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RESEARCH ARTICLE

THE SENSETIVITY OF LIVER, KIDNEY AND OVARIES OF PREGNANT RATS TO OXIDATIVE STRESS INDUCED BY BISPHENOL- A.

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Abstract

Bisphenol-A (BPA) is a worldwide used endocrine disruptor that is incorporated in many plastic industries. The exposure of humans to such substances starts early during the fetal life, postnatal life and extends throughout the life of the individual. Many agencies raised warnings against the excessive use of such substances. The aim of the present study is to evaluate the extent to which BPA can affect the liver, kidney and ovaries by measuring the oxidative stress induced by two different doses of BPA in these organs of pregnant rats; these rats were treated to different oral doses of BPA (25 mg/kg, 50mg/kg and 50mg/kg/b.wt). The oxidative stress arising from BPA was evaluated in liver, kidney and ovary tissues. In addition, serum urea and createnine levels as markers of kidney function together with the activities of MDA, catalase, GSH, GST as markers of liver function were measured. The results of the present study showed significant changes in the antioxidant mechanisms in the liver, kidney and morphological changes in ovaries with the lower (25 mg/kg, 50mg/kg for 9-days) and higher doses of BPA (500 mg/kg for 9-days). It could be concluded that BPA-induced toxicity is mediated by oxidative stress which was prominent in liver, kidney and ovaries after the exposure to both the higher and lower doses.

Introduction:-

BPA is found in polymers that are used in dental materials (Chapin et al., 2008).The ubiquitous and extensive use of BPA containing products results in a high human exposure worldwide (Vandenberg et al., 2010). It is one of the environmental contaminants widely used in the manufacture of polycarbonate plastic (e.g, water bottles, baby bottles), epoxy resins (e.g, inside coating in metallic food cans) and as a non–polymer additive to other plastics (Hernandez- Rodriguez et al., 2007). Hence, it becomes an integrated part of the food chain (Huang et al., 2011). It is thought that human exposure mainly occurs through diet as polymers containing BPA can be hydrolyzed under high temperature and acidic or basic conditions leading to leaching into food and drink containers (Welshons et al., 2006). The majority of studies on BPA have focused on their endocrine disrupting and potential adverse effects on the developing reproductive system. There are several reports suggesting reproductive toxicity of BPA in rats and mice (Takahashi and Oishi, 2001). Accumulation of BPA in female reproductive organs have some clinical implications since exposure to low doses of BPA during fetal life has been shown to decrease the efficiency of embryo production in the offspring of female rat (Tachibana et al., 2007). Lang et al. (2008) reported a significant relationship between urine concentration of BPA and cardiovascular disorders, type 2 diabetes and liver enzyme abnormalities in a representative sample of US population. Moreover, two studies on laboratory animals have shown adverse effects of BPA on brain, reproductive system, metabolic processes, including alterations in insulin homeostasis and liver enzymes (Richter et al., 2007; Lang et al., 2008). In addition, absorption of large amounts of BPA through skin has been shown to cause extensive damage to liver, kidney and other vital organs in human (Suarez et al., 2000). BPA has been shown to cause the formation of multinucleated giant cells in rat liver hepatocytes. It also causes degeneration of renal tubules in kidney of rat and mice (Nakagawa and Tayama, 2000). BPA semiquinone, a radical intermediate, was shown to be involved in DNA adduct formation along with

peroxidase and hydrogen peroxide in rat hepatocytes in vitro (Atkinson and Roy, 1995). Several studies reported the occurrence of oxidative toxicity after BPA exposure in rats and mice (Chitra et al., 2003; Gong and Han, 2006). It was suggested that BPA caused tissue injury in the liver, kidney, brain and other organs by the formation of reactive oxygen species (ROS) (Kabuto et al., 2004). Moreover, the study of Bindhumol et al. (2003) revealed that low doses of BPA generate ROS by decreasing the activities of antioxidant enzymes and increasing lipid peroxidation thereby causing oxidative stress in liver of rats. To date, there is a controversy about the toxicity of BPA. Newly emerging data has stirred discussion on urgency of more studies to make human health risk assessment of BPA exposure (Hugo et al., 2008) especially in developing countries where plastic usage has increased exponentially and certain population groups such as those suffering from malnutrition may be at higher risk than other populations (Aslan et al., 2006; Lahera et al., 2006). Accordingly, the aim of the present study was to investigate the effect of the administration (gestational day 8^{th} to 15^{th}) of different doses (lower doses and higher dose) of BPA (25 mg/kg 50mg/kg and 50mg/kg/b.wt) on the antioxidant status of the liver, kidney and morphological changes in ovaries of pregnant rats.

Materials and methods:-

Animals:-

Pregnant wistar albino rats weighing 250-300 g were used as experimental animals. The animals were obtained from the M/S Raghavendra Enterprises, Bangalore, India. They were maintained on stock diet and kept under fixed appropriate conditions of housing and handling. Different experimental groups of the animals were caged separately and an average of 6 animals per cage was maintained. The control as well as other treated groups of rats was given free access to standard chow and water ad libitum. All these animals were housed in wooden cages and provided water in glass bottles.

Chemicals:-

Pure bisphenol- A (BPA) powder was purchased from the Sd Fine chemicals, Bombay with 98.7% purity. Phosphate buffer and reagent kits were purchased from Hi Media Laboratories Pvt. Ltd., Mumbai, India.

Experimental Protocol:-

Female Wistar strain rats three months old, weighing 250 g to 300 g were used for the experiment.The status of estrous cycle stages were determined every morning between 7:00 am by collecting of vaginal smear. Vaginal smear cytology is the most practical approach to monitor estrous cycle normality. The vaginal smear was examined by the method described by Zarrow et al., (1964) and reviewed by cooper et al. (1993). Few drops of saline (0.9% NaCl) were introduced carefully into the vagina by means of specially made Pasteur pipette. The smear was carefully taken on a clean plain glass slide as a thin layer and observed under microscope for various stages of estrous cycle, and also to determine the pregnancy (based on the presence of sperm). Two females of pro-estrous stage were paired with a male overnight and the next morning, males were removed and females were assessed for the presence of sperm in the vaginal flush. Animals with positive sperm in the flushes are designated as day-1 of gestation. Six pregnant rats were used in each experimental group (test- 1, test- 2 and test-3 and Control group).

Experimental design:-

The animals were divided into 4 groups. Animals of group (1) served as control and received a daily oral administration of distilled water, Animals of group (2) were administered orally 25 mg/kg of BPA for 9days, Animals of groups (3) were administered orally 50 mg/kg of BPA for 9days and (4) were administered orally 500mg/kg of BPA respectively. The doses of BPA were administered 9 days (gestational day 8^{th} to 15^{th}). The lower dose of BPA (25mg/kg, 50 mg/kg) in this study was chosen on the base of previous studies (Bian et al., 2006; Richter et al., 2007).

Treatments:-

The mean (±SEM) body weight (250gms ± 300 gms) of the rats were randomly assigned to one of three groups, rats in group I (controls), II, III and iv (experimentals) received the distilled water and distilled water supplemented with 25mg BPA/kg body wt, 50mg BPA/kg body wt / day & 500mgBPA /kg body wt / day respectively. At gestational day 8^{th} (study initiation) to 15^{th}, Bisphenol-A (25mg/kg, 50mg & 500mg/kg body wt / day) was given orally through gavage. All pregnant rats were sacrificed on gestational day 15^{th} using a CO_2 inhalation chamber. Liver, kidney and ovaries were carefully dissected out, and serum samples were stored at -20^{o}c for further analysis of various proteins.

Preparation of serum:-
At the end of the experiment the animals were sacrificed (Gestational day 15th) after being fasted overnight. Blood samples were drawn from the retro-orbital venous plexus according to the method of Sorg and Buckner (1964). Blood samples were left to coagulate at room temperature, and then centrifuged at 986 g for 15 minutes, the clear non-hemolyzed supernatant serum was quickly removed and used for the estimation of serum enzymes.

Handling of tissue samples:-
The liver, kidney and ovaries of each animal was quickly removed and rapidly weighed and frozen until analyzed. The liver is homogenized in 10 ml and kidney in 6ml of ice cold phosphate buffer (50 mM pH 7.4, 0.1% triton X and 0.5 mM EDTA). The homogenates were centrifuged at 1753 g for 15 min at 4°C using a high speed cooling centrifuge (Type 3K-30, Sigma, Osterode-am-Harz). The clear supernatants were separated and used for analysis.

Determination of lipid peroxidation and reduced glutathione levels:-
Lipid peroxidation was assayed by determining the level of malondialdehyde (MDA) by measuring thiobarbituric reactive species using the method of Ruiz-Larrea et al. (1994). The thiobarbituric acid reactive substances react with thiobarbituric acid to produce a red colored complex that has peak absorbance at 532 nm. using a helios alpha thermospectronic. Reduced glutathione (GSH) was assayed by Ellman's method (1959). The procedure is based on the reduction of Ellman's reagent (5, 5-dithiobis- 2-nitrobenzoic acid) by –SH groups of GSH to form 2-nitro-5-mercaptobenzoic acid, which has an intense yellow color. The absorbance was measured at 412 nm and the GSH concentration was calculated by comparison with a standard curve.

Determination of enzyme activities:-
Glutathione-S-transferase (GST) activity was determined according to the method of Habig et al. (1974). 0.4 ml potassium phosphate buffer (50 mmol/l; pH 6.5), 0.1 ml of supernatant, 1.2 ml water and 0.1 ml CDNB (1-chloro-2, 4 dinitrobenzene, 30 mmol/l) were added and incubated in a water bath at 37°C for 10 min. After incubation, 0.1 ml of reduced glutathione (30 mmol/l) was added. The change in absorbance was measured at 340 nm at 1 min interval. Catalase activity was measured using Santacruz Company (Bangalore), which is based on the spectrophotometric method described by Aebi (1984). Catalase reacts with a known quantity of hydrogen peroxide and the reaction is stopped after 1 min with catalase inhibitor. In the presence of peroxidase, the remaining hydrogen peroxide reacts with 3,5- dichloro-2- hydroxybenzene sulfonic acid and 4- aminophenazone to form a chromophore with a color intensity inversely proportional to the amount of catalase in the sample. The absorbance was measured at 510 nm.

Serum enzymes assay:-
The concentration of urea and createnine was measured by Barham and Trinder (1972) method using reagent kits No: CA2120 purchased from Santacruz Company (Bangalore).

Statistical analysis:-
The data were expressed as means, standard error of mean (S.E.M.) All variables were tested for normal distribution and compared using the independent t-test. All analyses were performed using the Statistical Package for Social Sciences (SPSS) software in a PC-compatible computer and the significance was set at $p < 0.05$.

Results:-

The results of the present study revealed that lipid peroxidation level was significantly increased in the liver tissue after the daily oral administration of lower doses of BPA for 9 days, recording +47.81 % above the control level (Table-1). On the other hand, the only significant decrease in liver catalase activity was obtained after 9days of daily BPA administration at the low and higher doses of BPA. A significant decrease in the level of liver GSH was also evident after the administration of both the high (500 mg/kg) and lower (25 and 50 mg/kg) doses of BPA, respectively. BPA also resulted in an increase in the activity of GST after 9days of administration of the high dose (+15.45%) and after 9days of treatment with the low doses level (+31.74%). In the kidney (Table-2), significant changes in lipid peroxidation and GSH levels and in the activity of catalase occurred after the daily oral administration of BPA at the two dose levels. The only significant change obtained was an increase in GST activity after 9days of oral administration of of BPA. It is clear from table (3) that the oral administration of BPA at the dose of 25mg/kg, 50 mg/kg & 500 mg/kg for 9days caused a significant increase in urea and createnine activities as compared to the control. However, the two dose levels had significant effect on the activities of both urea and createnine after 9days compared to the control values. In control rats, the major ovarian follicles were secondary

follicles (S.F), and there was little corpus lutea (C.L). In contrast, there were more primary follicles (P.F) and scanty corpus lutea (C.L) in BPA treated rats that are lower and higher dosing (Figure-1).

Table (1): Effect of oral administration of bisphenol-A on some oxidative stress parameters in the liver of pregnant albino rats:

	Control	**BPA 25mg/kg/d**	**% d**	**BPA 50mg/kg/d**	**% d**	**BPA 500mg/kg/d**	**% d**
MDA (nmol/g tissue)	56.90 ±2.13(6)	83.58 ±9.01*(6)	47.81	36.07 ±3.08*(6)	-37.67	60.75 ±3.55(6)	-3.20
Catalase (U/g tissue)	8.01 ±0.17(6)	8.39 ±0.14(6)	-4.99	7.40 ±0.17(6)	-6.87	6.85 ±0.47*(6)	-14.36
GSH (nmol/g tissue)	1.48 ±0.19(6)	0.86 ±0.12*(6)	-42.00	0.70 ±0.11*(6)	-50.33	0.75 ±0.08*(6)	-48.67
GST (U/g tissue)	18.03 ±0.66(6)	25.04 ±0.69*(6)	31.74	18.10 ±0.89(6)	-4.83	20.97 ±1.05*(6)	15.45

Data are expressed as Mean ± S.E.M., wih the number of animals between parantheses.
*: Significant at $p < 0.05$.
%d: percentage difference.

Table (2): Effect of oral administration of bisphenol-A on some oxidative stress parameters in the kidney of pregnant albino rats:

	Control	BPA 25mg/kg/d	% d	BPA 50mg/kg/d	% d	BPA 500mg/kg/d	% d
MDA (nmol/g tissue)	46.65 ±4.95(6)	49.87 ±2.92(6)	-4.70	46.87 ±2.58(6)	-1.59	49.14 ±3.10(6)	-1.07
Catalase (U/g tissue)	4.87 ±0.14(6)	5.65 ±0.19(6)	-3.24	5.30 ±0.30(6)	-9.37	5.6 0±0.30(6)	-1.19
GSH (nmol/g tissue)	0.71 ±0.11(6)	0.60 ±0.08(6)	-15.28	0.59 ±0.06(6)	-16.67	0.50 ±0.03(6)	-27.78
GST (U/g tissue)	18.10 ±0.66(6)	25.04 ±0.13*(6)	20.11	16.68 ±0.09(6)	-8.04	17.36 ±0.09(6)	4.19

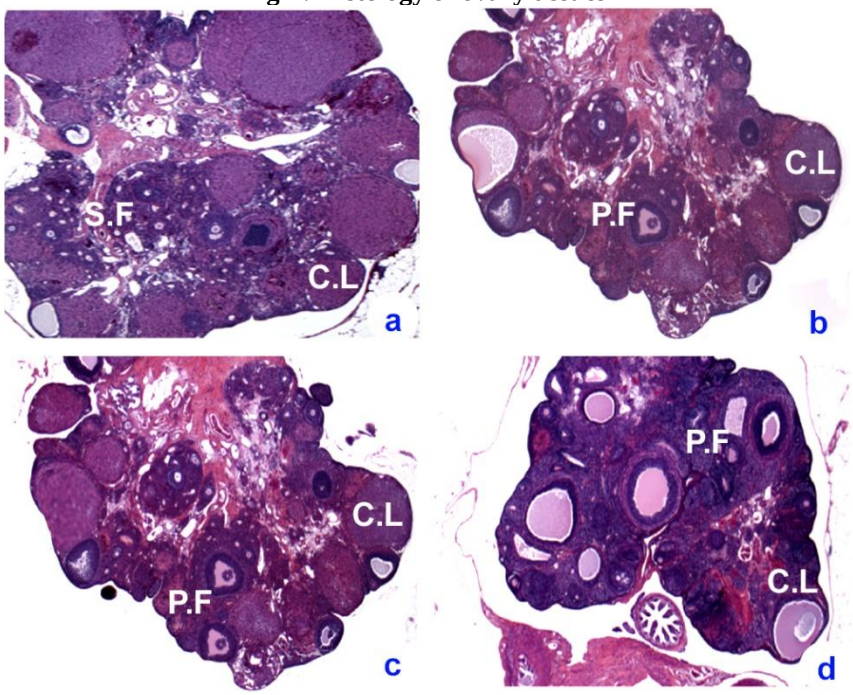
Data are expressed as Mean ± S.E.M., wih the number of animals between parantheses.
*: Significant at $p < 0.05$.
%d: percentage difference.

Table (3): Effect of oral administration of bisphenol-A on serum urea and createnine activity levels:

	control	BPA 25mg/kg/d	% d	BPA 50mg/kg/d	% d	BPA 500mg/kg/d	% d
Createnine (mg/kg/day)	24.11 ±2.66(6)	30.36 ±2.98*(6)	32.15	34.92 ±1.32(6)	6.31	46.75 ±2.20(6)	8.65
Uricacid (mg/kg/day)	1.52 ±0.12(6)	2.20 ±0.56*(6)	45.10	3.10 ±0.08*(6)	39.87	5.90 ±0.63(6)	27.45

Data are expressed as Mean ± S.E.M., with the number of animals between parantheses.
*: Significant at $p < 0.05$.
%d: percentage difference.

Fig-1: Histology of ovary tissues



a: control; **b:** 25 mg/kg/b.wt BPA; **c:** 50 mg/kg/b.wt BPA, **d:** 500 mg/kg/b.wt BPA

Discussion:-

BPA is not only widespread in the environment but also commonly ingested by humans. The growing interest by scientists and the public alike has placed BPA at the center of the debate over adverse effects of endocrine disruptors on fetal development, reproductive fecundity and carcinogenesis. The widespread consumption of BPA-containing products has raised concerns among scientists and regulatory agencies that human exposure to BPA may have adverse effects on different vital organs. In the present study, the recorded significant increase in lipid peroxidation and GST activity which was accompanied by a significant decrease in GSH in the liver of rats treated with BPA (25, 50 &5 00mg/kg) for 9days reflects a state of oxidative stress in liver cells. GSH is the most important freely available antioxidant, which acts directly as an antioxidant and also participates in catalytic cycles of several antioxidant enzymes such as glutathione peroxidase, glutathione reductase and glutahthione-S-transferase (Biswas and Rahman, 2009). In addition, GST catalyzes the conjugation of reduced glutathione – via a sulfhydryl group – to electrophilic centers on a wide variety of substrates (Douglas, 1987). This activity detoxifies endogenous compounds such as peroxidized lipids (Leaver and George, 1998) as well as breakdown of xenobiotics. Atkinson and Roy (1995) reported that BPA is oxidized to a reactive metabolite bisphenol-O quinone and major DNA adduct increased in rat liver DNA at the presence of peroxidase activation. BPA has been shown to decompose too many kinds of metabolites probably including BPA radical by a reaction of radical oxygen (Sajiki, 2001). Previous studies showed that macrophages generate ROS such as superoxide anion and hydrogen peroxide in liver after exposure to hepato-toxicants (McCloskey et al., 1992).

Accordingly, it could be concluded that the oxidative stress induced by BPA in liver of rats treated with lower and higher doses of BPA may be due to the formation of ROS arising from reduced mitochondrial fractions and the formation of quinine radical, one of the BPA metabolites. The raised activity of GST may be at the expense of the content of reduced GSH that acts as a catalyst for GST in pregnant rats. At the same time, GST catalyzes the conjugation of reduced glutathione to electrophilic centers of endogenous compounds to detoxify peroxidized lipids that recorded a significant increase in the present study. This could explain the decreased content of GSH and the increased activity of GST that were recorded in the liver after the higher dose exposure of BPA in early gestational rats. In the liver of rats treated with BPA at a dose level of 25 mg/kg, 50mg/kg & 500mg/kg for 9days, a significant decrease in GSH content and catalase activity has been observed. However, GST activity showed a significant increase. Bindhumol et al. (2003) suggested that the reduction in the activity of catalase may reflect the inability of liver mitochondria and microsomes to eliminate hydrogen peroxide produced after exposure to BPA. This may be

due to the enzyme inactivation caused by excess ROS production in mitochondria (Pigeolet et al., 1990). On comparing the effect of the different doses of BPA on liver lipid peroxidation, we observed that there was significant change after the lower doses of BPA, while the higher dose of BPA induced a significant increase in liver lipid peroxidation. Catalase is a common enzyme found in nearly all living organisms that are exposed to oxygen, where it catalyzes the decomposition of hydrogen peroxide to water and oxygen. Thus the reduced activity of catalase after 9days in the liver of rats treated with BPA which was accompanied by reduced GSH content and increased GST activity could explain the ability of these antioxidant mechanisms to prevent the increase in lipid peroxidation. The results of the present study are in agreement with the study of Bindhumol et al. (2003) who found that BPA significantly decreased the activities of antioxidant enzymes and increased lipid peroxidation in the liver thereby increasing oxidative stress. Pregnancy is one of the major physiological events in which the elimination process by glucuronidation in the liver is dramatically altered. In pregnancy, glucuronidation activities toward bilirubin, *p*-nitrophenol, and ethynylestradiol are attenuated to half to onethird (Luquita et al., 2001). BPA glucuronidation is also reduced in the rat liver microsomes during pregnancy (Matsumoto et al., 2002). Moreover, the expression of multidrug resistance associated protein (MRP) families which mediate transport of chemical glucuronide is limited in pregnancy (Cao et al., 2002).These findings led us to hypothesize that elimination of bisphenol-A from the liver may be curtailed in pregnancy. The potential public health hazards of bisphenol-A to the fetus remain known, but if hepatic glucuronidation of the chemical is retarded in pregnancy, then the level of exposure of the fetus is expected to increase accordingly.

Endocrine-disrupting chemicals (BPA) are exogenous agents with the ability to interfere with processes regulated by endogenous hormones. One such process is female reproductive function. The major reproductive organ in the female is the ovary. Disruptions in ovarian processes by EDCs can lead to adverse outcomes such as anovulation, infertility, estrogen deficiency, and premature ovarian failure among others. Clear adverse effects of BPA on the ovary (increased cystic follicles, depleted corpora lutea, and antral follicles), were observed at the lower & higher doses of BPA in this study. The cycling ovary contains ovarian follicles at different stages of development and, following ovulation, one or more corpora lutea depending on the species. The process by which the most immature follicles (primordial) develop into preovulatory follicles is termed folliculogenesis (Oktem & Urman (2010). BPA has various effects on the ovary. For instance, BPA exposure is associated with follicle loss (Souter, I et al., 2013). It caused lower antral follicle counts (Souter, I et al., 2013), decreased oocyte survival (Brieno-Enriquez, M.A et al., 2011), and even significant loss of primordial follicles by reducing ovarian follicular reserves in F3 generation females (Manikkam, M et al., 2015). Polycystic ovary syndrome (PCOS) is the most common endocrinopathy of women of reproductive age. Its cardinal features are hyperandrogenism, insulin resistance and chronic anovulation (Diamanti-Kandarakis, E et al., 1999). The distinctive clinical features are hirsutism, menstrual disfunction and infertility (Brown, M.A et al., 2007). Its etiology is not yet clear. Both human (Akın, L et al., 2015) and animal studies (Fernandez, M.; et al., 2010) have suggested a possible role of BPA in PCOS aetiopathogenesis. Recently, it was shown that women with PCOS had a significantly higher BPA level than women without PCOS (Kandaraki E.; et al., 2011). Neonatal rats exposed to 500 µg/day BPA developed PCOS-like syndrome in adulthood (Fernandez, M.; et al., 2010).

The present data showed significant changes in oxidative stress parameters in the kidney due to BPA treatment in early gestation rats. However, serum urea increased significantly after 9 days of the daily oral administration of BPA at both the higher (500 mg/kg) and lower (25 &50 mg/kg) dose levels. In the light of the present results, this increase in urea & createnine could be attributed to impairment in kidney function of pregnant rats. Due to limited information considering the toxic effect of different concentrations on kidney, our study used four different concentrations for evaluating the toxic effect of BPA on pregnant Wister Albino rat. Kidney function testes including urea and creatinine evaluated the presence of kidney damage or disease. Data presented in our study demonstrate that high dose of BPA 500mg/kg/ day significantly increased the urea and creatinine levels in treated group than control group. Urinary excretion of triclosan, and possibly BPA, decreased with decreasing renal function. The associations might differ by age or sex (You *et al*., 2011). The study that carried out by Reimschuessel, 2001 on fish showed that kidney, liver, brain and gills are the most vulnerable organs of a fish exposed to the medium containing any type of toxicant. They reported that after exposure to a variety of renal toxicants, effects in many fish species at all life stages, including rainbow trout, goldfish, tilipia and Zebrafish. The renal tubules are particularly sensitive to toxic influences, in part because they have high oxygen consumption and vulnerable enzyme systems, and in part because they have complicated transport mechanism that may be used for transport of toxins and may be damaged by such toxins. Also the tubules come in contact with toxic chemicals during their excretion and elimination by the kidneys (Tisher *et al*., 1989). Many chemicals had a direct nephrotoxic

action and excreted their effects principally on the proximal convoluted tubules. Zha *et al*., (2008) showed that Nonylphenol (at concentration from 37 to 150µg/l) caused a modification of the structure and function of the kidney and other organs such as the liver and gills (Bhattacharya *et al*., 2008; Zha *et al*., 2008).The organization of the urinary tubules and seminiferous tubules of the kidney was found altered (Roig *et al*., 2014). Tan *et al*., (2003) observed that BPA also caused the enlargement of the kidney and hydronephrosis. Jyothi *et al*., (2009) observed significant increases values in the concentration of serum creatinine when exposed with cyclophosphamide in rats on kidney. Okolie and Osagie (1999) have observed that serum urea and creatinine were significantly higher in the cyanide group relative to controls. Zurovsky and Haber (1995) have reported that sodium nitrate treatment, urea and creatinine were increased in the serum but decreased in the kidney, suggesting an impairment of kidney functions. In this study we observed that distribution of BPA at dose 500mg/kg/day for 15 days caused significant increasing in serum urea and cereatinine levels, suggesting an impairment of renal functions.

In the light of the present results, it could be concluded that high doses of BPA have serious effects on the liver, kidney and ovaries. These effects are mediated by the oxidative stress induced by BPA. Furthermore, the results of the present study suggest that in a population of high use of plastics where there is a great chance of exposure to BPA, the females may suffer from sexual disturbances due to the oxidative stress induced in the reproductive organs. Thus, the use of BPA in different plasticizers and other industries should be limited and the erroneous handling of plastic containers should be avoided to reduce the health risks resulting from exposure to these endocrine disruptors including BPA.

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