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Research Article

# Biological Effects of Chitosan against Bisphenol- A Induced Endocrine Toxicity and Androgen Receptor Gene Expression Changes in Male Rats

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**ABSTRACT**

Bisphenol A (BPA) is an endocrine disruptor which can mimic estrogen and has been shown to cause negative health effects in animal studies. The aim of this work was to study the protective effect of chitosan against BPA. Forty male albino rats were randomly divided into four equal groups. Group I, animals served as control. Group II, animals were injected by bisphenol A, intra-peritoneal, at a dose of 5 mg/kg body weight daily for 3 weeks. Group III, animals were gavaged with chitosan at a dose of 30 mg/kg body weight for 3 weeks. Group IV, animals were injected by bisphenol A, intra-peritoneal, at a dose of 5 mg /kg body weight and after two hours animals were gavaged with chitosan at 30 mg/kg body weight for 3 weeks. At the end of the experiment, blood samples were taken for subsequent biochemical analyses. Testosterone, estradiol, AST, ALT, alkaline phosphatase (Alk. ph.), bilirubin, plasma glutathione peroxidase (GPx) and malondialdehyde (MDA) were determined. In addition, sperm abnormalities, expression of androgen receptor (AR) gene in testis tissues and caspase 3 activity in liver samples were evaluated. Testis and liver specimens were dissected for histopathological examination. The results indicated a decrease in testosterone hormone level while estradiol increased after rats were injected by BPA. However, chitosan treatment restored the testosterone level. It was apparent that BPA caused dysfunction to hormonally regulated body systems. However, chitosan treatment adjusted the metabolic functions and controled fertility. The levels of AST, ALT, Alk. ph. bilirubin and (MDA) enzymes were significantly increased while glutathione peroxidase was significantly decreased in group II after injection with BPA. Chitosan treatment counteracted the effects of BPA. Chitosan decreased the liver inflammation and necrosis. Moreover, the results indicated that the frequency of the sperm abnormalities, induced by BPA treatment, decreased significantly with chitosan administration compared with BPA treatment alone. Also, the expression of AR gene was up-regulated significantly with chitosan treatment combined with BPA compared with BPA treatment alone. Furthermore, the high level of caspase-3 activity induced by BPA in liver tissues was significantly decreased with chitosan treatment compared with BPA treatment alone. Histopathological analysis indicated that BPA induced testis tissues degeneration as well as liver apoptosis and necrosis. Chitosan treatment ameliorated testis and liver damage. In conclusion, chitosan seams to act as an antioxidant against toxicity of the endocrine system, sperm abnormalities, alteration in the AR gene expression and liver apoptosis. The potential biological effect of chitosan may be due to its active ingredients.

**Key words**: Bisphenol A, chitosan, testosterone, liver enzymes, gene expression, caspase-3 activity.

## INTRODUCTION

Bisphenol A (BPA) is a carbon-based synthetic compound with the chemical formula (CH3)2C(C6H4OH)2 belonging to the group of diphenylmethane derivatives and bisphenols. BPA is used in the manufacture of a variety of consumer products, including polycarbonate plastics, epoxy resins, electronics, medical equipment, and dental sealants[1]. It qualifies as one of the world's leading volume production chemicals with an annual increase in demand of 6%–10%. The largest exposure humans have to BPA is by mouth from such sources as food packaging, the epoxy lining of metal food and beverage cans, and plastic bottles[2]. Inspite of no published data, in Egypt, about the levels of BPA that exists in the human plasma or urea, the probability of the exposure to BPA to human being is very high[3].. Because of incomplete polymerization and degradation of the polymer by exposure to higher than usual temperatures, BPA leaches out from food and beverage containers[4], as well as from dental sealants. In humans, free active unconjugated BPA (uBPA) is metabolized by rapid glucuro- or sulfo-conjugation and eliminated via renal clearance, as shown after a single oral dose in adults[5].

However, exposure to nanomolar concentrations of BPA in the environment is ubiquitous and continuous via different routes: oral, air or skin[5]. In rodents, fetal and perinatal exposure to such environmentally relevant doses of BPA has been shown to affect several organs including reproductive tract function[6]. The adverse effect of BPA on hormone-dependent cancer cells is believed to be a

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Table 1: Effect of Bisphenol A and chitosan on testosterone and estradiol and cholesterol levels in male rats.

| Groups<br>Parameters | Group I<br>(Control) | Group II<br>(BPA) | Group III<br>(Chitosan) | Group IV<br>(BPA+Chitosan) |
|---|---|---|---|---|
| Testosterone (ng/ml) | 1.96±0.36 [b] | 0.43±0.07 [c] | 3.1±0.49 [a] | 2.1±0.28 [b] |
| Estradiol (Pg/ml) | 0.79±0.13 [b] | 1.1±0.12 [a] | 0.86±0.1 [b] | 0.88±0.097 [b] |
| Cholesterol (mg/dl) | 74.8±7.6 [c] | 61.2±6.4 [d] | 92.5±7.9 [a] | 84.3±6.2 [b] |

*Data are presented as means ± SE. a, b, c and d means, in the same row, followed by different superscripts are significantly different (P≤0.05).*

Table 2: Effect of Bisphenol A and chitosan on AST, ALT, alkaline phosphatase and bilirubin in male rats.

| Groups<br>Parameters | Group I<br>(Control) | Group II<br>(BPA) | Group III<br>(Chitosan) | Group IV<br>(BPA+Chitosan) |
|---|---|---|---|---|
| AST(U/L) | 238.3±11.5 [c] | 311±16 [a] | 179.5±8.3 [d] | 261±9.7 [b] |
| ALT(U/L) | 83±9.8 [c] | 127.5±12.1 [a] | 64±8.1 [d] | 98.5±7.84 [b] |
| Alk. ph. (U/L) | 169±8.8 [c] | 205±11.6 [a] | 124.5±13.6 [d] | 183±8.6 [b] |
| Bilirubin(mg/dl) | 0.6±0.04 [c] | 1.4±0.2 [a] | 0.47±0.04 [d] | 0.75±0.07 [b] |

*Data are presented as means ± SE. a, b, c and d means, in the same row, followed by different superscripts are significantly different (P≤0.05).*

consequence of its endocrine disrupting effect, either estrogenic or anti-androgenic, through classical or non-classical pathways[7]. High levels of free active BPA have recently been correlated with obesity, diabetes and cardiovascular diseases and low sperm count[8]. However, there are controversies related to the real impact of BPA on human health. Clinical epidemiological studies concerning BPA remain scarce.

More specifically, BPA closely mimics the structure and function of the hormone estradiol with the ability to bind to and activate the same estrogen receptor as the natural hormone[9]. BPA mainly works by imitating the natural hormone 17B-oestradiol. In the past BPA has been considered a weak mimicker of oestrogen but evidence now indicates that it is an extremely potent mimicker. When it binds to oestrogen receptors it triggers alternative oestrogenic effects that begin outside of the nucleus. These alternative oestrogen receptor triggered pathways caused by exposure to BPA alter the function of key components involved in metabolism like pancreatic B cells and adipocytes[10].

BPA shows potential acute, short-term, and subchronic toxicity. There are no chronic organ toxicity studies on BPA. Several studies alert its effect on the reproductive system with inadequate reports on other tissues[11]. Reactive oxygen species (ROS) are cytotoxic agents causing oxidative damage by attacking cell membrane and DNA. Antioxidants are scavengers by preventing cell and tissue damage that could lead to cellular damage and disease. BPA can cause liver, kidneys, brain, and other organs injury by forming ROS. The liver has a range of antioxidant defense system. ROS are scavenged by the endogenous antioxidant defense system, including superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) in cells. The liver is a target organ; BPA could induce liver damage, affecting oxidant/antioxidant balance in rat liver[12].

Marine organisms produce many bioactive substances, which are having a lot of potential applications. Chitosan, an important polysaccharide of marine origin, is prepared from the shells of crustaceans. It has profound applications in the fields of clarification and purification, chromatography, paper and textiles, photography, food and nutrition, agriculture, pharmaceutical and medical, cosmetics, biodegradable membranes and biotechnology[13,14].

Chitosan is a copolymer of b-(1-4)-linked 2-acetamido- 2-deoxy-D-glucopyranose and 2-amino-2-deoxy-Dglucopyranose. This polycationic biopolymer is generally obtained by alkaline deacetylation from chitin, which is the main component of the exoskeleton of crustaceans, such as shrimps. The main parameters influencing the characteristics of chitosan are its molecular weight and degree of deacetylation, representing the proportion of deacetylated units. These parameters are determined by the conditions set during preparation. Chitosan is currently receiving a great deal of interest for medical and pharmaceutical applications. The main reasons for this increasing attention are certainly its interesting intrinsic properties. Indeed, chitosan is known for being biocompatible allowing its use in various medical applications such as topical ocular application[15], implantation[16] or injection. Moreover, chitosan is metabolised by certain human enzymes, especially lysozyme, and is considered biodegradable. Chitosan also promotes wound-healing[17] and has bacteriostatic effects[18]. Finally, chitosan is very abundant, and its production is of low cost and ecologically interesting[19].

Chitosan is a fiber which expands to form a gel in the acidic environment of the stomach. When chitosan is compared to common sugars, it was found that it has less toxic effects than these substances[20]. For safety purposes data gathered in mice is divided by 12 to get the human equivalent. Any breakdown of chitosan by our colon microflora would release D-glucoseamine which is itself a wonderfully beneficial nutrient for human[21].

The main objective of the current study was to evaluate the potential biological effects of chitosan against the toxic negative effects of the BPA on the endocrine and reproductive performances of rats. Thus, hormonal analysis, liver function, oxidative stress, sperm abnormalities, gene expression analysis of androgen receptor gene, activation of caspase-3 and histopathological studies were carried out in male rats.

Table 3: Effect of Bisphenol A and chitosan on glutathione peroxidase (GPx) and malondialdehyde (MDA) in male rats.

| Groups<br>Parameters | Group I<br>(Control) | Group II<br>(BPA) | Group III<br>(Chitosan) | Group IV<br>(BPA+Chitosan) |
|---|---|---|---|---|
| GPx (mmol/ml) | 24.3±1.34 [b] | 18.3±1.1 [d] | 27.3±2.3 [a] | 22±1.3[c] |
| MDA(mmol/ml) | 12.5±1.0 [b] | 15.3±0.98[a] | 8.5±0.97[c] | 11.5±0.83[b] |

*Data are presented as means ± SE. a, b, c and d means, in the same row, followed by different superscripts are significantly different (P≤0.05).*

## MATERIALS AND METHODS

Animals: Forty male albino rats weighing 130-150 g were obtained from the National Research Center, Egypt. The rats were acclimated on a stock diet and tap water that were allowed ad libitum.

Chemical treatment: Bisphnol A at a dose of 5 mg/kg body weight, dissolved in ethanol, was used for daily intra-peritoneal injection. Chitosan at a dose of 30 mg/kg of body weight dissolved in water for gavage. Both were purchased from Sigma Chemical Company.

Experimental design: Animals were randomly divided into four equal groups. Group I, animals served as a control group. Group II, animals were injected intra-peritoneally by bisphenol A at a dose of 5 mg/kg body weight daily for 3 weeks. Group III, animals were gavaged with chitosan at a dose of 30 mg/kg body weight for 3 weeks. Group IV, animals were injected intra-peritoneally at a dose of 5 mg/kg body weight with bisphenol A and after two hours it were gavaged with chitosan at a dose of 30 mg/kg body weight for 3 weeks. At the end of the experiment blood samples were withdrawn by cardiac puncture after anesthetization of the rats using diethyl ether. Blood samples were collected in clean dry test tubes and centrifuged at 3000 rpm for 10 minutes. Sera were then separated and kept frozen for subsequent biochemical analyses. Plasma was taken in anticoagulant test tubes for determination of glutathione peroxidase (GPx) only.

Biochemical analyses:

The collected sera were used for the determination of testosterone hormone level by the solid phase radioimmunoassay (RIA) using 125I according to the method of Wilson and Foster[22]. Estradiol was determined according to method of Burtis and Ashood[23]. Determination of total cholesterol by the enzymatic method as described by Allain et al.[24]. AST and ALT were determined according to the method of Reitman and Frankel[25]. Alkaline phosphatase was determined using the method described by Kind and King[26]. Bilirubin was determined according to the method of Doumas et al.[27]. Plasma glutathione peroxidase (GPx) was determined by the UV method according to Paglia and Valentine[28]. Malondialdehyde was determined by the colorimetric method of Saton[29].

Molecular Analyses:

1-Sperm abnormalities: Animals of each treated group were sacrificed by neck vertebra luxation to determine the sperm abnormalities[30]. The epididymides and testes from each rat were removed and weighed. Sperms were collected as quickly as possible when each rat was dissected. The cauda epididymides were cut in a pre-warmed Petri dish containing 1 ml of saline solution at 37 ◦C to release sperms. After mincing with scalpels, the suspension was stirred and dropped on grease-free clean slide to determine the motility of sperms using a Stereomicroscope (LABOMED, USA). Spermatozoa were counted using heamocytometer and a drop of a homogenate smeared on a cleaned slide and was allowed to air dry and stained with approximately 0.05% aqueous eosin Y. The slides were coded and used for the examination of sperm head and tails abnormalities. For each animal 500 sperms were examined for morphological abnormalities according to the criteria of Jeong et al.[31].

2-Expression of androgen receptor gene:

I. Isolation of total RNA: Total RNA was isolated from testis tissues of male rats by the standard TRIzol® Reagent extraction method. RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water by passing the solution a few times through a pipette tip. Total RNA was treated with 1 U of RQ1 RNAse-free DNAse (Invitrogen, Germany) to digest DNA residues, re-suspended in DEPC-treated water. Purity of total RNA was assessed by the 260/280 nm ratio (between 1.8 and 2.1). Additionally, integrity was assured with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis. Aliquots were used immediately for reverse transcription (RT).

II. Reverse transcription (RT) reaction: The complete Poly(A)+ RNA isolated from male rats tissues was reverse transcribed into cDNA in a total volume of 20 µl using RevertAidTM First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). An amount of total RNA (5µg) was used with a reaction mixture, termed as master mix (MM). The MM was consisted of 50 mM MgCl2, 5x reverse transcription (RT) buffer (50 mMKCl; 10 mMTris-HCl; pH 8.3; 10 mM of each dNTP, 50 µM oligo-dT primer, 20 U ribonuclease inhibitor (50 kDa recombinant enzyme to inhibit RNase activity) and 50 U M- MuLV reverse transcriptase. The RT reaction was carried out at 25 °C for 10 min, followed by 1 h at 42 °C, and the reaction was stopped by heating for 5 min at 99 °C. Afterwards the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for DNA amplification through semi-quantitative real time-polymerase chain reaction (sqRT-PCR).

III. Semi-Quantitative Real Time-Polymerase Chain Reaction (sqRT-PCR):

PCR reactions were set up in 25 µL reaction mixtures containing 12.5 µL 1× SYBR® Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd., Germany), 0.5 µL 0.2 µM sense primers, 0.5 µL 0.2 µM antisense primer, 6.5 µL distilled water, and 5 µL of cDNA template. The reaction program was allocated to 3 steps. First step was at 95.0°C for 3 min. Second step consisted of 40 cycles in which each cycle divided to 3 steps: (a) at 95.0°C for 15 sec; (b) at 55.0°C for 30 sec; and (c) at 72.0°C for 30 sec. The third

Table 4: Mean values of different types of sperm abnormalities induced by BPA and/or chitosan in male rats.

| Treatment | Sperm abnormalities* | | | | |
|---|---|---|---|---|---|
| | Head without hock | Amorphous head | Banana head | Coiled tail | Total mean |
| Group I (Control) | 0.5±0.1[b] | 0.0±0.0 | 0.3±0.1[b] | 0.3±0.1[b] | 1.1±0.1[c] |
| Group II (BPA) | 2.8±0.1[a] | 1.3±0.0[a] | 1.2±0.1[a] | 2.5±0.1[a] | 7.8±0.2[a] |
| Group III (Chitosan) | 0.5±0.0[b] | 0.0±0.0 | 0.4±0.1[b] | 0.3±0.1[b] | 1.2±0.1[c] |
| Group IV(BPA+Chitosan) | 0.8±0.1[b] | 0.7±0.1[b] | 0.6±0.1[b] | 0.5±0.1[b] | 2.6±0.1[b] |

**After treatment animals of each group were used to assess the sperm morphology.*
*a, b and c mean values, within columns, with different superscripts are significantly different (P≤0.05).*

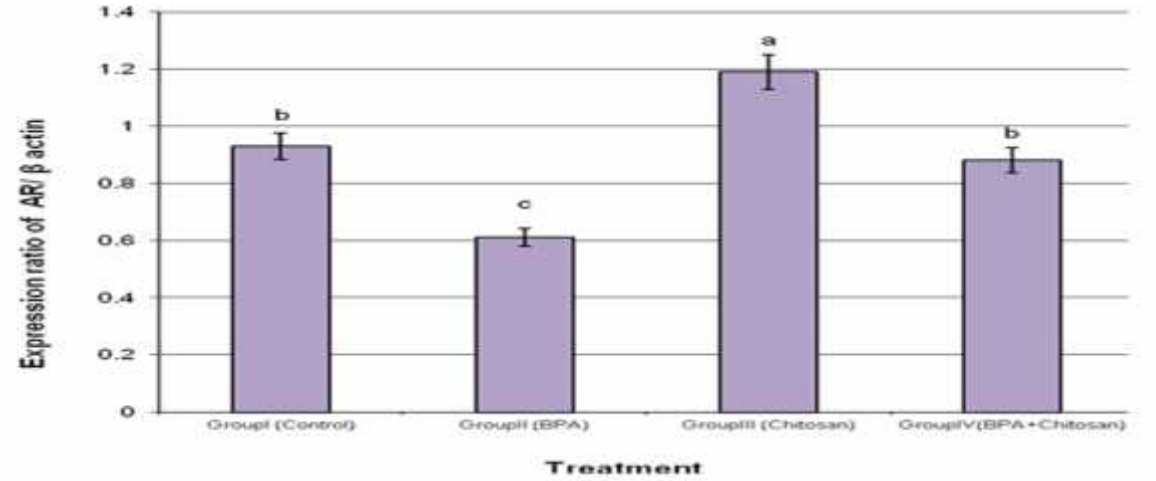



***Fig. 1:*** *Expression of AR gene in the testis tissues of rats injected with BPA and/or chitosan. a, b and c mean values with different letters differ significantly (P≤0.05).*

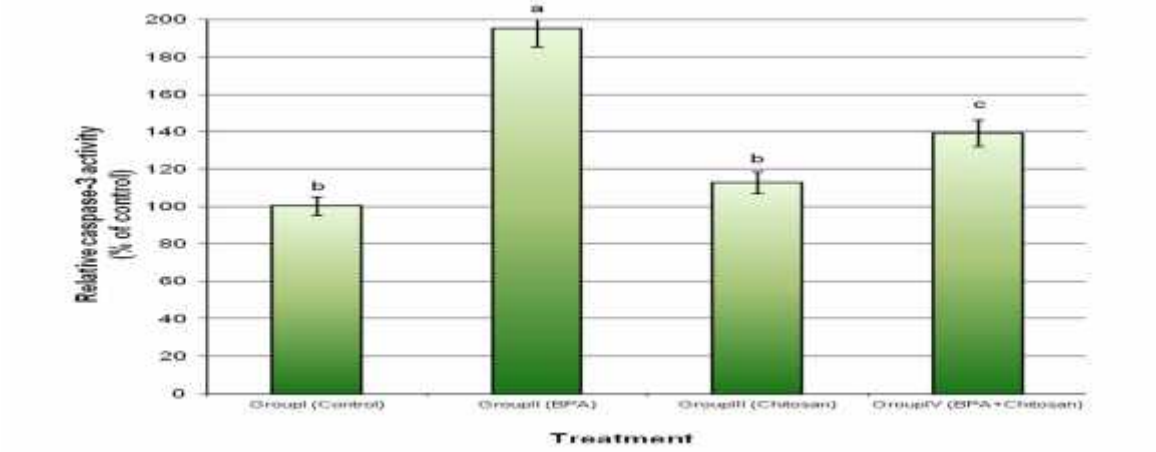



***Fig. 2:*** *Effect of BPA and/or chitosan on the caspase-3 activity in liver tissues of male rats. a, b and c mean values with different letters are significantly different (P≤0.05).*

step consisted of 71 cycles which started at 60.0°C and then increased about 0.5°C every 10 sec up to 95.0°C. At the end of each sqRT-PCR a melting curve analysis was performed at 95.0°C to check the quality of the used primers. Each experiment included a distilled water control.

The semi quantitative values of RT-PCR (sqRT-PCR) of Androgen Receptor (AR-F: 5′- GGA GAA CTC TTC AGA GCA AG-3′, AR-R: 5′- AGC TGA GTC ATC CTG ATC TG-3′,[32]) gene was normalized on the bases of ß-actin (ß-actin-F: 5′- GTG GGC CGC TCT AGG CAC CAA-3′, ß-actin-R: 5′- CTC TTT GAT GTC ACG CAC GAT TTC-3′,[33]) expression.

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

Calculation of Gene Expression: First the amplification efficiency (Ef) was calculated from the slope of the standard curve using the following formulae[34]:

Ef = 10-1/slope

Efficiency (%) = (Ef – 1) x 100

The relative quantification of the target to the reference was determined by using the

ΔCT method if E for the target (AR) and the reference primers (β-Actin) are the same[34]:

Ratio (reference/ target gene) = Ef CT(reference) – CT(target)

3-Determination of caspase-3 activity: Activation of caspase-3 that occurred during the apoptotic process in rat liver cells was assessed according to Doo et al.[35]. Caspase-3 activity was determined according to the user's manual for the caspase-3 assay kit in rats treated with bisphenol and/or chitosan. The supernatant obtained by the centrifugation of lysed cells was added to the reaction mixture containing dithiothreitol and caspase-3 substrate (Acetyl-Asp-Glu-Val-Asp p-nitroanilide) and incubated for 90 min at 37 °C. Absorbance was measured with a microplate reader at a test wavelength of 405 nm.

Histopathological study: Testis and liver specimens of different groups were dissected out and imbedded immediately in 15% formalin for histopathological examination. The tissue samples were fixed, stained and investigated by light microscope[36].

Statistical Analysis: All data were statistically analyzed as a one-way analysis of variance using the general Linear Model, SAS software[37]. Duncan, multiple range tests was used to separate the means when significant differences exist in biochemical analysis. followed by Scheffé-test to assess significant differences between groups in genetic analysis. Statistical significance was set at 0.05% probability.

**RESULTS**

Biochemical analyses: Testosterone and estradiol levels are represented in Table 1. The results showed that after Bisphenol A injection, the level of testosterone significantly ($P \leq 0.05$) decreased. However, by using chitosan treatment alone in group III, testosterone level significantly ($P \leq 0.05$) increased compared to the control group. In group IV, the level of testosterone returned to the control value due to chitosan treatment. Estradiol level significantly ($P \leq 0.05$) increased after injection by Bisphenol A (group II). While chitosan treatment (group IV) ameliorated this effect and the level become similar to that of the control group.

Data in Table 1 shows that cholesterol level was significantly ($P \leq 0.05$) decreased in group II after injection by Bisphenol A, while chitosan treatment in group III significantly ($P \leq 0.05$) increased the cholesterol level compared to the control. Also in group IV, chitosan counteracted the effect of BPA and the level of cholesterol returned to that of the control group.

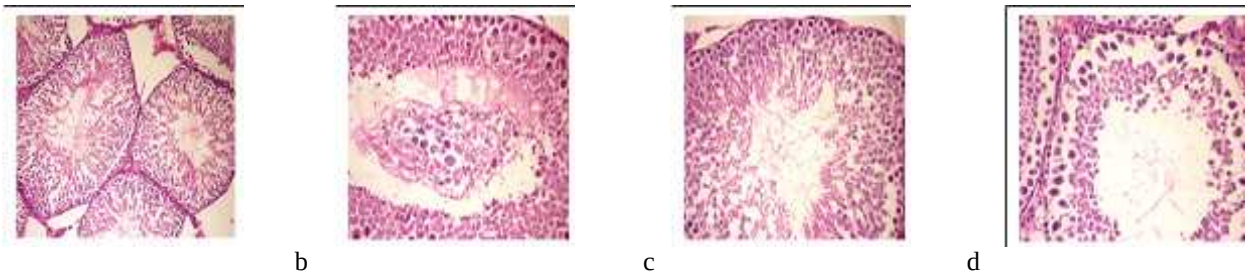
The results of liver enzymes (Table 2) indicates that in group II, the AST level was significantly ($P \leq 0.05$) increased after injection by Bisphenol A. However, in group III AST significantly ($P \leq 0.05$) decreased after treatment by chitosan compered to the control group. Chitosan in group IV significantly reduced the effect of Bisphenol A on AST activity. However, the AST level was still significantly ($P \leq 0.05$) higher than that of the control group. Also Bisphenol A caused significant ($P \leq 0.05$) increase in ALT (Table 2). In group III chitosan treatment caused significant ($P \leq 0.05$) decrease in ALT activity. In group IV chitosan decreased the effect of Bisphenol A but the level of ALT liver enzyme became significantly lower but did not reach the control group level.

Table 2 demonstrates that there was significant ($P \leq 0.05$) increase in alkaline phosphatase activity after injection with Bisphenol A. In group III, after chitosan treatment alone, a significant ($P \leq 0.05$) decrease was observed in alkaline phosphatase activity. Also, chitosan significantly decreased Alk. ph. level in group IV. However, Alk. ph. level was still significantly ($P \leq 0.05$) higher as compared to control group.

Data in Table 2 showes that in group II, Bisphenol A significantly ($P \leq 0.05$) increased the level of bilirubin. However, in group III, chitosan treatment significantly ($P \leq 0.05$) decreased the bilirubin level compared to the control group. However chitosan treatment in group IV significantly decreased the level of bilirubin but it was still significantly ($P \leq 0.05$) higher than the control group level.

Table 3 shows that the level of glutathione peroxidase (GPx) in group II after Bisphenol A injection was significantly ($P \leq 0.05$) decreased below the control group. However, treatment with chitosan alone significantly increased the GPx level compared to control level. Chitosan restored the GPx level, after BPA treatment, almost near the control level (group IV).

Data in Table 3 demonstrates that after Bisphenol A injection (group II), MDA enzyme was significantly ($P \leq 0.05$) increased. However, chitosan treatment alone (group III) significantly ($P \leq 0.05$) decreased MDA level



a b c d

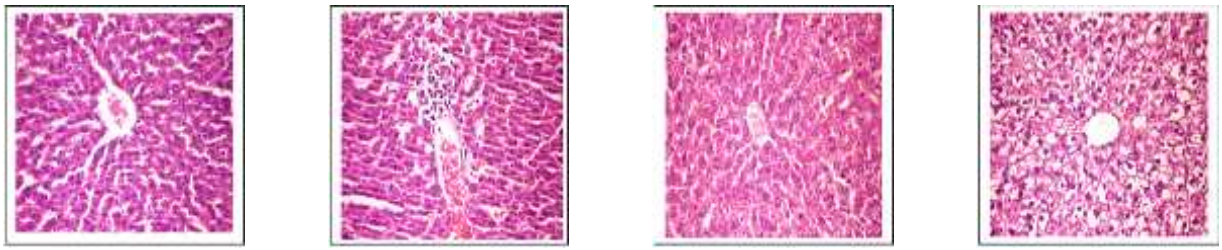
*Fig.3: Sections of rat testis (HandE×400)*

*(a) Testis of group I (control), untreated rat showing the normal histological structure of seminiferous tubules. Normal cells and complete spermatogenesis.*

*(b) Testis of a rat from group II ( BPA injected rats) showing degeneration of spermatogoneal cells lining seminiferous tubules, desquamation and accumulations of cells in lumen.*

*(c) Testis of a rat from group III ( chitosan treated rats) showing no histopathological changes.*

*(d) Testis of a rat from group IV (BPA+chitosan) showing slight degeneration of spermatogoneal cells lining seminiferous tubules.*



a b c d

*Fig. (4): Sections of rat liver (HandE×400)*

*(a) Liver of group I (control), untreated rat showing the normal histological structure of hepatic lobule.*

*(b) Liver of rat from group II (BPA injected rats) showing congestion of central vein, apoptosis of hepatocytes, focal hepatic necrosis replaced by leucocytic cells infiltration.*

*(c) Liver of rat from group III (chitosan treated rats) showing no histopathological changes.*

*(d) Liver of rat from group IV (BPA+chitosan) showing hydropic degeneration of hepatocytes.*

below the control level. Chitosan reduced BPA effect and MDA approached the control group value (group IV).

Molecular analyses:

Sperm abnormalities assessment: The mean values of different types of the abnormalities of sperm morphology are shown in Table 4. The most frequently observed abnormality was head without hock and coiled tail sperm. The results revealed that the frequency of the sperm abnormalities increased, significantly, with BPA injection compared with the control group. Whereas, the rate of total sperm abnormalities in group II (BPA injected rats) increased by 609% compared to the control group. However, chitosan treatment did not increase the frequencies of the morphologically abnormal sperms compared to the control group (Table 4).

The frequencies of head without hock and coiled tail sperm abnormalities found in chitosan group were similar to those in the control group. On the other hand, frequencies of the sperm abnormalities was increased in the rats injected with BPA. Whereas, treatment with chitosan after BPA injection, reduced the rate of sperm abnormalities in BPA-injected rats by 472% compared to the rats injected with BPA alone (Table 4).

AR gene expression in rats testis tissues: The effect of chitosan and/or BPA on the expression of the AR gene in the testis tissues of adult male rats is summarized in Figure 1. The level of AR gene decreased significantly with BPA injection compared with the control group (Fig 1). However, the expression level of the AR gene increased significantly with chitosan treatment above the control group. On the other hand, supplementation of BPA-injected rats with chitosan increased the expression level of AR gene compared with rats injected with BPA alone.

Determination of the apoptosis biomarker Caspase-3 activityin rats liver tissues: The activity of caspase-3 was examined in rats liver tissues (Fig. 2) because it is an important biomarker of the apoptosis process for induction of sperm abnormalities in male rats. The results of the present study indicated that the activity of caspase-3 in liver tissues significantly increased 195% in male rats injected with BPA as compared to the control group level. However, treatment of male rats with chitosan alone did not change the level of caspase-3 activity significantly above the control group level. Moreover, treatment of male rats with chitosan after BPA injection decreased the caspase-3 activity (induced by BPA injection). The level of caspase-3 activity in rats injected with BPA plus chitosan was 129% of the controls compared to 195% in rats injected with BPA alone (Fig. 2).

Histopathological analysis: Testis of group I (control), untreated rat showed the normal histological structure of seminiferous tubules, normal cells and complete spermatogenesis (Fig. 3a). Whoever testis of rat from group II (BPA injected rats) showed degeneration of spermatogoneal cells lining seminiferous tubules, desquamation and accumulations of cells in lumen (Fig. 3b).While testis of rat from group III (chitosan treated rats) showed no histopathological changes from the control group (Fig. 3c). Meanwhile testis of rat from group IV (BPA+chitosan) showed slight degeneration of spermatogoneal cells lining seminiferous tubules (Fig. 3d). Liver of group I (control), untreated rat showing the normal histological structure of hepatic lobule (Fig. 4a). Othewise liver of a rat from group II showing congestion of central vein, apoptosis of hepatocytes, focal hepatic necrosis replaced by leucocytic cells infiltration (Fig. 4b). Meanwhile liver of a rat from group III showing no histopathological changes (Fig. 4c). Whoever liver of a rat from group IV showing hydropic degeneration of hepatocytes (Fig. 4d).

## DISCUSSION

There is a large debate about human exposure to BPA, a widespread chemical pollutant with endocrine disrupting effects[38]. BPA is an endocrine disruptive chemical released in the environment, so most studies are focused on its effect on reproduction. Therefore the aim of the current study was to evaluate the potential toxicity of BPA to the endocrine system and the protective role of chitosan against BPA-induced reproductive disorders.

Bisphenol A (BPA) induced endocrine toxicity and reproductive damage: Our results indicated that testosterone (T) and cholesterol levels decreased while estradiol (E2) increased after BPA administration in male rats. No published data were found concerning the effect of BPA administration on cholesterol level.

Similar studies have reported declining in T levels, in humans, consistent with decreases in sperm concentration. It has been hypothesized that these changes might be the result, at least in part, of increasing human exposure to

endocrine-disrupting compounds[39]. Meeker et al.[40] examined a male population attending a fertility clinic. They reported inverse associations between urinary BPA concentrations and serum inhibin-B levels and the E2/T ratio. While, Vandenberg et al.[3] found that there are statistically significant positive correlations between BPA concentrations and total T and free testosterone (FT) levels in all subjects.

Most studies of the health effects of BPA have focused on its well-documented estrogenic activity, with reports of both estrogen agonist and androgen antagonist activity[1,41]. Suppression of aromatase activity has been detected as has binding to alternative nuclear receptors, including the aryl hydrocarbon receptor and estrogen-related receptor γ. Studies to clarify the mechanisms of these associations are clearly a priority. The previous results are important because they provide the first report that, there is association between elevated exposure to BPA and alterations in circulating androgen hormone levels[42]. Thuillier et al.[43] concluded that BPA may have been associated with adverse effects on Sertoli cells or their folicular stimulating hormone (FSH) receptors that led to altered inhibin-B production and reduced semen quality. Because estrogens and androgens can exert differential effects in function depending on the cell type and its stage of development, the consequences of BPA exposure on adult reproductive and somatic tissues merits further attention[44].

BPA binds to estrogen receptor (ER) in the pituitary gland, resulting in direct suppression of FSH secretion; this is based on studies that have found ERs in the pituitary gland and that E2 directly inhibits gonadotropin secretion at the pituitary level in men[45]. BPA is considered by some researchers to be one of the most potent reproductive toxicants[46]. Akingbemi et al.[47] described an inhibitory effect of BPA on testicular steroidogenesis at low exposure levels in pubertal rats, which they ascribed to an ER-mediated effect. BPA might also act as an androgen receptor antagonist, preventing endogenous androgens from regulating androgen-dependent transcription[1]. The disruption of the androgen receptor–androgen interaction has been speculated to be significant in eliciting adverse effects on the male reproductive system, including sexual dysfunctions[48].

Alternatively, there could be differential effects of BPA on the metabolism of testosterone and estrogen[49]. BPA also significantly decreased the activity of enzymes involved in the hydroxylation of testosterone, including the cytochrome P450 isoforms for testosterone 2 α-hydroxylase and testosterone 6 β-hydroxylase, CYP2C11/6 and CYP3A2/1, respectively, in isolated rat livers both of which could lead to a net increase in circulating testosterone[50].

Our results indicated that the frequency of the sperm abnormalities was significantly increased with the BPA injection compared with the control group. Our results are in agreement with a study released in 2013 which demonstrated that BPA, in weak concentrations, is sufficient to produce a negative reaction on the human testicle. Moreover, they revealed that BPA administration to male mice decreased sperm count and quality[51]. They also found that the main reason of sperm abnormalities was attributed to DNA strand breaks in germ cells of testis tissues due to enhancement of the apoptotic activity. Another study suggested that it is possible that BPA contributes to a reduction in the production of sperm and increase in the incidence of testicular cancer in human adults[52]. Regarding pubertal exposures, rodent studies have described a dramatic decrease in T levels and epididymal sperm counts after BPA exposure. Adult male mice showed a significant reduction in testicular sperm counts, as well as epididymal sperm counts. Also the plasma concentrations of T decreased and plasma concentrations of luteinizing hormone (LH) increased in BPA-treated male adult rats compared with control rats[53]. Also, Li et al.[54] reported that increasing urine BPA level in men exposed to BPA was significantly associated with decreased sperm concentration, decreased total sperm count, decreased sperm vitality, and decreased sperm motility. In rodent testes, the multiple types of germ cells are arranged in characteristic cellular associations that succeed each other in a given area of the seminiferous tubule, which are known as the stages of spermatogenesis[55]. Furthermore, BPA and estrogen have also been shown to induce blood–testis barrier restructuring, Sertoli cell junctional protein expression, and germ cell apoptosis, specifically at stages VII and VIII. Although we did not evaluate these changes, our data indicated that the BPA-induced sperm count reduction is highly associated with the disruption of spermatogenesis. Li et al.[48] reported several attempts to unequivocally demonstrate how spermatogenesis is disturbed by BPA exposure. Their results demonstrated that BPA-induced meiotic abnormalities in the male reproductive system in adult rats. In vivo, BPA administration at 200 μg/kg bw/d significantly decreased sperm counts in adult male rats. This was the result of spermiation inhibition, with no changes in relative body or reproductive organ weight, no alterations in sperm parameters and sperm apoptosis, and no impacts on sex hormone levels. The sperm count reduction was associated with a disruption in meiotic progression and apoptosis in testicular cells. Moreover, the adult BPA exposure adversely affects epididymal sperm counts[56].

Our present results revealed that BPA treatment decreased significantly the expression of androgen receptor (AR) gene in testis tissues of male rats compared with the control groups. AR, also known as NR3C4 (nuclear receptor subfamily 3), is a type of nuclear receptor[57] that is activated by binding of either of the androgenic hormones testosterone or dihydrotestosterone in the cytoplasm and then translocated into the nucleus. The main function of the androgen receptor is as a DNA-binding transcription factor that regulates gene expression; however, the androgen receptor has other functions as well[58]. Androgen regulated genes are critical for the development and maintenance of the male sexual phenotype. There are several factors that play a role in the inhibition mechanism of AR expression. A mutation of the androgen receptor gene, located on the X chromosome, namely androgen

insensitivity syndrome is one of these factors[59]. In addition, point mutations and trinucleotide repeat polymorphisms have been linked to a number of additional disorders[60]. So it could be possible that BPA treatment has a role to induce mutation in AR sequences which may suppress the expression of AR gene.

Also, the present study demonstrated that after BPA injection, the increase in liver enzymes; AST, ALT, Alk. ph. and bilirubin were associated with the apparent damage in liver structure. BPA induces mitochondrial dysfunction and structural changes in the rat liver. BPA increased hepatic oxidative stress and decreased antioxidant enzyme expression in vivo. All these findings suggest that a low dose of BPA can also induce hepatic dysfunction. We can postulate that BPA may induce hepatocyte damage, therefore, the hepatotoxic effects of BPA observed in liver function tests, including ALT, AST, and bilirubin. The high levels of AST, ALT and Alk. Ph. are attributed to liver damage[61].

The current study indicated that glutathione peroxidase (GPx) antioxidant increased while, malondialdehyde (MDA) decreased after BPA injection. Due to limited information concerning the effects of BPA on liver, the present study investigates whether BPA causes hepatotoxicity by the induction of oxidative stress in liver[62].Therefore, the damage at the cellular level by oxidants is attenuated by antioxidant enzyme such as GSHPx[63]. The increase in GSH level is important for GSHPx, which requires GSH as a cofactor, and the elevation in GSH level increases activity of GSHPx. The current study showed reduction in GSHPx levels. Therefore, our study confirmed that injection with of BPA causes oxidative stress by disturbing the balance between ROS and antioxidant defenses system in liver[64].

The cell has various defense mechanisms against oxidative stress, including scavenging enzyme systems such as GPx activities in the liver and kidney of male mice and the sperm of Wistar rats. It has been suggested that BPA exposure produces ROS by inhibiting antioxidant enzymes, or that antioxidant enzymes are depleted because of ROS production[12]. A low dose of BPA induces mitochondrial dysfunction in the liver, and this is associated with an increase in oxidative stress and inflammation. Razzoli et al.[65] reported that antioxidants reduce the cellular damage resulting from interaction between lipid, protein and DNA molecules and ROS. Regardless of the presence of this antioxidant system, an over or unbalanced production of ROS, due to contact with chemicals, may resulted in a number of clinical disorders. BPA can cause oxidative stress by disturbing the redox status in cells.

Recently, the oxidative stress was proposed as another adverse cellular effect of BPA in the liver[66]. BPA increased the generation of ROS and induced cellular apoptosis in hepatocytes[67]. Oxidative stress can induce mitochondrial damage, and damaged mitochondria can generate more ROS. Mitochondria are vulnerable to ROS due to impairment of the antioxidant and DNA repair enzyme systems. Accumulation of oxidative damage in the mitochondria induces mitochondrial dysfunction, mitochondrial DNA depletion, and cell apoptosis. BPA increased ROS production, as assessed by the measurement of MDA concentration[68].

Our results indicated that the activity of caspase-3 in liver tissues increased significantly in male rats after BPA injection. This may be because caspases are part of a highly conserved protein family that is central to the apoptotic pathway. Caspases are proteases activated after a cell has received a signal instructing it to undergo apoptosis. The key components that the caspases break down include DNA repair enzymes and structural proteins in the cytoskeleton[69]. Caspases can also activate other enzymes that degrade other parts of the cellular machinery by cleaving an inhibitory sequence on these enzymes. There is a loss of nuclear membrane integrity after disruption[69].

Protective effects of chitosan against BPA hazards: Chitosan is biocompatible, nonantigenic, nontoxic and biofunctional compound. It has received much attention as a new excipient and/or functional material of high potential in the pharmaceutical and food industries. Chitosan, derived from shrimp, has been recognized as a GRAS (Generally Recognized as Safe) for our knowledge no data have been published regarding the protective effect of chitosan against the reproductive toxicity[70].

Our results indicated that chitosan treatment, after BPA injection, enhanced testosterone level. This was in agreement with He et al.[71] who found that, in obese males chitosan exploring some aspects affecting fertility. Chitosan treatment alone was reported to adjust the metabolic functions controlling fertility. It was reported that chitosan could prevent the atherogenic process, associated with hyperlipidemia, by depressing blood level of cholesterol. It increased testicular weight. It decreased lipid content because chitosan has been reported to interact with cell membrane, enhancing peptide and protein uptake[71].

The current study indicated that chitosan treatment after BPA injection adjusted the levels of AST, ALT, Alk. ph. bilirubin and MDA enzymes. Chitosan play some roles in liver function, such as reducing the plasma cholesterol level and preventing liver diseases. Serum cholesterol decreased in mice group fed on diet containing chitosan. Moreover, several types of liver damage, such as inflammation, necrosis, concentration of lipid lobules and increase in ALT activity were elevated by chitozan treatment[72].

The actions of chitosan include interference of lymphatic absorption of cholesterol and fat[73], increased fecal excretion of neutral steroids and fat and improvement of liver function. Moreover, chitosan caused reduction in cholesterol, AST, ALT, Alk. ph activities[74]. MDA level is the most important factor indicating increased peroxidative level and may result in the destruction of membrane integrity. Chitosan decrease MDA which effectively protect membrane integrity. Chitosan increase GPx antioxidant enzyme and protects against oxidative stress induced by the depletion of enzymic and non-enzymic antioxidants[75]. In another study chitosan showed potent antioxidant properties in tissues by decreasing thiobarbtauric acids (TBARS) and increasing antioxidant

enzymes. This protective action against some hepatotoxic chemicals was also noticed by inhibiting MDA formation[76].

Our results are in agreement with the previous findings. It is suggested that treatment of male rats with chitosan decreased the frequencies of the sperm abnormalities in rats treated with BPA. Moreover, treatment of male rats with chitosan combined with BPA decreased the caspase-3 activity induced by BPA. Furthermore, expression of AR increased significantly with chitosan treatment combined with BPA compared with BPA alone.

The antioxidant efficiency was considerably improved when chitosan reacted with sugar, compared with chitosan or sugar alone[77]. An increase in the antioxidant activity of chitosan-sugar complex may be the same reason as described by Guérara and Sumaya-Martinez[78]. They also indicated that the study of the chromatographic profiles obtained before and after the Maillard reaction of glucose and protein hydrolysates ,with changes in absorbance at 280 nm, indicating molecular rearrangements with phenolic structure that could be involved in the improvement of the antioxidant activities. In addition, Hayase et al[79] showed that melanoidins were strongly active in scavenging active oxygen species. This may be another reason for its biological beneficial effects. Thus, the protective role of chitosan is most probably attributed to its antioxidant activity.

These active ingredients of chitosan can be considered as antioxidants against sperm abnormalities, alteration in the AR gene expression and liver apoptosis. The mechanism of action for the expression of the androgen receptors is direct regulation of gene transcription. Androgen receptors interact with other proteins in the nucleus, resulting in up- or down-regulation of specific gene transcription[80]. Up-regulation or activation of transcription results in increased synthesis of messenger RNA, which, in turn, is translated by ribosomes to produce specific proteins. One of the known target genes of androgen receptor activation is insulin-like growth factor I (IGF-1). Thus, change in levels of specific proteins in cells is one way that androgen receptors control cell behavior. So, in the current study we can suggest that chitosan and/or its active ingredients containing D-glucosamine and N-acetyl-D-glucosamine may act as promoters to regulate the expression of the AR gene[80].

## CONCLUSION

The current study indicated that BPA causes oxidative stress by disturbing the balance in the endocrine system causing several reproductive performance inhibition. Also BPA alters the balance between reactive oxygen species (ROS) and antioxidant defenses system in liver. Chitosan is nonantigenic, nontoxic and biofunctional compound. It has a protective potential effect against hepatic toxicity and reproductive disorders, induced by BPA injection. This may be due to its active ingredients of D-glucoseamine which is a wonderfully beneficial nutrient for human. Chitosan act as potent antioxidant against endocrine toxicity, sperm abnormalities, alteration in the AR gene expression and liver apoptosis.

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