the serotonin reuptake inhibitors or serotonergic antagonists eg., trazodone and nefazodone protected against the CCl_4- induced hepatic toxicity in rodents [20-22]. The mechanism by which these drugs decrease the toxin-induced liver damage is not clear, but increased central serotongeric activity [20], prevention of the metabolic derangement induced by CCl_4 [22], reduced platelet serotonin or decreased liver nitric oxide [21] have been suggested.

In this study, our aim was to investigate the possible modulatory effect of buspirone on the development of hepatic injury caused by CCl_4. The latter is a widely used industrial solvent which is known to cause heptotoxicity in humans and rodents. The acute administration of CCl_4 causes fatty degeneration, and hepatocellular death with the mechanism largely involving free radical-mediated oxidative damage to cellular biomolecules[23,24].

Materials and Methods

Animals

The study was conducted using Sprague–Dawley rats of both sexes (130–140 g of body weight). Rats were fed with standard laboratory chow and water *ad libitum*. The animal experiments were done in accordance to the Institutional Ethics Committee and in accordance with the recommendations for the proper care and use of laboratory animals (NIH publication No. 85–23, revised 1985).

Drugs and Chemicals

Carbon tertrachloride (BDH Chemicals, England) and buspirone hydrochloride (Bristol Global Napi Pharmaceutical Co. Cairo, A.R.E.) were used in the experiments. The rest of chemicals and reagents were of the analytical grade (Sigma, St Louis, MO, USA). The dose of CCl_4 used in the study was based on previous observations [20-22].

Experimental Design

Hepatic injury was induced by treating rats by

gavage with CCl_4–olive oil (1:1, v/v) at a dose of 2.8 ml/kg through an orogastric tube. The effect of buspirone given at doses of 10, 20 or 30 mg/kg on CCl_4-induced hepatic damage was then studied. Rats were divided into five equal groups (six rats each). Groups 1–4 were treated with CCl_4 in olive oil along with saline (group 1), or buspirone at doses of 10, 20 or 30 mg/kg (groups 2, 3, 4) once a day orally and treatments continued for 1 week. Rats were given half the initial dose of CCl_4 (0.14 ml/100 g body weight) 3 days after the first dose of CCl_4 in order to maintain the hepatic damage. A 5th group of rats (n=6) received only the vehicle (olive oil) at 2.8 ml/kg and this is followed 3 days later by an additional dose of 1.4 ml/kg olive oil. Rats were allowed free access to food and tap water during the study. Rats were euthanized by decapitation 7 days after the first dose of CCl_4.

Biochemical Studies

Serum Liver Enzymes

At the end of the study, retro-orbital vein plexus blood samples were obtained under light ether anaesthesia. The activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) in serum were then measured colorimetric ally using commercially available kits (BioMérieux, France).

Liver Lipid Peroxidation

The measurement of malondialdehyde (MDA) was used to determine the extent of lipid peroxidation in the liver tissue. The method used is that of Ruiz-Larrea et al. [25]. In this assay, thiobarbituric acid reactive substances (TBARS) react with thiobarbituric acid to produce TBA-MDA adduct with a red color that can be determined using spectrophotometer at 532 nm.

Liver Reduced Glutathione

The method used is that of Ellman et al. [26]. Ellman's reagent (DTNB; 5, 5'-dithiobis (2-nitrobenzoic acid)) is reduced by the sulfhydryl groups of GSH to produce 2-nitro-s-mercaptobenzoic acid. Absorption was measured at 412 nm using a spectrophotometer.

Liver Nitric Oxide

Nitric oxide levels were measured in tissue homogenates using the Griess reaction (Moshage et al. [27].

Paraoxonase 1 Activity

The arylesterase activity of paraoxonase was determined in liver supernatants and serum. In this assay, phenyl acetate used as a substrate is cleaved by the arylesterase/paraoxonase yielding phenol, the rate of its formation is determined by monitoring the increase in absorbance at 270 nm at a temperature of 25°C. One unit of arylesterase activity is considered equal to 1 µM of phenol formed per minute. The activity of PON-1 is expressed in kU/L (based on the extinction coefficient of phenol of 1,310 M^{-1} cm^{-1} at 270 nm, pH 8.0, and 25°C) [28].

Histopathological and Immunohistochemical Studies

The liver sections of each rat were fixed in 10% neutral-buffered formal saline for 72 hours at least and processed routinely for the microscopic examination. Serial sections (5 µm thick) were cut and stained with haematoxylin and eosin (Hx & E) and examined by light microscopy.

Caspase-3 immunohistochemical staining was performed with the use of streptavidin-biotin. In brief, deparaffinized sections (4 µm thick) were incubated for 30 min with fresh 0.3% hydrogen peroxide in methanol at room temperature, followed by incubation with anti caspase-3 antibodies (1: 100 dilution). The specimens were counter stained with H & E. In negative controls, normal mouse serum was substituted for anti caspase-3 antibodies. All sections were investigated by the light microscope. Adobe Photoshop version 8.0 was used for capturing and processing images.

DNA Ploidy Studies

For DNA analysis, we used Feulgen stained sections countered stained with Light green. Analysis was carried out using Leica Quin 500 image cytometry (Pathology Department, NRC, Cairo). For each section (100-120) cells were randomly measured [29].

Statistical Analysis

Data presented are means ± SE. For comparing values before and after CCl_4, paired Student's t-test was

used. For multiple group comparisons, ANOVA and Duncan test were used. P < 0.05 was considered statistically significant.

Results

Biochemical Results

Serum Liver Enzymes

Results are presented in Table 1. Rats treated with only CCl_4 exhibited markedly elevated alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) activities in plasma by 118.4% (122.3 ± 8.9 vs. 56.0 ± 3.1 U/L), 170.5% (184.2 ± 10.0 vs. 68.1 U/L), and 121.7% (159.6 ± 7.2 vs. 72.0 U/L), respectively, indicating the severity of hepatic injury caused by CCl_4. The administration of buspirone to CCl_4-treated rats resulted in a dose-dependent decrease in serum ALT by 17.5%, 19.5% and 34.3%, respectively, compared to the respective control value. Serum AST levels decreased by 24.2% and 31.4% by buspirone given at 20 and 30 mg/kg, respectively. Meanwhile, 21.7% decrease in serum ALP activity was observed after treatment with 30 mg/kg buspirone (Table 1).

Liver Oxidative Stress

The administration of only CCl_4 significantly increased liver MDA and nitric oxide by 99.2 % (71.7 ± 4.1 *vs* 36 ± 2.2 nmol/g.tissue) and 128.6 % (63.1 ± 3.4 *vs* 27.6 ± 1.40 µmol/g. tissue), respectively, compared to the corresponding control value. Meanwhile, GSH decreased by 53.5% (2.93 ± 0.10 *vs* 6.3 ± 0.38 µmol/g. tissue) compared with the vehicle treated group. In CCl_4-treated rats, buspirone given at 10 or 20 mg/kg had no significant effect on hepatic MDA, nitric oxide, or GSH. A significant decrease in liver MDA by 15.8% was, however, observed after treatment with 30 mg/kg of buspirone which also resulted in a significant decrease in nitric oxide level by 17.4% compared with the corresponding control value. Moreover, buspirone at 30 mg/kg elicited a significant increase in GSH by 20% compared with the CCl_4 control group (Table 2).

Serum and Liver Paraoxonase-1 Activity

In rats treated with CCl_4, the activity of

PON1 in the liver and serum was significantly depressed by 38.7% and 60.6%, respectively (Table 3). Treatment with buspirone had no significant effect on liver tissue PON1 activity. However, significantly increased serum PON1 activity by 43.9% and 53.5% was observed after treatment with 20 and 30 mg/kg buspirone, respectively (Table 3).

Histopathological Results

Sections from CCl_4 only-treated rats stained with Hx & E revealed severe damage of hepatocytes and liver tissue in the form of marked vacuolar degeneration and/or acidification of many cells, congestion of blood sinusoids and cellular infiltration (Fig. 1A). Buspirone given at 10 mg/kg showed no protection against CCl_4-induced liver damage as vacuolar degeneration of hepatocytes, cellular infiltration and nuclear changes were still observed (Fig. 1B) whereas a low ameliorating effect on the degree of liver damage was observed at buspirone dose of 20 mg/kg

Caspase-3 Immunostaining

The immunohistochemical investigations using anti-caspase-3 antibody revealed that CCl_4 caused apoptosis to many hepatocytes (Fig. 2, A). Buspirone had a very weak protecting effect against the damaging effect of CCl_4 at doses of 10 and 20 mg/kg as many positively stained hepatocytes were still observed (Fig. 2B & C). The highest dose of buspirone (30 mg) showed slight reduction of positively stained cells denoting mild protecting effect against CCl_4 induced apoptosis (Fig. 2D).

DNA ploidy Results

In the present study, the Qwine 500 image analyzer was used to evaluate the DNA content of examined cells. The image analysis system automatically expresses the DNA content of each individual cell measured then gives the percentage of each cell out of the total number of cells examined. Also, it classifies the cells into four groups; diploid (2C), proliferating cells (3C), tetraploid (4C) and aneuploid cells (>5C). The proliferating cells are further subclassified into; (<10%) low proliferation index, (10-20%) medium proliferation index and (>20%) high proliferation index [29].

Normal distribution of DNA content in the liver
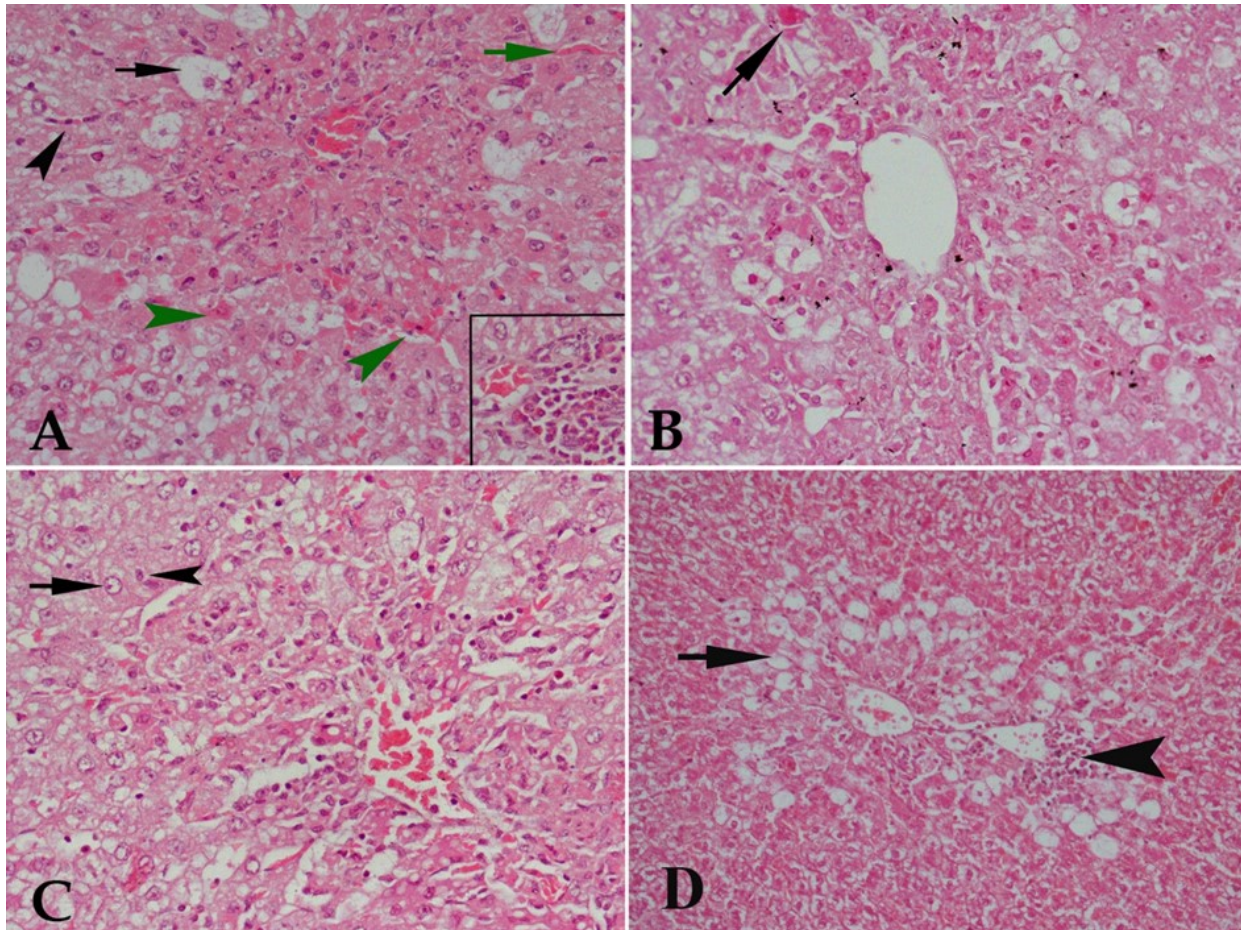
Fig. 1: Hx & E stained liver tissue from **(A)** control rat receiving CCl$_4$ showing severe degenerative changes in liver tissue in the form of marked vacuolar degeneration of many hepatocytes (black arrow), acidified hepatocytes (green arrowhead), congestion of blood sinusoids (green arrow) and cellular infiltration either diffuse in the center of the lobule or in blood sinusoids (black arrowhead). The lower right part of the figure shows focal aggregation of cellular infiltrates. **(B)** CCl$_4$ and 10 mg/kg buspirone showing no protective effect against the damaging effect of CCl$_4$ as acidified cells (arrow), vacuolar degeneration of most hepatocytes and cellular infiltration are still observed. **(C)** CCl$_4$ and 20 mg/kg buspirone showing minimal protection against the damaging effect of CCl$_4$ as some cells appear with normal nuclei (arrow) and others are with pyknotic nuclei (arrowhead), although many cells show vacuolar degeneration, cellular infiltration in the center of the lobule is still present and architecture of liver tissue is markedly deformed. **(D)** CCl$_4$ and 30 mg/kg buspirone showing mild protection of the drug against the damaging effect of CCl$_4$. Cellular infiltration is localized at focal areas beside central vein (arrowhead) with restriction of vacuolar degeneration to the area around central vein (arrow).

Table 1. The effect of buspirone on serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) activities in CCl_4-treated rats

Treatment group	ALT (U/l)	AST (U/l)	ALP (U/l)
Vehicle	56.0 ± 3.1	68.1 ± 4.2	72.0 ± 5.8
CCl_4 (control)	122.3 ± 8.9*	184.2 ± 10.0*	159.6 ± 7.2*
CCl_4 + buspirone (10 mg/kg)	100.9 ± 6.3*+ (-17.5%)	182.2 ± 5.7*	164.52 ± 10.6*
CCl_4 + buspirone (20 mg/kg)	98.4 ± 4.7+ (-19.5%)	139.6 ± 8.9+ (-24.2%)	165.8 ± 12.0*
CCl_4 + buspirone (30 mg/kg)	80.3 ± 3.2+ (-34.3%)	126.4 ± 7.2+ (-31.4%)	124.98 ± 8.7+ (-21.7%)

Results are mean ± S.E. Six rats were used per each group. The percent change from the CCl_4-control group is also shown in parenthesis. Data were analyzed by one way ANOVA and means of different groups were compared by Duncan's multiple range test. P < 0.05 was considered statistically significant. *: P<0.05 *vs* vehicle-treated group. +: P<0.05 *vs* the CCl_4 control group.

Table 2. The effect of buspirone on liver tissue levels of malondialdehyde (MDA), nitric oxide (NO), and reduced glutathione (GSH) in CCl_4-treated rats

Treatment group	MDA (nmol/g.tissue)	NO (mmol/g.tissue)	GSH (mmol/g.tissue)
Vehicle	36.0 ± 2.2	27.6 ± 1.4	6.3 ± 0.38
CCl_4 (control)	71.7 ± 4.1*	63.1 ± 3.4*	2.93 ± 0.10*
CCl_4 + buspirone (10 mg/kg)	68.56 ± 3.9*	62.1 ± 3.7*	2.99 ± 0.13*
CCl_4 + buspirone (20 mg/kg)	67.57 ± 2.8*	58.47 ± 2.0*	2.94 ± 0.14*
CCl_4 + buspirone (30 mg/kg)	60.38 ± 4.3+ (-15.8%)	52.1 ± 1.9+ (-17.4%)	3.52 ± 0.22+ (20.1%)

Results are mean ± S.E. Six rats were used per each group. The percent change from the CCl_4-control group is also shown in parenthesis. Data were analyzed by one way ANOVA and means of different groups were compared by Duncan's multiple range test. P < 0.05 was considered statistically significant. *: P<0.05 *vs* vehicle-treated group. +: P<0.05 *vs* the CCl_4 control group.

Table 3. The effect of buspirone on liver tissue and serum paraoxonase 1 activity (PON1) in CCl$_4$-treated rats

Treatment group	Vehicle	CCl$_4$ (control)	CCl$_4$ + buspirone (10 mg/kg)	CCl$_4$ + buspirone (20 mg/kg)	CCl$_4$ + buspirone (30 mg/kg)
Liver PON1	78.31 ± 4.1	47.96 ± 3.9*	58.68 ± 1.14*	48.72 ± 2.18	47.32 ± 4.5*
Serum PON1	231.0 ± 8.2	90.93 ± 3.9$_*$	99.6 ± 5.2*	130.84 ± 8.1^{*+} (43.9%)	139.56 ± 6.0^{*+} (53.4%)

Results are mean ± S.E. Six rats were used per each group. The percent change from the CCl$_4$-control group is also shown in parenthesis. Data were analyzed by one way ANOVA and means of different groups were compared by Duncan's multiple range test. P < 0.05 was considered statistically significant. *: P<0.05 *vs* vehicle-treated group. +: P<0.05 *vs* the CCl$_4$ control group.
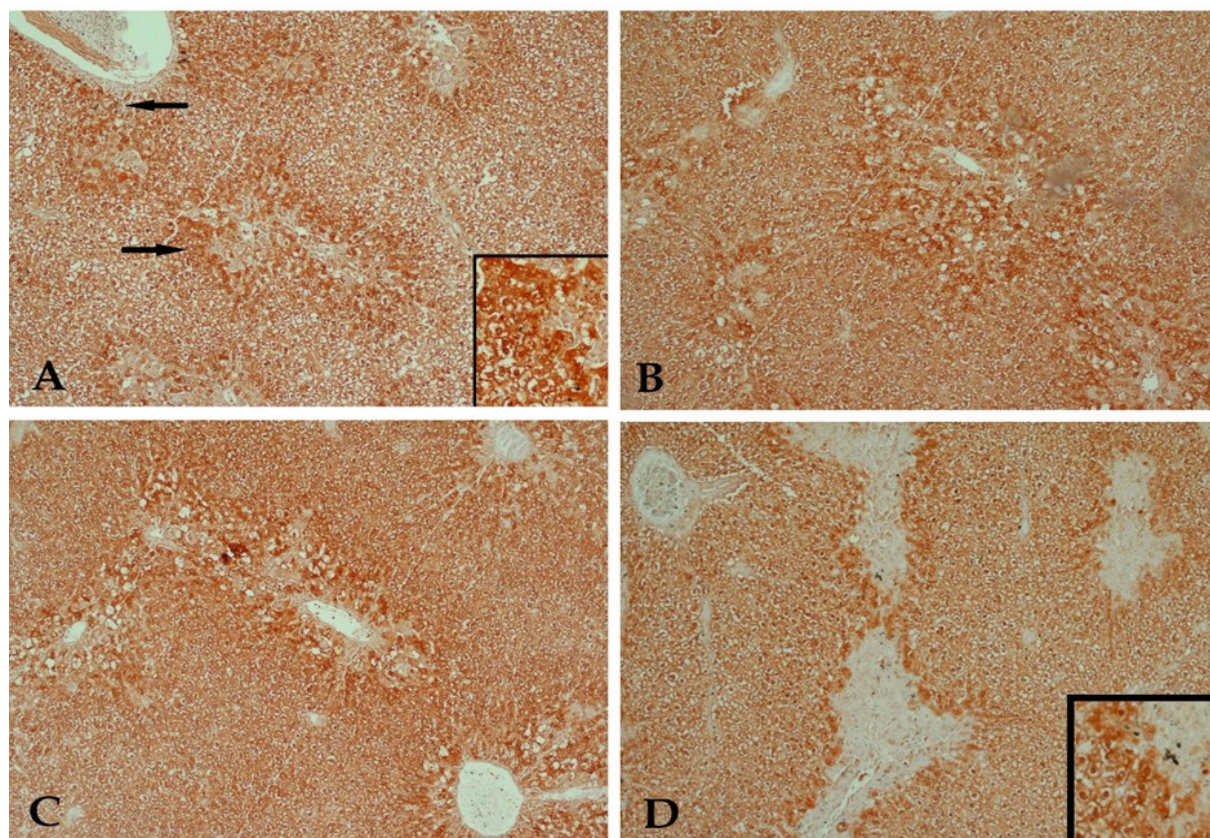
Fig. 2: A photomicrograph of a section of liver tissue stained with anti-caspase-3 antibody with streptavidin-biotin from **(A)** control rat receiving CCl$_4$ showing many hepatocytes with positive immune- reaction to the stain (arrow). **(B)** CCl$_4$ and 10 mg/kg buspirone showing a result close to that of the previous group. **(C)** CCl$_4$ and 20 mg/kg buspirone showing minimal reduction of the positively stained cells. **(D)** CCl$_4$ and 30 mg/kg buspirone showing mild reduction of the positively stained cells

cells of the control group (G. 1) showed that 13.2% of the examined cells contained DNA (<1.5C), 75.47% contained diploid DNA value (2C), 11.32% contained (3C) DNA value (medium proliferation index) and 0.0% of the examined cells at (4C) area (Fig. 3 & table 4).

Examination of cells from G. 2 (control +ve group) treated with CCl_4 showed that the cells contained DNA (<1.5C) were 89.74%, while 10.25% only contained DNA value (2C), which means decrease in DNA content (hypoploidy) compared to the control group. 0.0% of examined cells contained DNA value (3C) and (4C) (Fig. 4 A & table 4).

Examination of cells from the group treated with CCl_4 and buspirone at a dose of 10 mg/kg (G. 3) showed hypoploidy where cells contained DNA (<1.5C) were 80.39%, cells contained DNA value (2C) were 19.60% . Cells contained (3C) and (4C) values were 0.0% (Fig. 4 B & table 4).

On the other hand, 0.0% of the examined cells from the group treated with CCl_4 and 20 mg/kg of buspirone (G. 4) contained DNA value (<1.5 C), 11.0% of cells contained DNA value (2C), while 42.0% of the examined cells contained (3C) DNA value (high proliferating index) and 30.0% of the examined cells contained (4C) DNA value (Fig. 4 C & table 4).

Examination of cells from the group treated with CCl_4 and 30 mg/kg of buspirone (G. 5) showed that 24.77% of examined cells contained (< 1.5 C), 46.78% of examined cells contained (2 C) value of DNA. Cells contained (3C) value were 20.18%, while 6.42% of examined cells contained (4C) value of DNA (Fig. 4 D & table 4).

From the above results it was clear that CCl_4 caused hypoploidy in the examined cells as percentage of cells containing DNA value less than the normal was increased markedly, while percentage of cells containing the normal value of DNA was greatly reduced. Nearly the same results were obtained after treatment with the lowest dose of buspirone, whereas buspirone given at 20 mg/kg to CCl_4-treated rats caused hyperploidy as the percentage of cells containing DNA value higher than normal was increased at the expense of cells containing normal value of DNA. The highest dose of the drug showed mild amelioration of DNA values in hepatic cells as the percentage of cells containing normal values of DNA was increased, although the percentage of cells containing less or more DNA values was still higher than normal.

Discussion

The results of the present study indicate that treatment with buspirone was associated with a decrease in CCl4-induced hepatotoxicity. Buspirone treatment at 20-30 mg/kg resulted in a significant decrease in the activities of the hepatocellular enzymes ALT and AST in serum. It also reduced the leakage of ALP into the plasma. The release of these intracellular enzymes into the circulation is an important marker for detecting hepatocyte injury [30]. This enzyme is present at the hepatocyte's canalicular membrane and increased enzyme activity occurs whenever there is a stagnation of bile flow [31]. It is therefore clear that the decrease in the activity of these enzymes is a function of a decreased extent of liver damage and this notion was supported by histopathological examination of the liver and by the DNA studies. The administration of CCl4 caused marked vacuolar degeneration and/or acidification of many cells. Study of the DNA content of hepatic tissue indicated that CCl4 caused marked decrease in DNA content of hepatocytes (hypoploidy). Buspirone decreased hepatic vacuolar degeneration, the expression of the apoptotic marker caspase-3 and the CCl4-induced changes in DNA values in liver cells. The above findings collectively indicate a beneficial effect for the drug upon CCl4 hepatotoxicity.

The drug exerted an antioxidant action against the liver oxidative stress caused by CCl_4. The latter is a well known hepatic toxin in humans and in experimental animals. This is largely due to its metabolic activation by cytochrome (CYP)2E1, CYP2B1 or CYP2B2, and the formation of the trichloromethyl radical, CCl_3, capable of causing lipid peroxidation, protein and DNA damage [32]. In this study, the administration of CCl_4 caused a marked increase in liver content of the lipid peroxidation product malondialdehyde. This occurred along with depletion of the antioxidant reduced glutathione. In the cell, glutathione (L-g-glutamyl-L-cysteinyl-glycine) is the most abundant thiol in the cystosol, present mainly in its reduced form. Glutathione is a direct free radical scavenger and is also a co-factor for glutathione
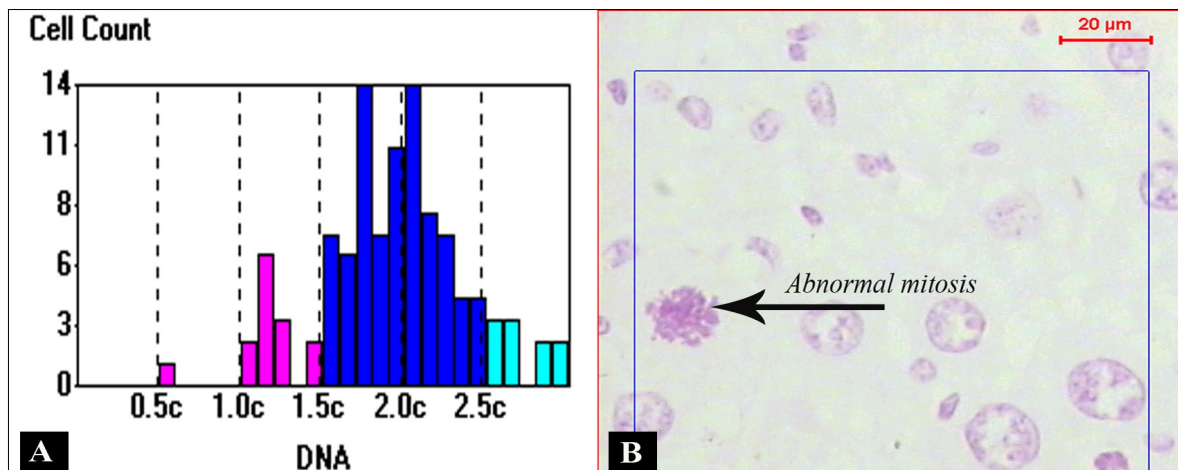
Fig. 3: (A) A chart showing the distribution of DNA content in normal hepatic cells. Notice that most of cells contain the normal content of DNA (2C). **(B)** Shows abnormal mitosis in Feulgen-stained sections of liver tissue.
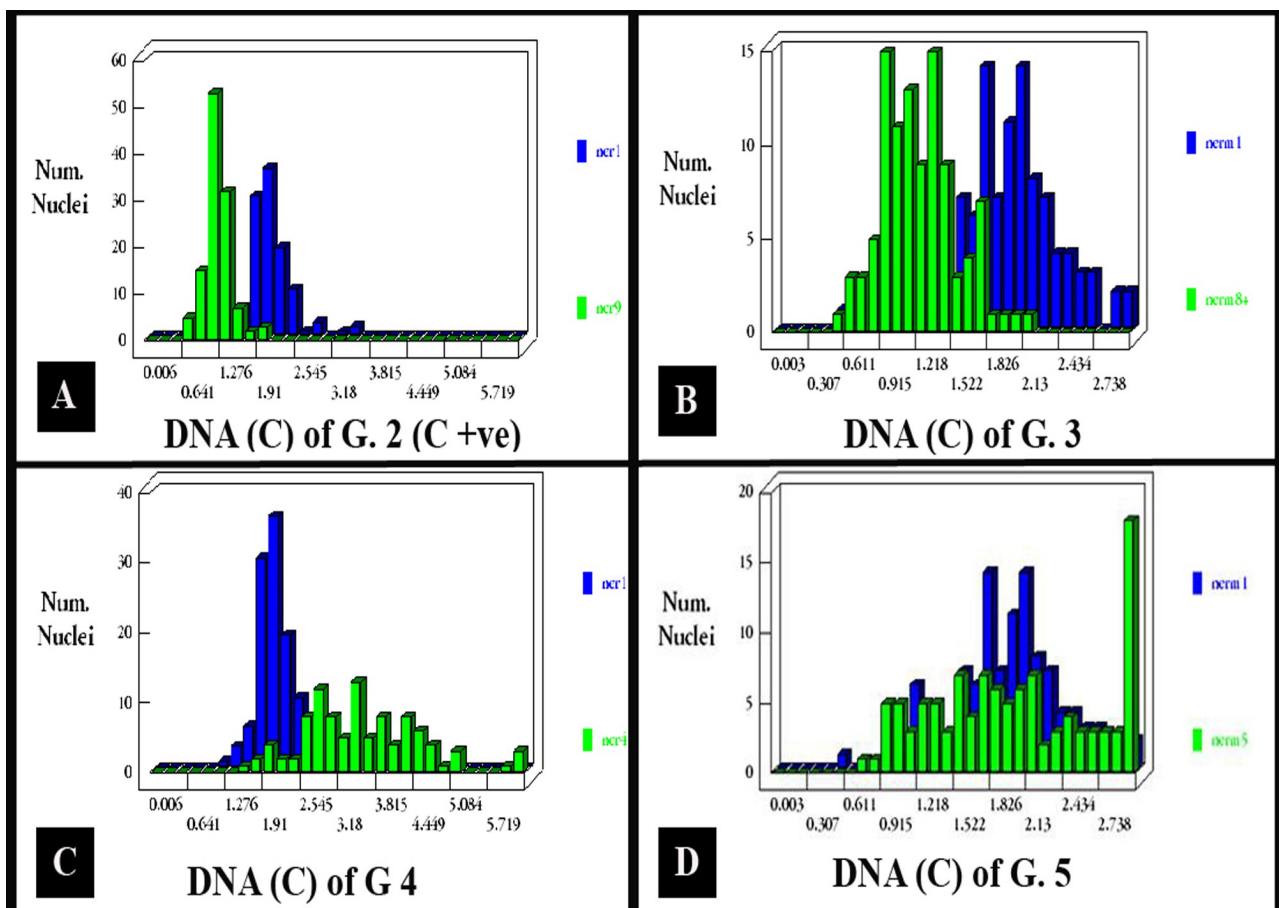
Fig. 4. (A) A chart of DNA content in liver cells of a rat treated with CCl_4 shows deviation to the left (<2C). (B) A chart of DNA content in liver cells of a rat treated with CCl_4 and 10 mg/kg buspirone shows the same result as the previous group. (C) A chart of DNA content in liver cells of a rat treated with CCl_4 and 20mg/kg buspirone shows deviation to the right (> 2C). (D) A chart of DNA content in liver cells of a rat treated with CCl_4 and 30 mg/kg buspirone shows increase in percentage of cells containing (2C value or less) and decrease in percentage of cells containing (>2C).

Table 4. The effect of buspirone on DNA content in CCl$_4$-treated rats

	DNA index (total)	< 1.5 C	DNA Index	1.5 – 2.5 C	DNA Index	2.5 – 3.5 C	DNA Index	3.5 – 4.5 C	DNA Index	>4.5 C	DNA Index
C -ve	1.00	13.208 ± 0.201%	0.603	75.472 ± 0.271%	1.009	11.321 ± 0.261%	1.404	0.0%	-	0.0%	-
C +ve	0.604	89.744 ± 0.189%*	0.576	10.256 ± 0.247%*	0.844	0.0%*	-	0.0%	-	0.0%	-
G 3	0.637	80.392 ± 0.198%*	0.576	19.608 ± 0.258%*	0.891	0.0%*	-	0.0%	-	0.0%	-
G 4	1.743	0.0%*#	-	11.0 ± 0.129%*#	0.998	42.0 ± 0.269%*+#	1.472	30.0 ± 0.270%*+#	1.936	17.0 ± 0.273%*+#	2.550
G 5	1.088	24.771 ± 0.099%*+#	0.589	46.789 ± 0.284%*+#	0.998	20.183% ± 0.260%*+#	1.497	6.422 ± 0.453%*+#	1.962	1.835 ± 0.275%*+#	2.558

C +ve: positive control (CCl$_4$ only); C-ve: negative control (saline); G3: CCl$_4$ + buspirone 10 mg/kg; G4: CCl$_4$ + buspirone 20 mg/kg; G5: CCl$_4$ + buspirone 30 mg/kg. *: P<0.05 *vs* vehicle-treated group. +: P<0.05 *vs* CCl$_4$ control group. #: P<0.05 *vs* G3.

peroxidase and glutathione reductase enzymes [33]. Glutathione is essential for hepatoceullar integrity. This is because cellular glutathione depletion resulted in hepatic steatosis, inflammation and cell death in mice [34]. On the other hand, the glutathione donor *N*-acetyl-cysteine was able to correct the biochemical and the pathological changes in the liver of glutathione deficient mice [35]. In this study, the increase in liver lipid peroxidation and the depletion of the liver tissue content of reduced glutathione was significantly decreased by the administration of 30 mg/kg of buspirone, possibly due to lowered level of oxidative stress by the drug.

Our results also indicated markedly increased hepatic nitric oxide content following CCl₄ administration. In the liver, nitric oxide that is constitutively formed by the action of endothelial isoform of nitric oxide synthase (eNOS) maintains hepatic microcirculation and the integrity of endothelium. In contrast, the increased generation of nitric oxide by the inducible form of NOS (iNOS) due to the action of inflammatory cytokines contributes to hepatocyte apoptosis, liver tissue damage and fibrosis under such conditions as ischaemic-reperfusion injury and also after exposure to CCl₄ [36,37]. Moreover nitric oxide synthase inhibitors were shown to prevent hepatic necrosis and to decrease the expression of tumour necrosis factor alpha (TNF-α) and cycloozygenase-2 in liver tissue of CCl₄ treated rats [38]. In this study, the administration of buspirone at 30 mg/kg was associated with a significant decrease in liver nitric oxide in CCl₄ intoxicated rats. The finding in the present study of the decrease in lipid peroxidation and nitric oxide as well as the increase in reduced glutathione after treatment with CCl₄ and buspirone might therefore indicate improved cell-redox state by the drug and/or a lower degree of tissue damage due to other mechanism of buspirone.

The present study also demonstrated markedly reduced paraoxonase 1 (PON1) activity in the liver tissue and serum from CCl₄-treated rats. This enzyme is synthesized in the liver and is found in plasma in association with high-density lipoproteins to prevent their oxidation. Paraoxonase 1 is also endued with xenobiotic metabolizing and antioxidant activities [39]. Paraoxonase-1 exerts an antioxidant and anti-inflammatory actions in the liver and is considered a

biomarker of liver diseases [40-42]. Mice deficient in paraoxonase 1 fed high fat and cholesterol diet exhibited increased extent of fatty degeneration as well as increased lipid peroxides and oxidative stress markers relative to their wild-type counterparts on the same diet [43]. It is conceivable that the serum level of PON1 depends on the ability of the liver to synthesize the enzyme [44]. The decrease in PON1 activity observed in the current study might thus reflects decreased synthesis by the intoxicated liver cells and the recovery in PON1 could be the result of reducing oxidative stress and improving the condition of hepatocytes by buspirone treatment.

In the peripheral tissues, synthesis of serotonin is carried out by the enterochromaffin cells of the gut. Serotonin is then released into circulation and most of this circulating serotonin is actively taken up, sequestrated within vesicles in platelets and released upon their stimulation [7, 45]. This platelet-derived serotonin has been implicated in both liver protection [46,47] and regeneration [14,15] but also in the development of hepatic injury [16,17]. Thus, in the aged rat stimulation of 5-hydroxytryptamine receptor 2 resulted in improving sinusoidal perfusion and in restoring the deficit in liver regeneration via vascular endothelial growth factor [46]. In mice, absence of peripheral serotonin caused increased acetaminophen-induced liver damage [47]. Moreover, mice lacking tryptophan hydroxylase 1 which is the rate-limiting enzyme for synthesizing serotonin in periphery showed blunted liver regeneration [48].

Serotonin administration increases hepatic glycogen synthesis and concentration [49]. This would provide a substrate for glycolysis and cellular ATP synthesis. On the other hand, in a murine model of non-cytopathic lymphocytic choriomeningitis viral infection, platelets recruited to the liver, and their activation resulted in reduced sinusoidal microcirculation, and delayed virus elimination while increasing liver damage. Fluoxetine, a SSRI resulted in a reduction of hepatocyte damage [15]. Platelet serotonin increases neutrophil recruitment to the sites of inflammation and this could be decreased by fluoxetine [16].

Studies have shown that the SSRIs inhibitors

fluoxetine, sertraline, citalopram, fluvoxamine or the serotonergic antagonists trazodone and nefazodone were able to protect against the hepatotoxic effect of CCl_4 [20-22]. These agents inhibit the serotonin transporter, thereby inhibiting the uptake of serotonin into platelets and impairing platelet aggregation [50].

The same mechanism could possibly be also involved in their hepatic protective effects described earlier by increasing the plasma serotonin and therefore serotonin availability for liver cells. Buspirone, however, does not affect the reuptake of serotonin into platelets and in healthy subjects causes an increase in plasma levels of free serotonin without affecting platelet serotonin or platelet aggregation [51].

In summary, the present study has demonstrated that the $5-HT_{1A}$ agonist buspirone reduced experimental liver injury induced by CCl_4 in the rat. Buspirone displayed antioxidant action, reduced apoptosis and improved the CCl_4-induced changes in DNA values in hepatic cells. These data suggest that the drug is safe in patients with liver disease.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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References

1. Loane C, Politis M. Buspirone: what is it all about? Brain Res 2012; 1461:111-8.

2. Barnes NM, Sharp T. A review of central 5-HT receptors and their function. Neuropharmacology 1999; 38(8):1083-152.

3. Bergman J, Roof RA, Furman CA et al. Modification of cocaine self-administration by buspirone (buspar (R)): potential involvement of D-3 and D-4 dopamine receptors. Int J Neuropsychopharmacol 1013; 16:445–458.

4. Kim SW, Fowler JS, Skolnick P et al. Therapeutic doses of buspirone block D3 receptors in the living primate brain. Int J Neuropsychopharmacol 2014; 17(8):1257-67.

5. Sagarduy A, Llorente J, Miguelez C et al. Buspirone requires the intact nigrostriatal pathway to reduce the activity of the subthalamic nucleus via 5-HT1A receptors. Exp Neurol 2016; 277:35-45.

6. Abdel Salam OME, Baiuomy AR. Effect of buspirone on inflammation, pain and gastric injury in mice: The Internet Journal of Pharmacology 2008; 6 (1).

7. Mohammad-Zadeh LF, Moses L, Gwaltney-Brant SM. Serotonin: a review. J Vet Pharmacol Ther 2008; 31 (3):187-99.

8. Shelton RC. Serotonin norepinephrine reuptake inhibitors: similarities and differences. Primary Psychiatry 2009; 16:5 (Suppl 4):25-35.

9. Scalori A, Pozzi M, Bellia V, Apale P et al. Interferon-induced depression: prevalence and management. Dig Liver Dis 2005; 37(2):102-7.

10. Cai W, Khaoustov VI, Xie Q et al. Interferon-alpha-induced modulation of glucocorticoid and serotonin receptors as a mechanism of depression. J Hepatol 2005; 42:880–887

11. Chojnacki C, Walecka-Kapica E, Stepien A et al. Serum and ascitic fluid serotonin levels and 5-hydroxyindoleacetic acid urine excretion in the liver of cirrhotic patients with encephalopathy. Advances in Medical Sciences 2013; 58(2): 251-256.

12. Lesurtel M, Soll C, Humar B et al. Serotonin: a double-edged sword for the liver? Surgeon 2012; 10 (2):107-13.

13. Papadimas GK, Tzirogiannis KN, Mykoniatis MG et al. The emerging role of serotonin in liver regeneration. Swiss Med Wkly 2012; 142:w13548

14. Naito K, Moteki H, Kimura M et al. Serotonin 5-HT2B receptor-stimulated dna synthesis and proliferation are mediated by autocrine secretion of transforming growth factor-α in primary cultures of adult rat hepatocytes. Biol Pharm Bull 2016; 39(4):570-7.

15. Lang PA, Contaldo C, Georgiev P et al. Aggravation of viral hepatitis by platelet-derived serotonin. Nat Med 208; 14(7):756-61.

16. Duerschmied D, Suidan GL, Demers M et al. Platelet serotonin promotes the recruitment of neutrophils to sites of acute inflammation in mice. Blood 2013; 121

(6):1008-15.

17. Kim DC, Jun DW, Kwon YI et al. 5-HT2A receptor antagonists inhibit hepatic stellate cell activation and facilitate apoptosis. Liver Int 2013; 33(4):535-43.

18. Tudhope SJ, Wang CC, Petrie JL et al. A novel mechanism for regulating hepatic glycogen synthesis involving serotonin and cyclin-dependent kinase-5. Diabetes 2012; 61 (1):49-60.

19. Pyroja S, Joseph B, Paulose CS. Increased 5-HT2C receptor binding in the brain stem and cerebral cortex during liver regeneration and hepatic neoplasia in rats. J Neurol Sci 2007; 254:3-8.

20. Abdel Salam OME, Sleem AA, Shaffie NM. Effect of the selective serotonin reuptake inhibitor fluoxetine on carbon tetrachloride induced hepatic damage in rats. J Pharmacol Toxicol 2006; 1: 395-406.

21. Abdel Salam OME, Sleem AA, Shafee N. Effect of trazodone and nefazodone on hepatic injury induced by carbon tetrachloride. Drug Discoveries & Therapeutics 2010; 4(4):285-297.

22. Abdel Salam OME, Sleem AA, Shafee N. The effect of serotonin reuptake inhibitors on hepatic injury induced by carbon tetrachloride. Comp Clin Pathol 2012; 21 (1): 77-89.

23. Ma JQ, Li Z, Xie WR et al. Quercetin protects mouse liver against CCl4-induced inflammation by the TLR2/4 and MAPK/NF-κB pathway. Int Immunopharmacol 2015; 28(1):531-9.

24. Xin Y, Wei J, Chunhua M et al. Protective effects of Ginsenoside Rg1 against carbon tetrachloride-induced liver injury in mice through suppression of inflammation. Phytomedicine 2016; 23(6):583-8.

25. Ruiz-Larrea MB, Leal AM, Liza M et al. Antioxidant effects of estradiol and 2-hydroxyestradiol on iron-induced lipid peroxidation of rat liver microsomes. Steroids 1994; 59(6):383-8.

26. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959; 82(1):70-7.

27. Moshage H, Kok B, Huizenga JR et al. Nitrite and nitrate determinations in plasma: a critical evaluation. Clin Chem 1995; 41(6 Pt 1):892-6.

28. Higashino K, Takahashi Y, Yamamura Y. Release of phenyl acetate esterase from liver microsomes by carbon tetrachloride. Clin Chim Acta 1972; 41:313–320

29. Danque PD, Chen HB, Patil J. Image analysis versus flow cytometry for DNA ploidy quantitation for tumors: A comparison of six methods of sample preparation. Mod Pathol 1993; 6:270-275.

30. Limdi JK, Hyde GM. Evaluation of abnormal liver function tests. Postgrad Med J 2003; 79: 307-312.

31. Dancygier H. Basic laboratory parameters. In "Clinical Hepatology Principles and Practice of Hepatobiliary Diseases" (Dancygier H, Ed.), Springer-Verlag, Berlin Heidelberg, 2010, Vol 1, pp. 319-441.

32. Weber LW, Boll M, Stampfl A. Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model. Crit Rev Toxicol 2003; 33(2), 105-136.

33. Lushchak VI. Glutathione homeostasis and functions: potential targets for medical interventions. J Amino Acids 202; 736837.

34. Chen Y, Yang Y, Miller ML et al. Hepatocyte-specific Gclc deletion leads to rapid onset of steatosis with mitochondrial injury and liver failure. Hepatology 2007; 45(5):1118-28.

35. Chen Y, Dong H, Thompson DC et al. Glutathione defense mechanism in liver injury: insights from animal models. Food Chem Toxicol 2013; 60:38-44.

36. Iwakiri Y. Nitric oxide in liver fibrosis: The role of inducible nitric oxide synthase. Clin Mol Hepatol 2015; 21(4):319-25.

37. Liu H, Li Q, Wang Y et al. Elevated nitric oxide levels associated with hepatic cell apoptosis during liver injury. Hepatol Res 2016; doi: 10.1111/hepr.12783. [Epub ahead of print]

38. Tipoe GL, Leung TM, Liong E et al. Inhibitors of inducible nitric oxide (NO) synthase are more effective than an NO donor in reducing carbon-tetrachloride induced acute liver injury.

Histol Histopathol 2006; 21(11):1157-65.

39. Kulka M. A review of paraoxonase 1 properties and diagnostic applications. Polish Journal of Veterinary Sciences 2016; 19(1): 225−232.

40. Desai S, Baker SS, Liu W et al. Paraoxonase 1 and oxidative stress in paediatric non-alcoholic steatohepatitis. Liver Int 2014; 34(1):110-7.

41. Ceron JJ, Tecles F, Tvarijonaviciute A. Serum paraoxonase 1 (PON1) measurement: an update. BMC Vet Res 2014; 10:74.

42. Wang B, Yang RN, Zhu YR et al. Involvement of xanthine oxidase and paraoxonase 1 in the process of oxidative stress in nonalcoholic fatty liver disease. Mol Med Rep 2017; 15(1):387-395.

43. García-Heredia A, Kensicki E, Mohney RP et al. Paraoxonase-1 deficiency is associated with severe liver steatosis in mice fed a high-fat high-cholesterol diet: a metabolomic approach. J Proteome Res 2013; 12(4):1946-55.

44. Fuhrman B (2012) Regulation of hepatic paraoxonase-1 expression. J Lipids 2012:684010.

45. Lesurtel M, Soll C, Graf R et al. Role of serotonin in the hepato-gastroIntestinal tract: an old molecule for new perspectives. Cell Mol Life Sci 2008; 65 (6):940-52.

46. Furrer K, Rickenbacher A, Tian Y et al. Serotonin reverts age-related capillarization and failure of regeneration in the liver through a VEGF-dependent pathway. Proc Natl Acad Sci USA 2011; 108(7):2945-50.

47. Zhang J , Song S, Pang Q et al. Serotonin deficiency exacerbates acetaminophen-induced liver toxicity in mice. Scientific Reports 2014; 5 (1): 8098.

48. Lesurtel M, Graf R, Aleil B et al. Platelet-derived serotonin mediates liver regeneration. Science 2007; 312(5770): 104−107.

49. Watanabe H, Akasaka D, Ogasawara H et al. Peripheral serotonin enhances lipid metabolism by accelerating bile acid turnover. Endocrinology 2010; 151(10):4776-86.

50. Anglin R, Yuan Y, Moayyedi P et al. Risk of upper gastrointestinal bleeding with selective serotonin reuptake inhibitors with or without concurrent nonsteroidal anti-inflammatory use: a systematic review and meta-analysis. Am J Gastroenterol 2014;109(6):811-9.

51. Lechin F, van der Dijs B, Jara H et al. Effects of buspirone on plasma neurotransmitters in healthy subjects. J Neural Transm (Vienna) 1998; 105 (6-7):561-73.