Pathological mechanisms of liver injury caused by oral administration of bisphenol A

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Abstract: Bisphenol A (BPA) is a widely produced, endocrine disrupting compound that is pervasive in the environment. BPA is a contaminant with increasing exposure to it and exerts both toxic and estrogenic effects on mammalian cells. Due to variability in study design, the disruptive effects of BPA have been proven difficult to experimentally replicate. BPA exposure causes oxidative stress leading to inflammation in the liver. However, its precise mechanisms are not fully elucidated. This study was designed to assess the molecular, biochemical and histological alterations behind inflammation and hepatic injury caused by BPA. We investigated the disruptive hepatotoxic actions of oral exposure to BPA by measuring changes in oxidative stress, cytokine expression and histopathology in the liver tissue of mice. Swiss albino mice were exposed to BPA via drinking water at doses of 1/50, 1/40, 1/30, 1/20 and 1/10 LD50 (48, 60, 80, 120 and 240 mg/kg b.w. respectively) for three weeks. Oral exposure to BPA caused dose-related hepatotoxic effects, including oxidative stress in terms of increase lipid peroxidation and decrease catalase antioxidant enzyme. The mRNA levels of liver pro-inflammatory cytokines IL-6, and IL-1β were up-regulated in a dose dependant manner by BPA. Our data demonstrated that BPA exposure causes liver injury, which is associated with remarkable inflammatory response, oxidative stress, and histopathological alterations.

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1. Introduction

Bisphenol A (BPA) is an estrogenic endocrine disrupting chemical (EDC) which has been utilized extensively in dentistry, food packaging, and lacquers (for coating food cans and water) (Colborn *et al.*, 1993). According to studies, even at very low doses, BPA's mimicry of estrogen resulted in an array of health maladies including prostate (Prins *et al.*, 2008) and breast cancer (Pupo *et al.*, 2012). The adverse effects of BPA are largely related to its estrogenic activity (Hiroi *et al.*, 1999; Kurosawa *et al.*, 2002), and result in disturbances in reproductive function (Takeuchi *et al.*, 2004). However, BPA has other effects such as inflammatory cytokines dysregulation (Wetherill *et al.*, 2007; Ben-Jonathan *et al.*, 2009) and increase of oxidative stress (Nakagawa and Tayama, 2000; Bindhumol *et al.*, 2003; Asahi *et al.*, 2010), which is independent of estrogenic activity. More recently, it has been reported that BPA may interfere with and disrupt the immune system via a variety of cytokine signals (Segura *et al.,* 1999; Yamashita *et al.,* 2005). Therefore, dysregulation of cytokine signaling can cause a variety of diseases, including allergy, autoimmune diseases, inflammation, and cancer (Tamiya *et al.,* 2011).

The liver is the major organ for the metabolism and detoxification of xenobiotics, including BPA (Knaak and Sullivan, 1966). Therefore, the liver could be largely exposed to BPA, and could be susceptible to lower doses, than other organs (Moon *et al.,* 2012). There are some reports revealed that high doses of BPA altered liver weight in mice or rats (Tyl *et al.,* 2002; Tyl *et al.,* 2008) and decreased the viability of rat hepatocytes (Nakagawa and Tayama, 2000). Kupffer cells (KCs), the hepatic macrophages reside in the lumen of the liver sinusoids, on activation, KCs release various cytokines and play important role in the pathogenesis of various liver diseases (Wu *et al.,* 2010). KCs have been implicated as the source of the inflammatory response, because they are known to produce proinflammatory cytokines, such as interleukin (IL)-1beta and IL-6 when activated (Kopf *et al.,* 2010). Increased levels of proin-flammatory cytokines disturb the homeostasis of oxidants/anti-oxidants and DNA repair enzymes, all of which appear to be involved in BPA-associated inflammatory processes (Yongvanit *et al.,* 2012). The production of these mediators leads to a second phase of liver injury, including endothelial cell adhesion molecules that mediate the adhesion and transmigration of neutrophils from the vascular space into the hepatic parenchyma (Colletti *et al.,* 1996). These accumulation release oxidants and proteases that directly injure hepatocytes and vascular endothelial (Jaeschke and Smith, 1997). Furthermore, oxidative stress can induce many kinds of negative effects including membrane peroxidation, protein cleavage,

and DNA strand breakages, which could lead to cancer (Collins and Harrington, 2002; Mittler, 2002).

The aim of the present study to evaluate the hepatotoxic effects of BPA administrated in drinking water to male mice in a dose dependent manner using different assays. Parameters of oxidative status (catalytic activity of catalase and malondialdehyde, MDA) were evaluated in liver homogenates. Moreover, the extent of inflammation was determined by assessment of proinflammatory cytokine gene expression for both interleukin-1β (IL-1β) and interleukin-6 (IL-6) in liver. Finally histological examination of liver sections from all studied groups was performed.

2. Methodology

1. Chemical reagents:

Bisphenol A: CAS registry no. 80-05-7, Agarose, absolute ethanol 99.5%, 37% formaldehyde, glacial acetic acid, Tris-(hydroxymethyl)-amino methane (Tris–base), ethylenediaminetetracetic acid disodium salt (Na2EDTA), Triton X-100, ethidium bromide (EtBr) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were purchased from local standard companies and were of reagent grade or better.

2. Experimental animals:

Swiss Albino male mice at three weeks of age were used. The animals were bred in the animal facility at the Department of Zoology, Faculty of Science, Cairo University, Egypt. The experiments performed herein were in accordance with research protocols established by the animal care committee of the National Research Center, Egypt. The mice were divided randomly into experimental groups. They were housed in an air-conditioned room at a temperature of 26°C and 50–70% humidity with a 12/12 h-light/dark cycle throughout the experiment. The doses were selected based on the LD50 value of BSA (LD50 oral mouse 2400 mg/kg body weight) (MSDS, 2004).

3. Experimental protocol:

The mice were divided into seven groups (I–VII) each consisting of five animals. BPA was finally dissolved in 1% ethanol and delivered to animals in drinking water according to the dosing schedule of each group. Fresh solutions were prepared every 2 days. Thirty five male mice were randomly divided into seven groups. Group (I) served as negative control group received tap water. The vehicle control group (II) treated with water containing only the concentration of ethanol (1%) used as a diluent for the BPA solutions. Groups (III-VII) received BPA solutions at concentrations calculated to deliver approximately daily intake of (1/10, 1/20, 1//30, 1/40 and 1/50 the LD50), respectively. The experimental period for all groups was 21 days.

At the end of the experiment, mice were killed by cervical dislocation. Liver were removed, cleared of adhering tissues and washed in ice-cold saline solution. Small pieces of the tissue were preserved for histological studies in appropriate fixatives and the rest of the portions finally stored at -80 °C for further biochemical analysis and RNA extraction.

4. Histopathological investigations for hepatic tissues:

Autopsy samples were taken from the liver of mice in different groups and fixed in 10% formol saline for twenty four hour. Washing was done in tap water then serial dilutions of ethyl alcohol were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 degree in hot air oven for twenty four hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns by slidge microtome. The obtained tissue sections were collected on glass slides, deparaffinized and stained by hematoxylin and eosin stains for histopathological examination (Banchroft *et al.,* 1996).

5. Measurement of a biomarker of oxidative stress:

Lipid peroxidation level—The weighed amount of the hepatic tissue was homogenized (10%) in ice-cold 0.9% saline (pH 7.0) with a Potter Elvejhem all glass homogenizer (Belco Glass Inc., Vineland, NJ, USA) for 30 sec and the levels of the lipid peroxidation products in the homogenate was determined as Thio-Barbituric Acid Reactive Substances (TBARS) according to the method as employed by (Chattopadhyay *et al.,* 2003). In brief, the homogenates were mixed with trichloro acetic acid-thiobarbituric acid-hydrochloric acid (TBA-TCA-HCl) reagent and mixed thoroughly and heated for 20 min at 80°C. The tubes containing the samples were then cooled to room temperature. The absorbance of the pink chromogen present in the clear supernatant obtained after centrifugation at 1200 × g for 10 mins at room temperature was measured at 532 nm using a UV-VIS spectrophotometer (SmartSpec Plus, BioRad, Hercules, CA, USA). Tetraethoxypropane (TEP) was used as standard. The values were expressed as nmoles of TBARS per g wet tissues.

6. Assay of an antioxidant enzyme:

Catalase—Catalase was assayed by measuring the breakdown of hydrogen peroxide (H_2O_2) according to the method of (Mukherjee *et al.,* 2010) with some modification. The weighed amounts of the hepatic tissue were homogenized in 5% ice-cold 50 mM phosphate buffer pH 7.2. The homogenates were then centrifuged at 12000 × g for 12 min. The supernatant thus obtained was then carefully collected and incubated with 0.01 ml of absolute ethanol at 4°C for

30 min. Thereafter, 10% Triton X-100 was added to have a final concentration of 1%. The sample, thus obtained, was used to determine the catalase activity by measuring the breakdown of H_2O_2 spectrophotometrically at 240 nm. The enzyme activity was expressed as units per g wet tissues.

7. mRNA expression of IL-1β, IL-6, and GAPDH genes by reverse transcriptase polymerase chain reaction (RT-PCR) analysis:

Cytokine transcript levels of IL-1β and IL-6 in the liver tissues of experimental mice were measured using a reverse transcriptase (RT)-PCR technique. Total RNA was extracted from the liver using the TRIsure™ Sample Preparation Reagent (BIO-38032, BIOLINE) and according to the manufacturer's instructions. RNA concentrations and purity were determined by measuring the absorbance A260/A280 ratios. RT-PCR was performed according to the recommended protocol using the One Step RNA PCR Kit (AMV) (RR024A, TAKARA) and primers sequences shown in Table 1. The amplification profile consisted of an initial denaturation at 94 °C for 5 min followed by denaturation at 94 °C, annealing from 55°C to 60 °C and extension at 72 °C for 30 s. Expression of the housekeeping gene GAPDH served as the control. The number of amplification cycles was determined using individual primer sets to maintain exponential product amplification (28–36 cycles). Amplicons were separated by electrophoresis in 2% agarose gel, visualized by staining with ethidium bromide (0.5 µg/ml) and the bands intensities on the gels were calculated by image J (National Institute of Health, MD, USA). All signals were normalized to mRNA levels of the house keeping gene, GAPDH, and expressed as a ratio.

Table 1: primers sequences and product size for the three genes.

Factors	Primer sequences (F)	Primer sequences (R)	Size of product (bp)
IL-1β	GCC CAT CCT CTG TGA CTC AT	AGG CCA CAG GTA TTT TGT CG	230
IL-6	AGT TGC CTT CTT GGG ACT GA	TCC ACG ATT TCC CAG AGA AC	159
GAPDH	GTG GAT GGC CCC TCT GGA AA	GGC CTC TCT TGC TCA GTG TC	495

8. Statistical analysis:

All results were expressed as mean ± standard error (SE). The results of the toxicological evaluation and measurements of oxidative status parameters and expression levels of inflammatory cytokines were compared between the treatment groups and the control group using one-way ANOVA and Dunnett's test (at significance level $p < 0.05$) in GraphPad Prism 5 (GraphPad Software, USA).

3. Results

1. Histopathological analysis:

Light microscopic study of liver of group one of mice kept as control showed no histopathological alteration. (Fig.1) shows normal liver architecture with the central vein and radiating cords of normal hepatocytes possessing central rounded nuclei. Liver sections of mice administrated alcohol (vehicle group) show congestion in the central and portal veins, some cell infiltration in the portal area with normal liver architecture which appeared nearly similar to the control group (Fig.2). light microscopic examination of group administrated 1/50 LD50 revealed light abnormal pathological change compared with the control, dilatation and congestion of the central vein, portal vein and hepatic sinusoids with diffuse kupffer cells proliferation in between the hepatocytes (Fig.3).

In the group of mice administrated 1/40 LD50 the trabecular structure of the liver is blurred ,the cytoplasm is light, foamy and filled with vacuoles, dilatation of sinusoids and kupffer cells proliferation in between the degenerated hepatocytes (Figs. 4&5). Microscopic examination of group administrated 1/30 LD50 revealed light histopathological changes, the central and the portal vein were dilated and congested associated with the diffusion of kupffer cells in dilated sinusoids between the hepatocytes (Fig.6). Group of mice administrated 1/20 LD50 show mononuclear cellular infiltration, some enlarged nuclei while others were shrinkage with diffuse proliferation of the kupffer cells between the degenerated hepatocytes (Fig.7).

The significant histopathological changes were observed in the group of mice administrated 1/10 LD50 showing dilatation and focal congestion in the portal vein at the portal area (Fig.8) with mononuclear cellular infiltrate in the portal tract, few hepatocytes appeared more or less preserved but most hepatocytes appeared degenerated or exhibit vacuolated cytoplasm (Figs.9 &10) . Cell infiltration was observed in focal manner surrounding the dilated bile duct (Fig.11) some hepatocytes around central vein appeared necrotic (Fig.12).

2. Effect of BPA on hepatic oxidative status parameters:

2.1. Lipid peroxidation:

The effect of BPA in lipid peroxidation is presented in Fig. 13. Oral BPA administration for 21 days, in all selected doses (48, 60, 80, 120, 240 mg/kg) corresponding to (1/10, 1/20, 1//30, 1/40 and 1/50 the LD50), respectively, increased TBARS levels (a lipid peroxidation marker) in the liver (P

<0.05) as compared to control groups. The sham or vehicle group showed no significant variations in these parameters as compared to the control group.

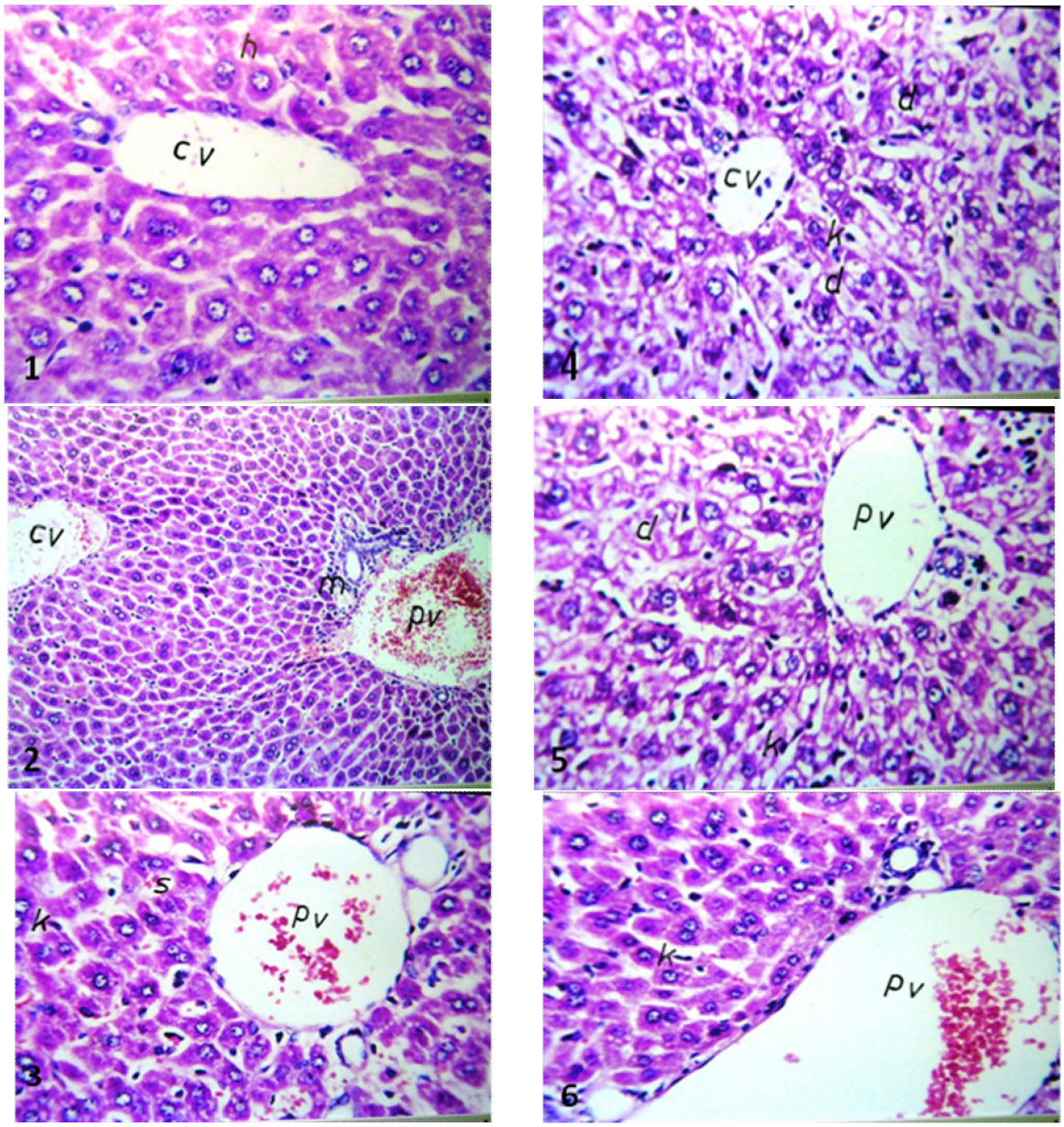


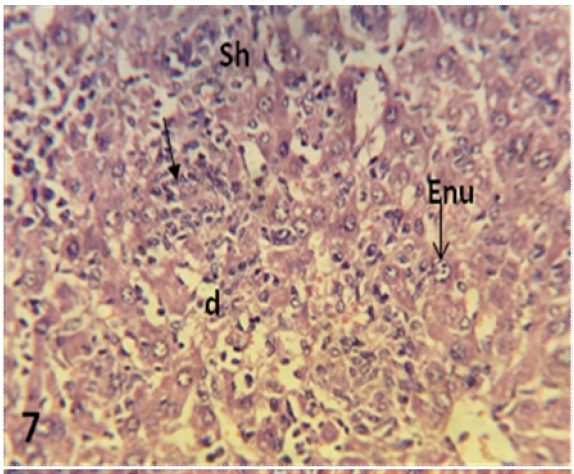
Fig. 1: Photomicrograph of a liver section of mice showing normal histological structure of central vein (cv) and surrounding hepatocytes (h). **H & E. X800**.

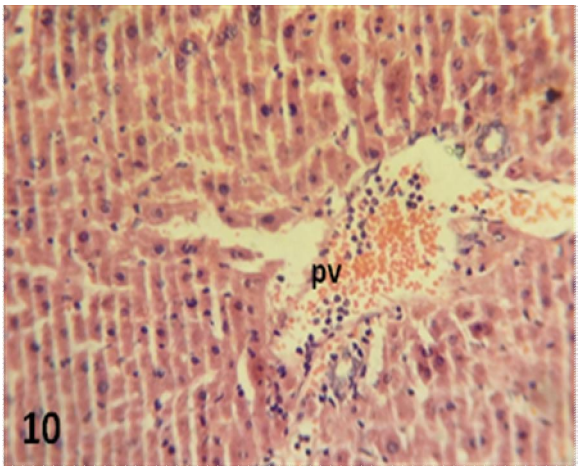
Fig. 2: Photomicrograph of a liver section of mice showing congestion in the portal (pv) and the central (cv) veins with inflammatory cells infiltration (m) in portal area and in between the hepatocytes (arrow). **H & E. X400.**

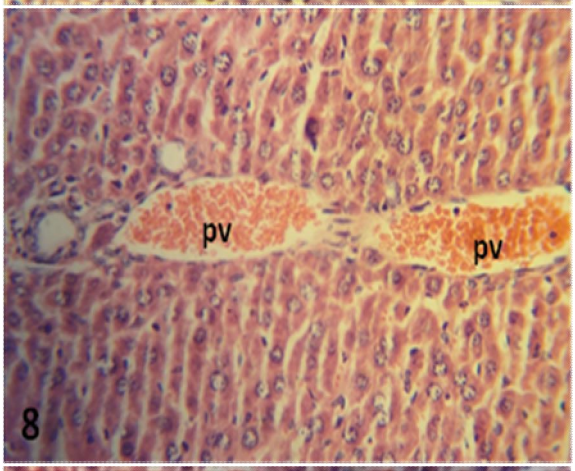
Fig. 3: Photomicrograph of a liver section of mice showing dilatation and congestion of the portal vein (pv) and hepatic sinusoids(s) with diffusion of kupffer cells (k) proliferation in between the hepatocytes .**H & E. X800**.

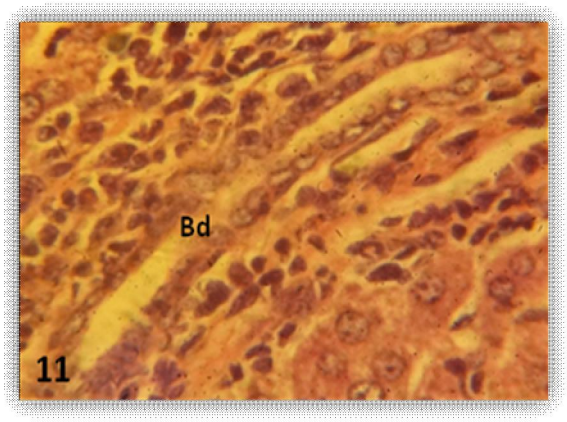
Figs. 4 &5: Photomicrographs of liver sections of mice showing dilatation of hepatic sinusoids and portal vein (pv), the cytoplasm is light, foamy and filled with vacuoles, ,kupffer cells(k) proliferation in between the degenerated hepatocytes. **H & E. X800**.

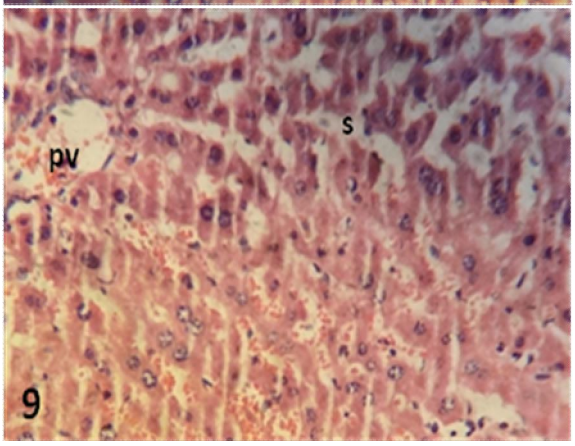
Fig. 6: Photomicrograph of a liver section of mice showing light histopathological changes the central and the portal vein (pv) were dilated and congested associated with the diffusion of kupffer cells (k) in dilated sinusoids between the hepatocytes. **H & E. X800.**











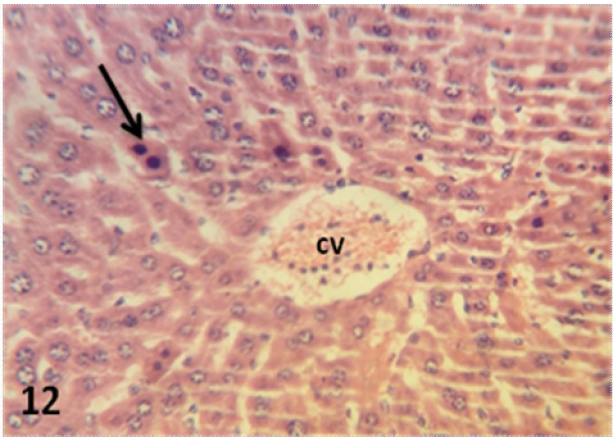


Fig. 7: Photomicrograph of a liver section of mice showing cellular infiltration (arrow) some enlarged nuclei (Enu) while others were shrinkage (Sh) with diffuse proliferation of the kupffer cells between the degenerated hepatocytes (d). H & E. X400.

Fig. 8: Photomicrograph of a liver section of mice showing dilatation and focal congestion in the portal vein (pv) at the portal area. H & E. X400.

Fig. 9: Photomicrograph of a liver section of mice showing cellular infiltration in the portal tract, with dilated sinusoids (s), few hepatocytes appeared more or less preserved but most hepatocytes appeared degenerated. H & E. X400.

Fig. 10: Photomicrograph of a liver section of mice showing congestion in the portal vein (pv), most hepatocytes appeared degenerated (the architecture of the liver is blurred). H & E. X400.

Fig. 11: Photomicrograph of a liver section of mice showing the cell infiltration appeared in a focal manner surrounding the dilated bile duct (Bd). H & E. X1000.

Fig. 12: Photomicrograph of a liver section of mice showing congestion in the central vein (cv), some hepatocytes appeared necrotic (arrow). H & E. X400.

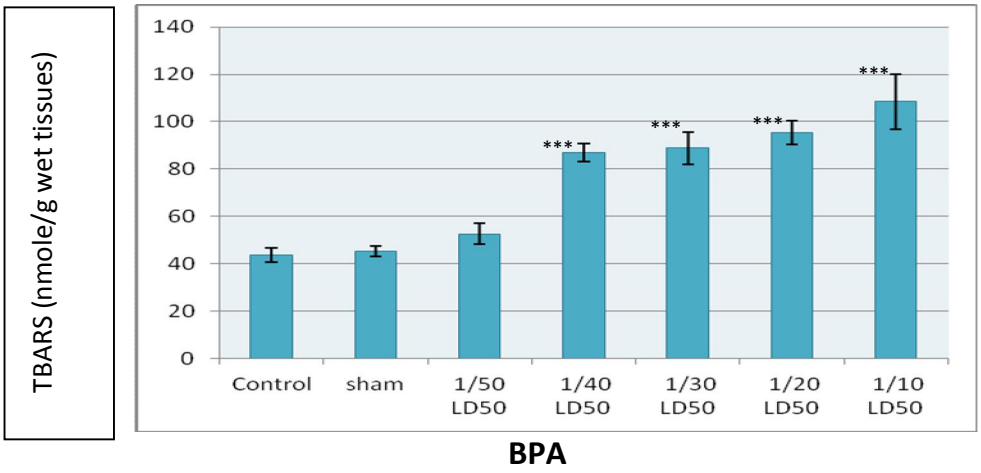


Fig. 13: Levels of TBARS (lipid peroxidation level) in liver of adult mice treated with vehicle or BPA (1/10, 1/20, 1//30, 1/40 and 1/50 the LD50) for 21 days. The means ± SE for three animals from each group are shown. *** P < 0.05 when compared to controls (ANOVA and Dunnett's test).

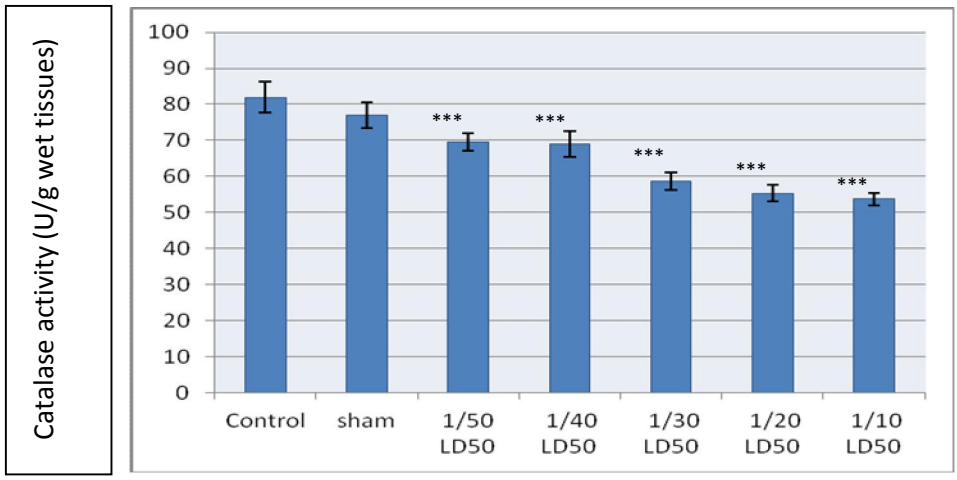


Fig. 14: Levels of catalase activity in liver of adult mice treated with vehicle or BPA (1/10, 1/20, 1//30, 1/40 and 1/50 the LD50) for 21 days. The means ± SE for three animals from each group are shown. *** P< 0.05 when compared to controls (ANOVA and Dunnett's test).

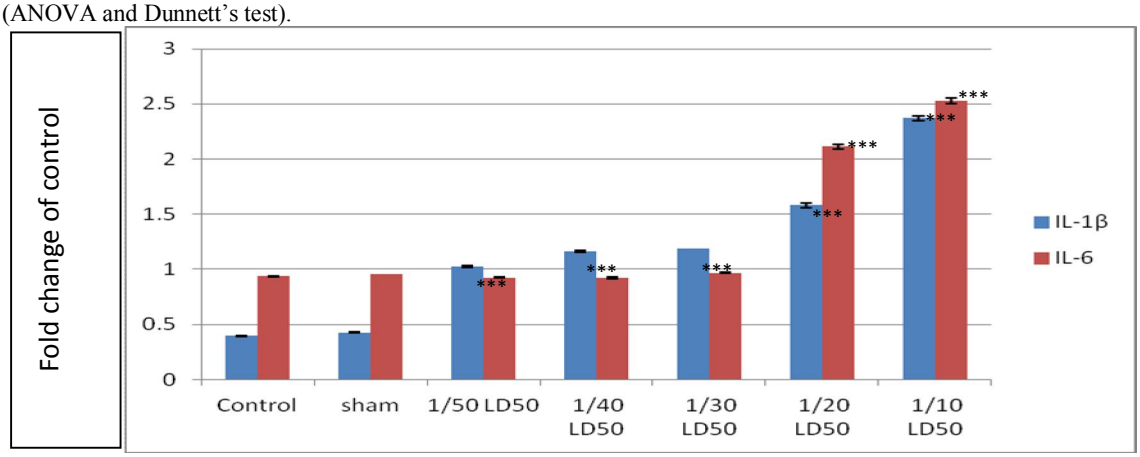


Fig. 15: Expression levels of both IL-1β and IL-6 cytokines in liver of adult mice treated with vehicle or BPA (1/10, 1/20, 1//30, 1/40 and 1/50 the LD50) for 21 days. The means ± SE for three animals from each group are shown. *** P< 0.05 when compared to controls (ANOVA and Dunnett's test).

MDA concentrations representing oxidative stress also increased compared to the controls, even in the mice treated with the lower dose of BPA, although this apparent difference was not statistically significant (Fig. 13).

2.2. Catalase Activity

Catalase antioxidant enzyme activity levels in the liver of control and tested groups are shown in Fig. 14. In BPA groups, a significant decrease in catalase activity was observed in liver, as compared to control group. The sham or vehicle group showed no significant variations in these parameters as compared to the control group.

3. Effect of BPA on the expression of inflammatory cytokines in the liver

Because inflammatory cytokines can induce oxidative stress, we measured the hepatic expression of IL-1β and IL-6. Expression levels of both cytokines in the liver of control and tested groups are shown in Fig. 15. IL-1β expression increased significantly in all selected doses when compared with control (P<0.05). The expression of IL-6 was increased significantly only at higher doses (1/20 and 1/10 LD50) while remain unchanged at low doses (1/50, 1/40 and 1/30 LD50) (P<0.05).

4.Discussion

The present study demonstrated that BPA exposure for male swiss albino mice, increased hepatic oxidative stress and proinflammatory cytokines, and decreased the antioxidant catalase enzyme activity. Oral administration of BPA induces inflammatory cellular infiltration and increased number of KCs and other histopathological changes in the mouse liver. All these findings suggest that there is a dose dependent manner of the hepatotoxic effects of BPA administrated in drinking water for male mice.

Recently, the oxidative stress was proposed as another adverse cellular effect of BPA in the liver (Bindhumol *et al.,* 2003). BPA increased the generation of reactive oxygen species (ROS) and induced cellular apoptosis in hepatocytes (Asahi *et al.,* 2010). (Moon *et al.,* 2012) indicated that BPA can induce hepatic damage and mitochondrial dysfunction by increasing oxidative stress in the liver. In this study, BPA increased ROS production in a dose dependent manner, as assessed by the measurement of MDA concentration which is in accordance with the finding of a previous study that found MDA levels in the tissues increases after BPA administration (Kabuto *et al.,* 2003). At low dose of BPA there was no significant increase in MDA concentration.

The cell has various defense mechanisms against oxidative stress, including scavenging enzyme systems such as catalase. Our finding of the decreased activity of CAT for all selected doses of BPA corroborates with that of earlier findings by (Chitra *et al.,* 2003) and (Sangai *et al.,* 2012). This indicates that H_2O_2 was most probably present in high levels, since CAT is involved predominantly in the detoxification of high H_2O_2 levels (Hermes-Lima, 2004). Reduction in the activity of catalase may reflect inability of liver mitochondria and microsomes to eliminate hydrogen peroxide produced after exposure to bisphenol A (Bindhumol *et al.,* 2003).

The results demonstrate that exposure to BPA severely disrupts the liver of male mice which are caused by mechanisms that include oxidative stress and inflammation. Inflammation is a complex process mediated by proinflammatory cytokines and free radicals produced by macrophages. Several studies have reported that macrophages in liver generate ROS such as superoxide anion and hydrogen peroxide on exposure to hepato-toxicants (Pilaro and Laskin, 1986; McCloskey *et al.,* 1992). Although ROS can increase proinflammatory cytokines (Dong *et al.,* 1998), proinflammatory cytokines themselves can induce oxidative stress (Babbar and Casero, 2006). Furthermore, cytokines are involved in the physiology and physiopathology of the liver, and the proinflammatory cytokines such as (IL-1beta and IL-6) are produced in several types of liver injury (Zimmermann *et al.,* 2012). The release of IL-1β and IL-6 can active macrophages during infection, injury, and inflammation (Kim *et al.,* 2007; Li *et al.,* 2012). Examination of these interleukins in the present study shows that IL-6 expression was elevated only at high doses of BPA (240 and 120 mg/kg/day) compared to the control, while IL-1β expression levels were increased significantly at all selected range of doses. Even though the reason for this will be required further study, IL-1β expression can be probably established through another signal pathway of TNF-α and IL-6.

Our data is in consistent with the findings of previous studies revealed that both IL-1β and IL-6 have also been implicated in the mechanism of cadmium hepatotoxicity (Kayama *et al.,* 1995; Marth *et al.,* 2000; Yamano *et al.,* 2000). Moreover it was suggested that IL-6 might play a pathogenic role in BPA-induced ROS generation and mitochondrial dysfunction (Moon *et al.,* 2012). In addition, IL-1beta triggers the production of other cytokines and can also recruit inflammatory cells (Tilg and Diehl, 2000). Furthermore, IL-6 is a mediator of the acute-phase response and is also essential for liver repair (Streetz *et al.,* 2000). Thus, it is not surprising that gene

expression of these cytokines is elevated during the inflammatory response.

Additionally, we observed histopathological changes in liver indicating variable damage after BPA administration. Our microscopic examination revealed that liver could be susceptible to low doses this result was reported by several authors, (Moon *et al.,* 2012; Mourad and Khadrawy, 2012). In present study; it has been observed that BPA showed degenerative changes in hepatic cells this also was reported by (Boshra and Moustafa, 2011; Roy *et al.,* 2011). A study by (Verma and Sangai, 2009) showed that treatment with bisphenol A leads to cell rupture and membrane damage of human erythrocytes which may be due to the oxidative stress. Moreover, light microscopic examination revealed signs of inflammatory cellular infiltration, vacuolated hepatocytes, dilated sinusoids, and congested blood vessels, increased in number of Kupffer cells and necrosis. It has been reported by previous findings that BSA causes cell infiltration and necrosis (Boshra and Moustafa, 2011; Mourad and Khadrawy, 2012), vacuolated hepatocytes (Roy *et al.,* 2011), liver damage (Hassan *et al.,* 2012).

The degree of cellular infiltration and number of KCs is gradually increased with elevated levels of administrated BPA. It was evidenced that hepatic macrophages KCs are essential players in the propagation of acute liver damage. These cells attracted much attention lately in the context of chronic liver inflammation due to their dual pro- and antifibrotic qualities (Zimmermann and Tacke, 2011). It has been proposed that KCs are involved in the indirect component of the mechanism of Cd toxicity (Hoffmann *et al.,* 1975;Sauer *et al.,* 1997;Yamano *et al.,* 1998;Yamano *et al.,* 2000). Experimental murine models of liver injury highlighted the importance of hepatic macrophages, for initiating and driving this inflammatory response by releasing proinflammatory cytokines including IL-1β and IL-6 (Wang *et al.,* 2003).

In conclusion, oral administration of BPA for 21 days in mice causes oxidative stress; it increases MDA levels and decreases CAT activity in liver of mice. Furthermore, it induces elevation of hepatic proinflammatory cytokines. In addition, it alters the normal histopathology of liver.

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1/2/2013