Keywords: Bisphenol A; Liver; Oxidative stress; Hepatoprotective; Silymarin; Curcumin.

ABSTRACT

Background: Bisphenol A [BPA] is widely used in industrial processes. It persists in the environment and exerts a harmful action on human health, through endocrine-disruption. Many natural compounds are suggested to have a preventive effect on BPA-induced human health hazards.

Aim of the work: This study aimed to evaluate the effect of Silymarin/Curcumin in ameliorating the hepatotoxic effect induced in albino rats following oral administration of BPA.

Patients and Methods: Forty adult male albino rats were included and randomly categorized into four equal groups. The control group and BPA-exposed [50 mg/kg] group. Groups 3 and 4 for rats received oral BPA [50 mg/kg] plus [100 mg/kg] of curcumin or silymarin. Serum liver enzymes, pro-inflammatory cytokines [TNF-α & IL-1,6,8] and the oxidant/antioxidant profile were assessed in the serum. SOD, GPX and CAT levels, MDA and H2O2 were assessed. Histopathological changes of the liver, immunohistochemical detection of caspase 3 and area density of collagen fibres were also assessed.

Results: Bisphenol-A administration for 30 days was associated with a significant increase in the number of pro-inflammatory markers as interleukin 1,6,8 and tumor necrosis factor-α [TNF-α]. It also, stimulates oxidative stress with structural changes, apoptosis and fibrosis in hepatic cells. Treatment with silymarin or curcumin for 30 days lead to a reduction of pro-inflammatory mediators and significant amelioration of structural changes.

Conclusion: Silymarin/Curcumin had an ameliorative of harmful effects of BPA on the liver cells through potent anti-inflammatory, antiapoptotic and antioxidant effects.
INTRODUCTION

Canned foods are widely consumed, as they contain high-quality food stuffs. However, the direct contact with packaging materials results in the contamination by some undesirable substances, such as bisphenol A [{BPA}]. Bisphenol-A is an endocrine-disrupting compound. It is broadly used in the production of many industrial compounds. The annual consumption of BPA was approximately 8 million tons worldwide, and around 100 tons may be released within one year into the environment. Several studies have revealed that BPA is widely distributed in foods, environment, and even human body fluids. Thus, different tissues and organs are affected by the toxic effects of BPA. These included liver, respiratory system, kidney, mucous membranes, immune system and the skin.

The liver is more vulnerable than other organs and tissues to BPA-induced toxic effects. BPA metabolism is achieved by the CYP2C liver cytochrome family, through two main metabolic pathways. The first is the elimination by conjunction with a glucuronide and/or a sulfate. The second by hydroxylation to a catechol and then alteration to an o-quinone. These is associated with oxidative stress due to interaction redox cycle and high production of reactive oxygen species [ROS]. In addition, exposure to low or high doses of BPA increased oxidative stress through increased malondialdehyde [MDA] and decreasing reduced glutathione [GSH] production in the liver, which was confirmed by a previous study.

Different herbal products were used to alleviate the toxic hepatic effects induced by BPA. The wide spectrum of used herbs is related to the nature of used compounds, its safety, efficacy and cultural acceptance. However, many natural compounds gained wide acceptance for treatment of BPA-induced harmful effects on the liver. For example, curcumin [CUR] has potential therapeutic actions on different toxic effects, exerted on different body systems. In addition, silymarin is a well-known hepatoprotective compound enriched with polyphenols and flavonoids [e.g., silybin, silychristin, silybinin, and silydianin], with antioxidant therapeutic effects.

The potential hepatoprotective effects of CUR or silymarin against hepatoxic effects induced by BPA was not widely discussed on the molecular and structural levels. Thus, the current study was designed and aiming to assess the potential protective effects of curcumin or silymarin on the BPA-induced hepatotoxicity in albino rats by histopathological, molecular and biochemical studies.

MATERIALS AND METHODS

Chemicals: BPA [99% pure], was acquired from Sigma-Aldrich [St Louis, Missouri, USA]. Curcumin [CUR] was obtained in the powder from Sigma Chemical Company, St Louis, Missouri, USA, CAT No. 458-37-7. Then, it was dissolved in corn oil. Silymarin [SL] was provided by SEDICO Company, Egypt CAT No. 65666-07-1, prepared in sachets. Then, dissolved in distilled water. All were administered through oral route by intragastric tube.

Animals: Forty adult male albino rats, weighing 120-160 g, were included. Housing was achieved in cages made of polypropylene, with 12 hours of light and dark cycles, at a temperature of 22±2°C and 10% humidity. The free access to water and standard diet pellets was permitted ad libitum.

Ethical approval: The study protocol was reviewed and accepted by the institutional research and ethics committee [Damietta Faculty of Medicine, Al-Azhar University, Egypt] [IRB 0001267-21-07-005]. The study also adheres to Declaration of Helsinki code of ethical retrace conduct and reporting for experimental studies. The data is available on reasonable request.

Experimental design and drug administration: Forty adult male albino rats were left in the experimental room for 1 week prior to the study for acclimatization, then, animals were divided into four equal groups [10 rats in each group, 5 rats in a cage]. Group I [control group] received the standard rat chow diet and given normal saline [1 ml/kg body weight [BW]/day] by gastric tube for 30 days. Group 2 [BPA group], rats received BPA dissolved in corn oil and given orally in a daily dose of 50 mg/kg; Groups 3 and 4 for rats received oral, freshly-prepared BPA [50 mg/kg] plus [100 mg/kg] of silymarin or curcumin respectively for 30 days.

Blood and tissue sampling: At the 30th day of the experiment, a retro-orbital plexus was used to obtain blood samples. Samples were centrifuged at 1200g for 20 minutes, serum was separated, collected in Eppendorf tubes and kept at -20°C till the time of analysis.

Serum biochemical analyses: Serum concentrations of liver enzymes were examined by an automated hematology analyzer.

Determination of ROS: A piece of liver tissue (0.3 g) was homogenized in ice phosphate buffer saline using a Teflon pestle connected to a homogenizer motor (25 strokes per minute at 1000 rpm), the liver homogenate was diluted to yield 10% (w/v) then centrifuged at 13000 rpm for 30 min at 4 °C to remove cell debris and nuclei. The supernatant was used for biochemical determination of renal malondialdehyde (MDA) and H2O2.

Determination of antioxidants: Activity levels of the extra-cellular values of superoxide dismutase [SOD], glutathione peroxidase [GPx], and catalase [CAT] were measured by enzyme linked immunosorbet assays according to manufacturer’s instructions [Shanghai Enzyme-linked Biotechnology Co. Ltd., Shanghai, China].

Determination of inflammatory markers: The levels of Interleukin [IL]-1β, IL-6, IL-8, and tumor necrosis factor-α [TNF-α] contents were determined using enzyme-linked immunosorbet assay kits [Shanghai Enzyme-linked Biotechnology Co. Ltd., Shanghai, China].
Assessment of histological and immunohistochemical changes: The liver specimens were fixed in 10% formalin for at least 24 hours. Then, processed and sectioned at a thickness of 5 μm, and stained by hematoxylin and cosin [Hx&E] for the detection of histopathological [structural] changes. Masson trichrome for detection of fibrosis and immunostain for caspase-3 for detection of apoptosis. The slides were examined by light microscopy, photographed & the percentage area density of collagen fibres and caspase-3 were measured by a Raywild E5 microscope with a Raywild M-300 digital camera equipped with an image-analyzing system [Mvi-mage program v12].

Statistical analysis: The arithmetic means and standard deviation [SD] were used to represent numerical data. Groups were compared by one way analysis of variance [ANOVA] test with least significant differences [LSD] as a post-hoc analysis for multiple comparison. The statistical package for social science [SPSS] version 16 [SPSS Inc., Chicago, IL, USA], running on Microsoft windows personal computer, was used for all analysis. Statistical significance was set at P < 0.05.

RESULTS

Liver enzymes, antioxidants and ROS: Bisphenol-A induced toxicity produced a significant increase of liver enzymes in the study than the control group, but, the concomitant administration of BPA with silymarin or curcumin produced a significant reduction of liver enzymes than BPA-exposed group, however, the liver enzymes remain significantly high when compared to the control group [Table 1].

Bisphenol-A group showed a significant reduction of SOD, GPX and CAT than the control group, confirming the role of redox system in pathogenesis of BPA-induced toxicity. The concomitant administration of BPA with silymarin or curcumin showed a significant increase of SOD, GPX and CAT levels, when compared to the BPA-exposed group. however, values remain significantly higher than the control group. Otherwise, bisphenol-A induced toxicity group showed a significant increase of MDA and hydrogen peroxide than the control group. however, curcumin or silymarin concomitantly administered with BPA was associated with a significant decrease of these levels, when compared to BPA-exposed group. however, it was significantly increased than the control group [Table 2].

Pro-inflammatory and apoptotic cytokines: Values of IL-1β, IL-6, IL-8 and TNF-α in the BPA group were markedly higher than the control group. these cytokines were significantly reduced in the groups treated with silymarin or curcumin than the BPA only group [Table 3].

The morphometric measurement of the liver sections revealed a significant increase of the collagen content and the hepatic pro-apoptotic protein caspase-3 levels in the BPA-intoxicated group than the control group. These levels were significantly decreased in groups treated with silymarin or curcumin than the BPA only group [Table 4].

Table 1: Assay of serum liver enzymes levels in the different studied groups

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>BPA</th>
<th>BPA +SIL</th>
<th>BPA +CUR</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT [U/L]</td>
<td>30.8±4.72</td>
<td>82.63 ± 4.24*</td>
<td>67.71 ± 3.27#</td>
<td>60.44± 21#</td>
</tr>
<tr>
<td>AST [U/L]</td>
<td>52.26±6.34</td>
<td>95.64±7.26*</td>
<td>68±7.62*</td>
<td>65.32±5.18#</td>
</tr>
<tr>
<td>ALP [mg/dl]</td>
<td>350.64±40.67</td>
<td>615.74±54.43*</td>
<td>432±47.25#</td>
<td>419±36.41*</td>
</tr>
</tbody>
</table>

Table 2: Assay of oxidative/antioxidative stress parameters different groups

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>BPA</th>
<th>BPA +SIL</th>
<th>BPA +CUR</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD [ng/ml]</td>
<td>7.06±0.37</td>
<td>3.31±0.43*</td>
<td>5.13±0.42*</td>
<td>4.74±0.61*</td>
</tr>
<tr>
<td>GPX [ng/ml]</td>
<td>6.82±0.42</td>
<td>2.81±0.27*</td>
<td>3.84±0.24*</td>
<td>4.06±0.31*</td>
</tr>
<tr>
<td>CAT [ng/ml]</td>
<td>154.64±8.3</td>
<td>82.36±4.91*</td>
<td>98.34±14.36*</td>
<td>127.98±5.1*</td>
</tr>
<tr>
<td>MDA [ng/ml wet tissue]</td>
<td>76.06±3.49</td>
<td>298.3±11.24*</td>
<td>114.61±11.24*</td>
<td>108.57±11.24*</td>
</tr>
<tr>
<td>H2O2 [ng/ml wet tissue]</td>
<td>1.42±0.35</td>
<td>4.26±0.74*</td>
<td>2.78±0.34*</td>
<td>2.38±0.18*</td>
</tr>
</tbody>
</table>

Table 3: Assay of serum inflammatory markers levels in the different studied groups

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>BPA</th>
<th>BPA +SIL</th>
<th>BPA +CUR</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β [pg/ml]</td>
<td>96.06±15.27</td>
<td>217.31±21.83*</td>
<td>145.57±17.68*</td>
<td>138.31±13.81*</td>
</tr>
<tr>
<td>IL-6 [pg/ml]</td>
<td>1.51±0.38</td>
<td>2.74±0.51*</td>
<td>1.81±0.41*</td>
<td>1.76±0.22*</td>
</tr>
<tr>
<td>IL-8 [pg/ml]</td>
<td>104.64±9.3</td>
<td>52.36±7.91*</td>
<td>78.34±12.46*</td>
<td>72.9±9.53*</td>
</tr>
<tr>
<td>TNF-α [ng/l]</td>
<td>112.14±1.74</td>
<td>236.57±9.57*</td>
<td>145.39±7.41*</td>
<td>139.72±10.44*</td>
</tr>
</tbody>
</table>

Table 4: Assessment of Liver fibrosis and apoptosis markers in the different studied groups

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>BPA</th>
<th>BPA +SIL</th>
<th>BPA +CUR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen [μm]</td>
<td>1.36±0.27</td>
<td>3.57±0.83*</td>
<td>2.57±0.42*</td>
<td>2.31±0.74*</td>
</tr>
<tr>
<td>Caspase-3 [μm]</td>
<td>0.82±0.32</td>
<td>2.81±0.27*</td>
<td>1.21±0.31*</td>
<td>1.16±0.54*</td>
</tr>
</tbody>
</table>

* Significant differences between the BPA and the control group; # significant differences between BPA plus SIL or CUR-treated groups than BPA group.
Histopathological results

Hematoxylin and Eosin-stained sections results: The hepatic sections from the control group revealed normal hepatic structure in the form of presence of central vein with radiating hepatocyte cords separated by the blood sinusoids. The hepatocytes had centrally located vesicular nucleus with acidophilic cytoplasm. The portal triad had normal appearance and contain a large venule, a hepatic arteriole and bile ductule. In the BPA-treated group, hepatocytes revealed different levels of destruction with vacuolated cytoplasm, deeply stained shrunken nuclei and dilated congested blood sinusoids with many Kupffer cells. The portal area had marked cellular infiltration, congestion of the bile duct and hepatic arteriole. The use of silymarin or curcumin showed restoration of normal shape of most hepatocytes. There were a less dilated congested sinusoids with fewer kuppfer cells. Nearly normal portal triad, portal venule, a hepatic arteriole and a bile ductule was observed, with low cellular infiltrations [Figures 1 & 2].

Masson trichrome stained sections: The collagen deposition in the liver showed as significant increase \( [p < 0.005] \) in BPA-induced hepatotoxic rats compared to the control, whereas it significantly decreased in BPA + Sily/CUR groups in comparison to the BPA group with no significant difference between them [Figure 3].

Immunohistochemical assessment of caspase-3: Liver sections of BPA-exposed group showed marked deposition of caspase 3 than the control group. The use of curcumin or silymarin reduced the deposition of caspase-3 when compared to BPA-exposed groups. However, the difference was statistically non-significant [Figure 4].

![Diagram](image-url)

Figure 1. (A): The liver sections of the control group, showed normal hepatic structure in the form of presence of central vein [C] with radiating cords of hepatocytes separated by blood sinusoids [thick arrow], the hepatocytes showed centrally located vesicular nucleus with acidophilic cytoplasm [thick arrow]. (B): the liver of the BPA exposed group showed; the hepatocytes appear destructed with vacuolated cytoplasm and deeply stained shrunken nuclei [thick arrow] and dilated congested blood sinusoids with many Kupffer cells [notched thick arrow]. (C, D): the liver sections of BPA + Sily/CUR groups respectively showed; Most of the hepatocytes restore their normal shape [thick arrow], less dilated and no or minimally congested blood sinusoids with less Kupffer cells [notched thick arrow]. A: control group; B: Bisphenol A group; C: Bisphenol A + Silymarin group; and D: Bisphenol A + Curcumin group. [Hx.&E. X400] Scale bars, 200 pixel.
Figure [2]: [A]: The liver sections of the control group, showed the portal triad [P] was of normal appearance containing a large portal venule, a hepatic arteriole and bile ductile. [B]: In the BPA exposed group; the hepatocytes appear destructed with vacuolated cytoplasm and deeply stained shrunken nuclei [thick arrow] and dilated congested blood sinusoids with many Kupffer cells [notched thick arrow], the portal area [P] showed marked cellular infiltration, congested bile duct and hepatic arteriole, [C,D] BPA + Sily/CUR groups respectively; Most of the hepatocytes [thick arrow] restore their normal shape, less dilated and congested blood sinusoids with less kupffer cells [thick notched arrow], almost normal portal triad [P]: portal venule, a hepatic arteriole and a bile ductule with few cellular infiltrations can be observed A: control group; B: Bisphenol A group; C: Bisphenol A + Silymarin group; and D: Bisphenol A + Curcumin group. [Hx.&E. X400] Scale bars, 200 pixel.

Figure [3]: Photomicrographs of M. Trichrome stained liver sections for the caspase-3 [A] control group showing weakest deposition of collagen fibers while the highest expression of collagen fibers in Bisphenol A group [B]; weak deposition of collagen fibers in treated groups with Bisphenol A + Silymarin/Curcumin group [C, D]. A: Control group; B: Bisphenol A group; C: Bisphenol A + Silymarin group; and D: Bisphenol A + Curcumin group; [M. Trichrome stain. X400] Scale bars, 100 µm.
DISCUSSION

Bisphenol A is a commonest environmental pollutant. It is commonly used in different industries [e.g., epoxy resins, food containers, and many plastic products]. This led to high BPA concentrations in several body fluids [e.g., blood, amniotic fluid, and breast milk]. In addition, it is deposited in different organs [10]. Bisphenol-A is usually metabolized by the liver. This exposes the liver to the toxic actions of BPA through induction of oxidative stress [11,12]. Preventions of such damage is crucial to keep the health of liver. The current experiment aimed to assess the preventive or ameliorative effects of silymarin or curcumin on the hepatotoxicity induced by BPA in albino rats. Results revealed that, bisphenol A toxicity was associated with a significant increase of liver enzymes than the control group. Previous research has reported elevated liver enzymes on exposure to BPA, which reflected a damage of liver tissues [13]. The cellular damage of the liver is associated with a reduction of the permeability of cell membranes. Thus, increasing the serum levels of transaminases. The high levels of ALP may be due to cholestasis and indicating hepatocellular damage [14].

In the current work, the cellular hepatic damage suggested by increased liver enzymes on exposure to BPA was confirmed by the microscopic examination of hepatic sections. It was reflected as hepatocyte destruction with marked vacuolated cytoplasm, deeply stained shrunken nuclei and dilated congested blood sinusoids with abundant Kupffer cells. The portal area had a marked cellular infiltration, congested bile duct and hepatic arteriole. This was in line with a previous study reported that BPA-induced hepatotoxicity and cardiotoxicity in rats [15]. Nakagawa and Tayama [16] also reported hepatocyte cytotoxicity, cell damage and lysis in BPA-exposed rats.

In the present study, the liver fibrosis was assessed by Masson trichrome stain for detection of collagen deposition in hepatocytes. The oral administration of BPA was associated with hepatic fibrosis in the form of increased collagen deposition in the liver tissue. These results come in accordance with Elswefy et al. [17], who reported liver fibrosis in BPA-treated rats. They also reported that BPA exerted this harmful action through increased production of the proinflammatory cytokine [IL-1b] and reduction of the hepatocyte antioxidants [GSH, CAT] and hepatocyte fibrotic markers.

In the current work, the associated inflammation in the liver exposed to BPA was detected by assessment of the inflammatory markers [TNF-α, IL-6, IL-1β, and IL-8]. These substances were significantly increased. A recent study reported that BPA exposure was associated with significantly increased levels of NF-κB, TNF-α, IL-6, IL-1β, and COX-2 [10]. Those pro-inflammatory cytokines are indicators of acute inflammatory response, liver fibrosis and other ROS-connected disorders [18].

In this study, caspase-3 was used to assess the liver apoptosis. Results revealed that, oral BPA administration stimulated an increase in caspase-3 immunoreactivity. A previous animal study examined the oral exposure of rats to BPA showed a significant increase in the caspase-3 levels [17]. The mechanisms of BPA-induced apoptosis...
may be explained by mitochondrial actions. These actions play a chief role in induction of apoptosis through liberating intermembrane space proteins, like cytochrome-c, a principal mediator of apoptosis by the cytoplasmic activation of caspases [19].

Oxidative stress had a significant role in apoptosis. Consistent with this assumption, BPA was associated with a significant increase of oxidative stress and stimulate hepatocyte cellular apoptosis. This was confirmed in the current work by a notable decrease in the activity of antioxidant enzymes [e.g. CAT, SOD, and GPX] and remarkable increase in MDA and H$_2$O$_2$ levels. This was in agreement with the results of recent researches which reported that BPA exposure decrease the activation of antioxidant enzymes [10, 20]. These antioxidants offer a cellular defense action against ROS-induced oxidative stress damage [21]. Also, the harmful effects of BPA might be related to increased production of reactive intermediate substances during BPA metabolism [13]. Moreover, several researches have reported that the exposure to BPA induces free radicals as [MDA and H$_2$O$_2$], which usually leads to disruption of antioxidant enzymes [CAT, SOD, and GPX] defense mechanism [2, 11].

Those antioxidant markers have a main role in the cellular defense mechanism against oxidative stress produced by ROS. This could be explained by the following: CAT catalyzes the hydrogen peroxide into oxygen and water. Thus, defends hepatocytes against oxidative injury of hydroxyl radicals [22]; SOD plays a protective role against damage of free radicals and eradicates them by decreasing their toxicity [23]; GPX is a selenoenzyme that contribute in minimizing levels of H$_2$O$_2$ and fatty acid hydroperoxides. Thus, protecting the mammalian cells against oxidative stress as well as other peroxides [24]. Lastly, MDA is an end product of polyunsaturated fatty acids peroxidation in the cellular membrane. The MDA high level is a chief indicator of lipid peroxidation. However, the level of MDA indirectly reveals the degree of cellular destruction and tissue damage [25]. Results of the current work confirmed by previous study indicating that, curcumin and silymarin are natural products, with high levels of antioxidants, and previous study indicating that, curcumin and silymarin are protective actions of curcumin and silymarin concomitant administration significantly alleviates the BPA-induced apoptosis. However, curcumin has a more anti-apoptotic effect than silymarin. These results are in line with a previous studies revealed a strong anti-apoptotic effect of curcumin by reducing the immune-expression of caspase-3 [16, 32].

**Conclusion:** BPA causes hepatotoxic effects through disturbance of the redox system and increased ROS production leading to a condition of oxidative stress. Oral administration of curcumin or silymarin ameliorates the harmful effects of BPA by anti-inflammatory, anti-apoptotic and antioxidant effects.

**Financial and Conflict of interest disclosure:**

None

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