The Possible Protective Effects of Some Antioxidants against Growth Retardation and Malformations Induced By Bisphenol-A in Rats

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Abstract: Bisphenol A is a widely used industrial plasticizer with known estrogenic properties. It is omnipresent in the environment and widely distributed and unavoidable. It accumulates in pregnant adult females and its continued exposure during gestation is likely to have an impact on the development of the fetus. It is well known that Omega-3 fatty acids, garlic and Zn have a potent antioxidant activity and play an important role in normal growth and development. The aim of the present work was to evaluate the possible protective effects of some antioxidants against growth retardation and malformations induced by Bisphenol A in rats. Bisphenol A was administered orally from 6th day of gestation to 15th day in a dose of (300mg/kg, P.O.). Omega-3 (1200 mg/kg P.O), garlic (500 mg/kg P.O) or Zn (32 mg/kg P.O) was administered prior to BPA (300 mg/kg P.O) during the same period of gestation. The study indicated that administration of Bisphenol A significantly elevated the rate of resorption, significantly decreased fetal growth as well as induction of external and skeletal malformations. Also, it caused diffuse liquifactive necrosis and suppurative inflammatory reaction as well as pus cells infiltration and massive inflammatory cells infiltration mainly neutrophils were noticed in the giant cells and underlying labyrinth layer of placenta. Omega-3 and garlic protected from fetal resorption, growth retardation, external, skeletal malformations and placental necrosis induced by BPA. On the other hand zinc protected from external malformations only, but has no effect on resorption, growth retardations, and skeletal malformations as well as placental necrosis induced by Bisphenol A.

[Hebatalla I Ahmed, Essam EzzEldin, Amany A Ahmed, Azza A Ali. **The Possible Protective Effects of Some Antioxidants against Growth Retardation and Malformations Induced By Bisphenol-A in Rats**. *Life Sci J* 2013; 10(4): 1575-1586]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 208

Key wards: Bisphenol A, Teratogenicity, Malformation, Omega-3, Garlic, Zn, Rats.

1. Introduction

Bisphenol A (BPA) is a widely used industrial plasticizer with known estrogenic properties. It is used in the manufacture of epoxy resins and polycarbonate plastics, which in turn find application in a wide variety of domestic products. BPA is present in dental fillings, plastic food and water containers, baby bottles, food wrap, as well as in the lining of beverage and food cans, presenting a large number of routes for human exposure **(Le *et al.*, 2008, Vandenberg *et al.*, 2009).**

It was confirmed that BPA can leach from food containers. Normal use such as storage, brushing and dishwashing can also result in polymer degradation leading to release of BPA **(Goodson *et al.*, 2004).**

The hazardous effects of BPA may lead to heart disease, insulin resistance syndrome, type II diabetes, dyslipidemia, obesity and high blood pressure and liver toxicity **(Elobeid and Allison, 2008, Newbold *et al.*,2009).**

Bisphenol A accumulates in pregnant adult females likely because of accumulation in fat **(Fernandez *et al.*,2007),** also is particularly potent during fetal and neonatal development because the liver has limited capacity to deactivate BPA in fetuses and newborns. Exposure of the developing fetus to BPA is of particular concern as the compound readily crosses the placental barrier and accumulates both in the placenta and in the fetus **(Takahashi and Oishi, 2000).**

It was shown that prenatal exposure to BPA is associated with changes in hypothalamic pituitary gonadal axis function, mammary development and cognitive function as well as sex-specific behaviors in the offspring **(Markey *et al.*, 2001, Vandenberg *et al.*, 2009).** Also continued exposure to BPA during gestation is likely to have an impact on the development of the fetus and may lead to intra-uterine growth retardation **(Markey *et al.*,2002).**

Studies have been conducted in 2009 showing association between BPA exposure and oxidative stress, which might contribute to some of its toxic effects **(Hong *et al.*, 2009).**

Omega-3 polyunsaturated fatty acids (PUFAs) such as eicosahexanoic acid (EPA) and docosahexaenoic acid (DHA) are found in high

proportion in fish oil, and it has been proposed that these molecules may have anti-inflammatory, antithrombotic, antioxidant, and immunomodulatory functions and neuroprotective effects on the synaptogenesis and biogenesis of the neuronal membrane **(Ramirez-Ramirez *et al.*, 2013).** EPA and DHA play a crucial role in brain function as well as normal growth and development. Both have been shown to have multiple beneficial effects, including improving childhood development when ingested during pregnancy **(Jensen, 2006).**

Allium sativum L. (Liliaceae), whose common name is garlic, has been known to mankind for centuries. It is widely used around the globe as a spice, food and home remedy **(Lawson, 1998).** Several studies have shown that garlic has a beneficial effect on cardiovascular risk factors such as dyslipidemia, high blood pressure and glucose levels. In addition, it has an antioxidative effect, an antiplatelet aggregation effect, and enhances fibrinolytic activity (**Rahman, 2007; Ried *et al.*, 2010).** Moreover, garlic has protective effects during pregnancy that may be attributed to inhibition of enzymes involved in lipid synthesis, prevention of lipid peroxidation, LDL oxidation by scavenging superoxide and inhibiting the formation of lipid peroxides**(Dillon *et al.*,2003)**and increasing antioxidants level **(Rahman and Lowe, 2006).**

Zn is a key nutrient during embryogenesis, foetal growth and development, and mammary gland function for milk synthesis and secretion **(Maret, 2009).** Dietary Zn intake prevents alterations in the body status of lipids, so protect against some toxic effects, including oxidative damage to the cellular membranes and atherogenic action **(Rogalska *et al.*, 2009).** Also, the administration of Zn enhances the bone alkaline phosphatase activity and prevents bone resorption, so dietary intake of Zn may have a protective influence on the skeleton **(Brzóska *et al.*,2007).**

In the light of what was mentioned, the present work was undertaken to assess the teratogenic effects of BPA which is widely distributed and unavoidable and to study the possible protective effects of omega-3, garlic and Zn (as potent antioxidants) against the teratogenic effects induced by BPA.

2. Materials and Methods:

2.1. Animals:

Female Wistar albino rats weighting 160-180 g and three months age were used in this study. They were obtained from NILE Co. for Pharmaceutical and Chemical Industries, Cairo, Egypt. Animals were kept under the same adequate environmental conditions. Rats were housed in stainless steel cages, three to four rats per cage. The animals were kept on standard pellet diet and had free access to water.

2.2. Drugs and chemicals

BPA was purchased from Sigma -Aldrich Chemical Co., St. Louis, USA.) It was freshly suspended in corn oil. Omega- 3 fatty acids were purchased from Egyptian market as fish oil capsules. Zinc sulfate was purchased as white powder from El Nasr Chemical Co., Abu Zaabal, Cairo, Egypt. It was freshly dissolved in distilled water. A preparation of whole crude garlic aqueous extract was carried out as follows: Fresh peeled cloves of garlic purchased from Egyptian native market were sliced into small pieces and ground in a clean mortar using a mortar pestle to produce a fine paste, then suspending the paste in distillate water and daily administered orally **(Singh *et al.*,1995).** All other chemicals were of the highest analytical grade.

2.3. Experimental protocol

Preliminary studies showed that BPA in dose 100 mg/kg is not teratogenic and 1000 mg/kg is highly toxic showing complete resorption or even death of dams, While BPA in 300 mg/kg was found to be the teratogenic dose.

Pregnant females were divided into five groups; each group contained 6 pregnant females which were subjected to the following schedule of drug treatment: rats of the 1st group received vehicle (distillated water and corn oil) orally. Rats of the 2nd group received BPA alone (300 mg/kg P.O.) **(Kim *et al.*, 2001)**. Rats of the 3rd group received omega-3 (1200 mg/kg P.O) **(Arterburn *et al.*,2000)** prior to BPA (300 mg/kg P.O). Rats of the 4th group received garlic (500 mg/kg P.O) **(Singh *et al.*,1995)** prior to BPA (300 mg/kg P.O). Rats of the 5th group received Zn (32 mg/kg P.O) **(Piechal *et al.*,2012)** prior to BPA (300 mg/kg P.O). All drugs were daily administered orally in dose volume not exceeding 0.5ml/100g body weight starting from 6th to 15th day of gestation.

2.4. Experimental Procedures

Female rats in pro oestrus and oestrus phases (time of ovulation), as determined by examination of vaginal smear were housed with males; three females were housed with one male in its cage. The following morning was designed as day one of pregnancy for females with sperms in the vaginal smears. Prior to delivery, at day 21 of pregnancy, females were anesthetized by ether and the fetuses were removed by cesarean operation. The fetuses were identified as living or dead by the presence of movement after stimulation, sexed **(Kelsey, 1974)** and the number of corpora lutea were counted for both ovaries after removal of ovaries bursa and the number of resorptions were counted. Sex ratio, Placental weight, growth parameters (Including body weight and crown rump length) were recorded for each fetus. The off

springs were removed and examined for external malformations **(Calderon *et al.*, 1992).**

Internal examination:

Fetuses were fixed in Bouin's solution for seven days **(Beck, 1977).**

The head of the fetus was removed and head transverse sections were made through the nasal region, the orbital region and between frontal and parietal bones. The abdominal wall of the fetuses was opened and internal organs were examined for any gross malformations. In addition, transverse sections were made through the kidney **(Wilson, 1973).**

Examination of the skeleton:

The examination of the skeleton of fetuses was carried out according to Alizarin Red-S staining technique. This technique involves fixation of the fetuses, clearing of the soft tissues, staining of the skeleton and removal of excess dye from the soft tissues **(Staples and Schnell, 1964).**

Histopathology of placenta:

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

2.5. Statistical analysis:

Results are presented as mean ± standard error (SE). Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons tests(using GRAPHPAD INSTAT 3 (ISI software) computer program (2006) for placental weight, fetal body weight and length and Chi Square test for percent of resorption, sex ratio, external and skeletal malformations**(Zar, 1999).**

3. Results:

3.1. Incidence of Resorption:

BPA (300 mg/kg) significantly increased the rate of resorption from 4.3% (incidence percentage) to 17.5%. Administration of omega-3(1200mg/kg) or garlic (500mg/kg) prior to BPA (300 mg/kg) decreased significantly the rate of resorption from 17.5% to 2.2 % and 1.9% respectively. Administration of Zn (32 mg/kg) prior to BPA (300 mg/kg) insignificantly decreased the rate of resorption as compared to BPA alone treated group (Figure 1).

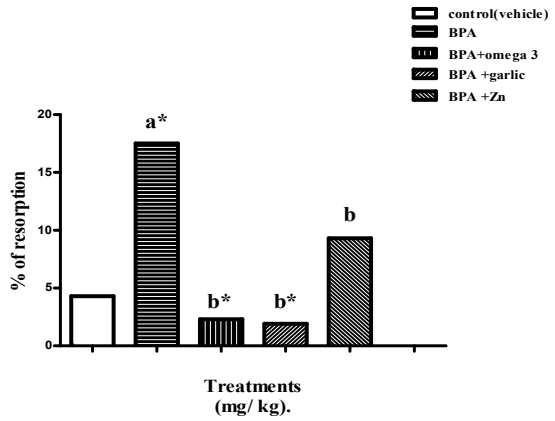


Figure (1): **Effect of omega-3, garlic and Zn on resorption induced by BPA.**

Statistical analysis was carried out using Chi-Square test (n of dams = 6).

(a) Compared to the corresponding control group.

(b) Compared to the corresponding group receiving BPA (300 mg/kg).

(*) Significantly different at P<0.05.

3.2. Placental changes: administration of BPA in 300 mg/kg, insignificantly decreased the mean placental weight to 0.408 ±0.014 g from 0.452 ±0.044 g as compared to the corresponding control group. Administration of omega-3 (1200mg/kg), garlic (500mg/kg) or Zn (32 mg/kg) prior to BPA (300 mg/kg) did not significantly alter the mean placental weight as compared to BPA alone treated group (Figure 2).

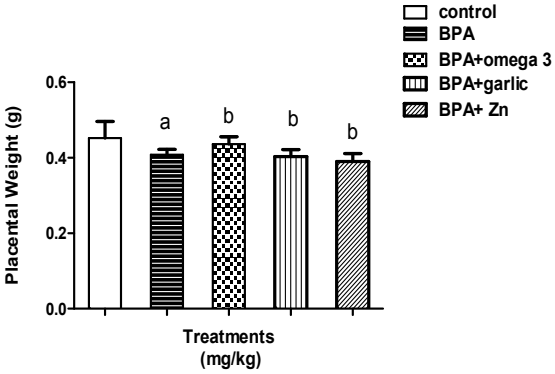


Figure (2): **Effect of BPA, omega-3, garlic and Zn on Placental Weight.**

Data are expressed as mean ± SE (n of dams=6)

Statistical analysis was carried out using one way ANOVA analysis test followed by Tukey- Kramer multiple comparisons test .

(a) Compared to the corresponding control group. (b) Compared to the corresponding group receiving BPA(300 mg/kg).

3.3. Effect on sex ratio: administration of BPA in 300 mg/kg, insignificantly decreased the sex ratio to 57.1% from 69.2% as compared to the corresponding control group. Administration of omega-3 (1200mg/kg), garlic (500mg/kg) or Zn (32 mg/kg) prior to BPA (300 mg/kg) did not significantly alter the sex ratio as compared to BPA alone treated group (Figure 3).

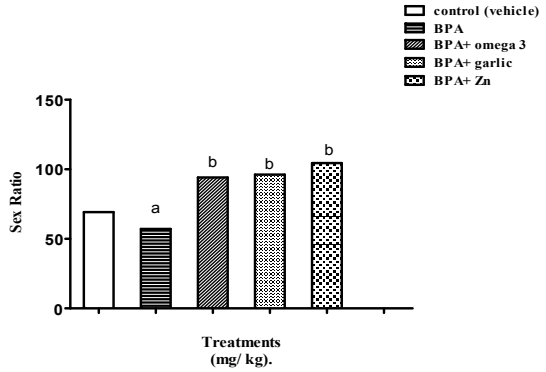


Figure (3): Effect of BPA, omega-3, garlic and Zn on sex ratio.

Statistical analysis was carried out using Chi-Square test (number (n) of dams = 6).
(a) Compared the corresponding control group. (b) Compared to the corresponding group receiving BPA (300 mg/kg).

3.4. Fetal growth: This is evaluated by the body weight and crown-rump length (body length). Aadministration of BPA in 300 mg/kg, significantly decreased the mean body weight and body length from the corresponding control group. Co treatment of omega-3 (1200mg/kg) or garlic (500mg/kg) with BPA (300 mg/kg) significantly increased the mean body weight and body length as compared to BPA alone treated group. Administration of Zn (32 mg/kg) prior to BPA (300 mg/kg) did not significantly alter the mean body weight or body length as compared to BPA alone treated group (Figures 4a, 4b).

3.5. Incidence of Malformations:

BPA (300 mg/kg) significantly increased the rate of external malformation (The types of malformations were phocomelia, edema, coiled tail and hematocele) from 0% to 14.9 % as compared to control group (Table 1). The rate of internal malformation (The mainly examined internal malformations were hydronephrosis and hydrocephellus) did not significantly change following administration of BPA (300 mg/kg). Skeletal malformations upon administration of BPA (300 mg/kg) increased significantly from 23.3% (spontaneous skeletal malformations of control group) to 60.7%. Most skeletal malformations reside in the ribs, sternebrae, metacarpals, metatarsals, parietal, inter-parietal, frontalas well as supra occipital bones (Tables 2, 3). Administration of omega-3 (1200mg/kg) or garlic (500mg/kg) prior to BPA (300 mg/kg) significantly decreased the rate of external and skeletal malformations from group receiving BPA (300 mg/kg) alone while Zn (32 mg/kg) prior to BPA (300 mg/kg) significantly decreased the rate of external malformations only. Following administration of omega-3 (1200 mg/kg), garlic (500mg/kg) or Zn (32 mg/kg) prior to BPA (300 mg/kg), there was no incidence of hydronephrosis or hydrocephellus.

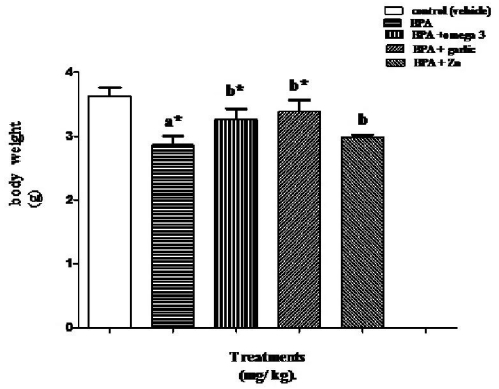


Figure (4a): Effect of omega-3, garlic and Zn on body weight decrease induced by BPA.

Data are expressed as mean ± SE (n of dams =6).

Statistical analysis was carried out using one way ANOVA analysis test followed by Tukey- Kramer multiple comparisons test.

(a) Compared to the corresponding control group. (b) Compared to the corresponding group receiving BPA 300 mg/kg.

(*) Significantly different at P<0.05.

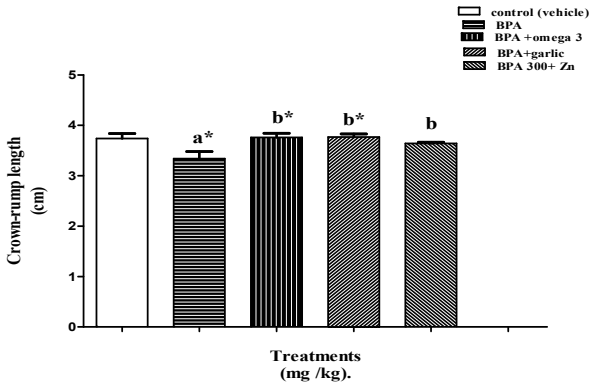


Figure (4b): Effects of omega-3, garlic and Zn on crown-rump length decrease induced by BPA.

Data are expressed as mean ± SE (n of dams=6).

Statistical analysis was carried out using one way ANOVA analysis test followed by Tukey- Kramer multiple comparisons test.

(a) Compared to the corresponding control group. b) Compared to the corresponding group receiving BPA mg/kg.

(*) Significantly different at P<0.05.

Table(1): Incidence of foetal external malformations following administration of BPA, omega-3, garlic and Zn treatment.

Groupnumber	Treatments (mg/kg)	No. of examined fetuses	Frequency of malformations	No. of Malformed fetuses	% of malformations (No. of malformed fetuses/ examined fetuses)	Average of malformations (No. of malformations/ No. of fetuses)
1	Control (vehicle)	44	0	0	0	0
2	BPA 300 mg/kg	47	7	7	14.9[a*]	0.15
3	BPA 300 mg/kg+omega-3(1200 mg/kg)	44	2	1	2.3[b*]	0.05
4	BPA 300 mg/kg+Garlic 500 mg/kg	51	1	1	1.9[b*]	0.02
5	BPA 300 mg/kg+Zn 32 mg/kg	51	1	1	1.9[b*]	0.02

Statistical analysis was carried out using Chi-Square test (n of dams= 6).
(a) Compared to the corresponding control group. (b) Compared to the corresponding group receiving BPA 300 mg/kg.
(*) Significantly different at $P<0.05$.

Table(2): Incidence of foetal skeletal malformations following administration of BPA, omega-3, garlic and Zn treatment.

Subgroup number	Treatments (mg/kg)	No. of examined fetuses	No. of malformed fetuses	% of malformation
1	**Control (vehicle)**	30	7	23.3
3	**BPA 300 mg/kg**	28	17	60.7a*
4	**BPA 300 mg/kg+omega-3(1200 mg/kg)**	26	1	3.8[b*]
5	**BPA 300 mg/kg+ garlic 500 mg/kg**	33	2	6.1[b*]
6	**BPA 300 mg/kg+Zn 32 mg/kg**	33	17	51.5[b]

Statistical analysis was carried out using Chi-Square test (n of dams= 6).
(a) Compared to the corresponding control group. (b)Compared to the corresponding BPA 300 mg/kg group.
(*) Significantly different at $P<0.05$.

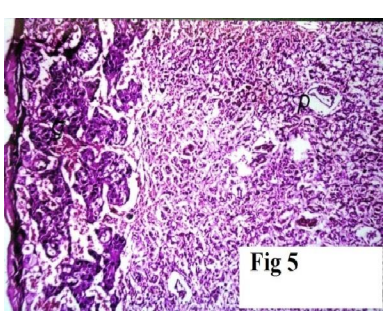
Table (3): Types of fetal skeletal malformations induced following administration of BPA, omega-3, garlic and Zn treatments.

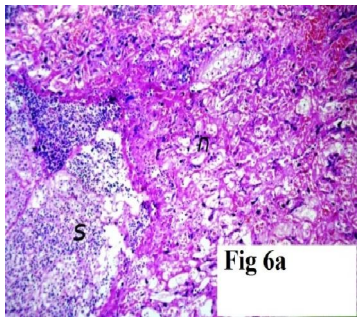
Types of the delay	**Control (vehicle)**	**BPA 300 mg/kg**	**BPA300 mg/kg+omega-3(1200 mg/kg)**	**BPA 300 mg/kg+Garlic 500mg/kg**	**BPA 300 mg/kg+ Zn 32 mg/kg**
Number of examined fetuses	30	28	26	33	33
Short ribs	-	-	-	-	3
Rudiment ribs	-	-	-	-	1
Poorly ossified supraoccipital	4	5	1	1	5
Poorly ossified interparietal		4	-	1	-
Poorly ossified parietal	3	10	-	1	3
Poorly ossified frontal		8	-	-	2
Poorly ossified sternebrae	-	15	-	1	13
Poorly ossified metacarpals	-	3	-	1	2
Unossified metacarpals		3			
Poorly ossified metatarsals	-	13	-	2	11
Unossified metatarsals		3			
Absence of vertebrae, ribs	-	1	-	-	-
Number of malformed fetuses	7	17	1	2	17
Frequency of malformations	7	65	1	7	40
Average malformations/ Fetus	0.23	2.3	0.04	0.21	1.2
% of malformations	23.3	60.7[a*]	3.8[b*]	6.1[b*]	51.5[b]

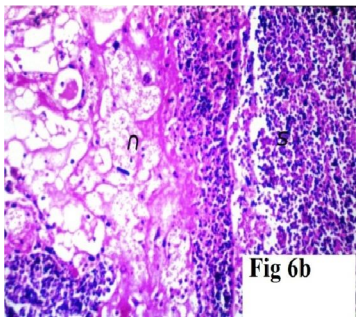
Statistical analysis was carried out using Chi-Square test (n of dams= 6).
(a) Compared to the corresponding control group. (b)Compared to the corresponding BPA 300 mg/kg group.
(*) Significantly different at $P<0.05$.

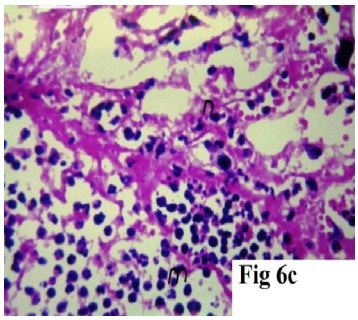
3.6. Histopathological findings: There was no histopathological alteration observed in the placenta of control group and the normal histological structure of the giant cell layer with blood vessels and the underlying labyrinth with villi and trophospongium were recorded in (Fig. 5) . Administration of BPA (300 mg/kg) resulted in diffuse liquifactive necrosis and suppurative inflammatory reaction as well as pus cells infiltration and massive inflammatory cells infiltration mainly neutrophils in the giant cells and underlying labyrinth layer (Figs. 6a, b, c). Administration of omega-3 1200mg/kg prior toBPA (300 mg/kg) showed no histopathological alteration in the giant cells, blood vessels and the underlying

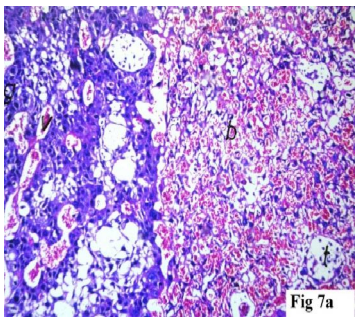
labyrinth layer with the trophospongium and villi (Figs. 7a, b, c). Administration of garlic 500 mg/kg prior to BPA (300 mg/kg) showed no histopathological alteration and the normal histological structure of the giant cell layer with blood vessels and the underlying labyrinth with villi and trophospongium were recorded(Figs. 8a, b). Administration of Zn 32 mg/kg prior to BPA (300 mg/kg) showed necrosis and liquifaction in the labyrinth layer, giant cells, trophospongium and villi associated with massive number of pus cells and other inflammatory cells infiltration mainly neutrophils (Figs. 9a, b, c).

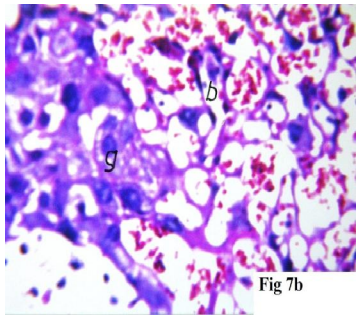












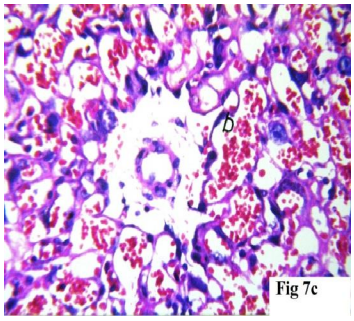


Figure (5): Fetal placenta of control rats showing normal histological structure of giant cell layer(g) with the underlying labyrinth layer with fetal villi(p).(H&E stain, x16).

Figure (6a): Fetal placenta of rats receiving BPA (300 mg/kg) showing diffuse liquifactive necrosis with suppurative inflammatory reaction as well as pus cells infiltration in the giant cells layer (s) and underlying labyrinth layer (n).(H&E stain, x16).

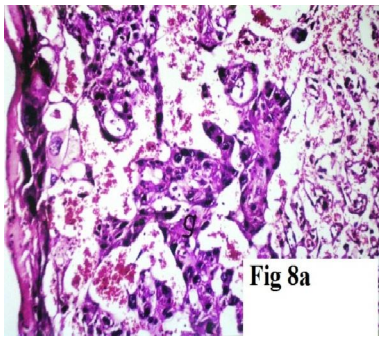
Figure (6b): Fetal placenta of rats receiving BPA (300 mg/kg) showing massive number of inflammatory cells infiltration and pus cells(s) in the giant layer.(H&E stain, x40).

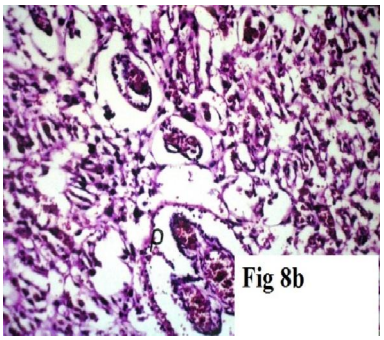
Figure (6c): Fetal placenta of rats receiving BPA (300 mg/kg) showing the magnification of figure (6b) to identify the different leucocytes inflammatory cells and pus cells(m) in liquifactive necrosis of placental layer.(H&E stain, x160).

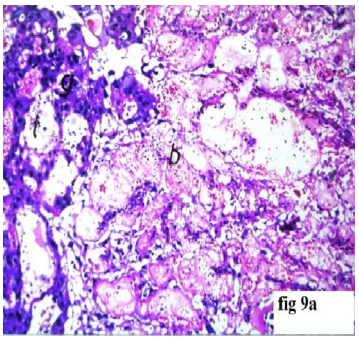
Figure (7a): Fetal placenta of rats receiving omega-3 1200mg/kg prior to BPA (300 mg/kg) showing normal histopathological structure of giant cells(g) with blood vessels (v)and the underlying labyrinth layer(b) with the trophospongium (t).(H&E stain, x16).

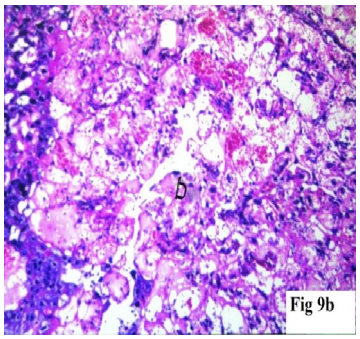
Figure (7b): Fetal placenta of rats receiving omega-3 1200mg/kg prior to BPA (300 mg/kg) showing the magnification of fig. (7a) to identify the giant cells layer(g).(H&E stain, x40).

Figure (7c): Fetal placenta of rats receiving omega-3 1200mg/kg prior to BPA (300 mg/kg) showing the magnification of fig.(7a) to identify the labyrinth cell layer(b).(H&E stain, x40).









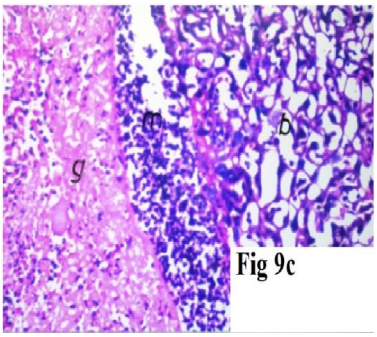


Figure (8a): Fetal placenta of rats receiving garlic 500mg/kg prior to BPA (300 mg/kg)showing normal giant cells(g) and blood vessels(v).(H&E stain, x40).
Figure (8b): Fetal placenta of rats receiving garlic 500mg/kg prior to BPA (300 mg/kg) showing the villi (p) in the labyrinth layer by the magnification.(H&E stain, x40).
Figure (9a): Fetal placenta of rats receiving Zn 32mg/kg prior to BPA (300 mg/kg) showing diffuse necrosis in labyrinth layer and trophospongium(b).(H&E stain, x16).
Figure (9b): Fetal placenta of rats receiving Zn 32mg/kg prior to BPA (300 mg/kg) showing magnification of fig. (9a).(H&E stain, x40).
Figure (9c): Fetal placenta of rats receiving Zn 32mg/kg prior to BPA (300 mg/kg) showing liquifactive necrosis in giant cells (g) with underlying massive inflammatory cells aggregation(m).(H&E stain, x40).

4. Discussion

Data of the present investigation revealed that administration of BPA (300mg/kg) orally from 6th day of gestation to 15th day significantly elevated the rate of resorption, significantly decreased fetal growth as well as induction of external and skeletal malformations as compared to control ones.

Increase the rate of resorption is in agreement with results obtained by **Kim *et al.* (2001)** showing that foetal death and resorption were increased in pregnant Sprague-Dawley rats administrated a high BPA level during the entire gestational period. **Roy George and Malini (2012)** supported the present results showing that administration of BPA orally (600mg/kg) to pregnant rats from days 0 - 15 of gestation induced some abnormal changes in gestation such as resorption of fetuses and teratogenicity. Also **Varayoud and his co-workers (2011)** reported that the neonatal exposure to BPA affected the number of implantation sites. BPA not only increased the number of rats with more than one resorption site but also the increased number of resorption sites per rat relative to control group.

It is well defined that progesterone has an important role in maintenance of pregnancy by decreasing the contractility of the gravid uterus, thus allowing decidual cells to develop in the uterine endometrium, and these cells then play an important role in the nutrition of the early embryo **(Hamilton and Mossman, 1986).** It is well known that the reproductive axis is composed anatomically of the hypothalamus, pituitary, ovaries in females and testes in males and physiologically by gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone, and inhibin. GnRH, a peptide hormone, stimulates the pituitary gland to produce and secrete LH and FSH **(Sokol *et al.*, 2002). Peretz *et al.* (2011)** have shown in vitro follicle culture system that BPA inhibits follicle growth and decreases steroidogenesis resulting in decrease in estradiol, estrone, testosterone,

androstenedione, dehydroepiandrosterone sulfate and progesterone levels produced by the follicles.

However, **Morrissey *et al.* (1987)** reported no increase in percentage resorptions per litter whose mothers were exposed to BPA during gestation. This difference may be due to the difference in dose and time of administration.

It was shown in our results that administration of BPA (300mg/kg) orally from 6th day of gestation to 15th day significantly decreased growth rate. This result is similar to that obtained from **Kim *et al.* (2001);** they reported that administration of a high BPA level (300 mg/kg) during the entire gestational period in Sprague-Dawley rats reduced the weight of the fetuses.

Prenatal or neonatal exposure to BPA was correlated with adverse effects on fetal growth parameters such as low birth weight (LBW) and small for gestational age as reported by **Manikkam *et al.* (2004)** showing that exposure to endocrine-disrupting compounds in utero caused fetal growth retardation and low birth weight offspring in sheep. This combined with the fact that in utero exposure to an estrogenic agents, is associated with intrauterine growth restriction (IUGR) **(Bamigboye and Morris, 2003)** as BPA mimics estrogen in its actions, continued exposure to BPA during gestation is likely to have an impact on the development of the fetus.

In addition, it was suggested that exposure of placental cells to low doses of BPA may cause detrimental effects, leading *in vivo* to adverse pregnancy outcomes such as preeclampsia, IUGR, prematurity and pregnancy loss **(Benachour and Aris, 2009).**

Li *et al.* (2010) suggested that BPA demonstrates developmental toxicity to rat embryos in vitro which may be attributed to directly damaging embryos, inducing cell death, and inhibiting cell proliferation and differentiation. Moreover, BPA can cause abnormal expression of inducible nitric oxide synthase (iNOS) in the embryonic cells, which might be a potential mechanism for its developmental toxicity in cultured rat embryos.

Nitric oxide (NO) is an important second messenger. NO is not only involved in natural biochemical and physiological processes, but may also be involved in embryonic development and teratogenesis. iNOS is regarded as an effective biomarker in the screening of developmental toxicants in teratological research. Nitric oxide synthase (NOS) is a multifunctional and widely distributed isoenzyme that plays an important role in a series of tissue and organs **(Bloch *et al.*, 2001,).** When cells respond to abnormal conditions, the expression of iNOS is induced, which result in large quantities of synthesized NO from its precursor L-arginine **(Tsutsui *et al.*, 2006**). Increasing NO can interfere with growth and development of embryos in multiple ways.

However, certain studies provided conflicting results, reporting an increased weight in offspring whose mothers were exposed to BPA during gestation **(Rubin *et al.*, 2001, Newbold *et al.*, 2007).**

The present data also revealed that administration of BPA in dose (300mg/kg) orally from 6th day of gestation to 15th day had no effect on placental weight but caused diffuse liquifactive necrosis and suppurative inflammatory reaction as well as pus cells infiltration and massive inflammatory cells infiltration mainly neutrophils in the giant cells and underlying labyrinth layer of placenta. These results were in agreement of that showing low dose exposure to BPA leads to apoptosis and necrosis in primary human placental cytotrophoblast cells **(Benachour and Aris, 2009).** Though the mechanism of BPA toxicity is still unclear, previous studies with BPA have demonstrated its ability to alter DNA methylation patterns **(Dolinoy *et al.*, 2007)** suggesting an epigenetic component to its toxicity.

Regarding skeletal malformations, BPA administration (300mg/kg) orally from 6th day of gestation to 15th day significantly increased the incidence of fetal skeletal malformations. This result is in agreement with results obtained by **Kim *et al.* (2001)** reporting that skeletal ossification sites were decreased in fetuses exposed to1000mg/kg BPA group, but it was suggested to be a delay in ossification rather than anomalies due to maternal toxicity. Also, **Li *et al.* (2010)** reported that high-levels of BPA in vitro can influence cell growth and morphological differentiation resulting in malformed embryos with small forebrain and midbrain, small forelimb bud and abnormal optic, and abnormal flexion. These irregularities clearly demonstrate that BPA is developmentally toxic to rats.

In addition, BPA may act via reducing calcitonin secretion and plasma calcium levels, while suppressing directly osteoblasts and osteoclasts among vertebrates as it suppresses Tartrate-resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP) activities that are markers of osteoclasts and osteoblasts, respectively as reported in goldfish by **Suzuki *et al.* (2003).**

However, **Morrissey *et al.* (1987)** revealed that post-implantation exposure to BPA by oral gavage or intra-peritoneal injection did not cause any skeletal malformations at doses that caused significant maternal toxicity in rats or mortality in mice or rat.

The present data revealed that omega -3(EPA and DHA) administration in a dose 1200 mg/kg orally from 6th day of gestation to 15th day showed marked significant decrease in the rate of resorption, incidence

of external and skeletal malformations and significant increase in growth rate as compared to the corresponding BPA group as well as improvement of placental histopathology. These results are in agreement with **Jones *et al.* (2013)** showing that omega 3PUFA supplementation in pregnant rats reduces placental oxidative stress and increases placental and foetal growth. Studies in developing countries showed that a higher omega 3 PUFA intake or supplementation during pregnancy may result in improvement in birth weight, length and gestational age. However, there is no evidence of improvement in growth following omega 3 PUFA supplementation in children >2 years of age **(Huffman *et al.*, 2011).**

Generally essential fatty acids appear to have the following beneficial effects for bone: increased calcium absorption from the gut, reduced urinary excretion of calcium, increased calcium deposition in bone, improved bone strength and enhanced synthesis of bone collagen **(Kruger and Horrobin, 1997).**

In addition, dietary fat may influence bone metabolism by altering prostaglandins (PGs) biosynthesis **(Watkins *et al.*, 1997).** The PGE2 produced by osteoblasts stimulates Insulin like growth factor-I (IGF-I) synthesis and affects its action to support an anabolic response in bone **(McCarthy *et al.*, 1991).**In support of the relationships between dietary PUFA, PGs, and bone metabolism, **Watkins *et al.* (1997)** reported that animals given long-chain n-3 fatty acids (omega 3) modulated vivo bone PGE2 production and the concentration of IGF-I in bone tissues, and led to an increased rate of bone formation, suggesting a stimulatory effect on osteoblastic activity. While **Iwami-Morimoto *et al.* (1999)** reported that Dietary fish oil reduced osteoclastic activity (its number was only 60% of control) and subsequent bone resorption (80% of control). Moreover, the activity of serum bone-specific alkaline phosphatase was greater in rats given a diet high in n-3 fatty acids, which further supports the positive action of these fatty acids on bone formation **(Watkins *et al.*, 2000),** also EPA appeared to stimulate collagen synthesis **(Watkins *et al.*, 1996).**

The present study revealed that garlic administration in a dose 500 mg/kg orally from 6th day of gestation to 15th day showed marked significant decrease in the rate of resorption, incidence of external and skeletal malformations and significant increase in growth rate as compared to the corresponding BPA group as well as improvement of placental histopathology.

These results are in agreement with results obtained by **El-Sayyad *et al.* (2010**) showing that garlic supplementation during pregnancy ameliorated decreased numbers, body weight and congenital abnormalities as external malformations of fetuses in hypercholesterolemic dams. In addition it restored the progress of bone formation and partially improved ossification. They attributed these protective effects to the ability of garlic to scavenge free radicals which protect membranes from damage and maintain cell integrity via lowering of cholesterol, anti-platelet activities, and thromboxane formation.

The effects of garlic and its derivatives have been shown to play a positive role in reducing bone resorption **(Mukherjee *et al.*, 2004).** In addition, garlic extract has anti-oxidative properties that can protect osteoblast from oxidative damage induced by free radicles, thus diets rich in garlic might improve bone matter and stability **(Ehnert *et al.*, 2012).**

The present work showed that Zn administration in a dose 32 mg/kg orally from 6th day of gestation to 15th day showed non-significant decrease in the rate of resorption, incidence of skeletal malformations and non-significant increase body weight and body length as compared to the corresponding BPA group. In addition to its failure in protection from necrosis and liquifaction in the labyrinth layer, giant cells, trophospongium and villi associated with massive number of pus cells and other inflammatory cells infiltration mainly neutrophils induced by BPA in placenta. These results are supported by a study in Iranian pregnant women found an inverse relationship between maternal serum zinc at delivery and neonatal birth weight **(Badakhsh *et al.*, 2011).**

In contrast to **Lee *et al.* (2011**) suggesting a positive relationship between maternal zinc status or intake at mid-pregnancy and infant birth weight and height.

The importance of Zn in fetal growth may be attributed to stimulation of insulin like growth factor (IGF) which has a critical role in fetal growth as supported by **Hanna *et al.* (2010).**

These conflicting studies may be attributed to inhibitory effects of Zn of excessive peroxidation via increasing superoxide dismutase activity and metallothionein type I (MT-1) mRNA expression **(He *et al.*, 2009).** Metallothioneins have a number of complex cellular functions, including gene expression, proliferation and differentiation, regulating intracellular zinc homeostasis, detoxification of heavy metals such as Cd and Hg and mitigating oxidative stress **(Maret, 2009).**

Regarding skeletal malformations, conflicting studies showed that presence of a large amount of zinc in bone tissue suggesting that zinc plays an important role in the development of the skeletal system and Zn has a stimulatory effect on bone formation and mineralization **(Yamaguchi, 1998)**, also zinc increase bone alkaline phosphatase activity and DNA content **(Yamaguchi and Inamoto, 1986).**

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10/22/2013