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Teratogenicity of Bisphenol-A (BPA) in Pregnant RAT

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Abstract: Bisphenol- A (BPA) is an organic compound with two phenol functional groups. 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. This study was designed to evaluate and assess the possible teratogenicity of BPA administrated to pregnant rats. Sixty pregnant *Wistar albino* rats (10 RAT/group) were administrated oral doses of Bisphenol- A (300 & 600 mg/kg /day) from 6th up to both 15th and 19th days (GD15 & GD19) of gestation. While, control groups received Olive oil only. The obtained data revealed that, pregnant rats treated with Bisphenol- A showed states of instability, nervousness, twitching of head, agitation, hazy movement and marked reduction in food intake as well as reduction in the body weight. Uteri were removed and dissected to evaluate the intrauterine growth retardations as well as skeletal malformation. BPA doses induced reduction in implantation sites, foetal body weight, with a symmetrical distribution of embryos in both uterine horns. Increase in both mortality and resorption rates were reported. BPA also induced decrease in the embryonic crown rump values and induced severe skeletal anomalies on both 15th and 19th days of gestation. In conclusion, this study shaded more light on the teratogenicity of BPA on embryonic development and the adverse effects on the pregnant female rats.

Key words: Bisphenol-A · Embryo Teratogenicity · Skeletal Malformations · Pregnant Rats

INTRODUCTION

Estrogenic endocrine disruptors (EEDs) are environmental compounds that are able to bind to estrogen receptors (ERs) and evoke biological responses. Several authors exerts a great efforts to demonstrate the estrogenic activity of EED *in vitro* and *in vivo*. One of these EED is bisphenol-A (BPA) [1].BPA is classified as an endocrine disruptor with weak estrogenicity [2]. Its estrogenic potency was estimated to be 10,000-fold less than that of 17β-estradiol (E2) [3, 4], which may reflect the affinity of BPA for the classical nuclear estrogen receptors (ERs) [5-6]. However, numerous studies demonstrate that BPA at concentrations that are too low to efficiently activate nuclear ERs also have cellular effects [7-9].

BPA reportedly have the potential to produce widespread adverse effects through their endocrine disrupting activity, such as carcinogenicity, neurotoxicity, immunotoxicity, interference with the cardiovascular system, reproductive abnormalities, developmental toxicity and so on [8,10]. Many studies in animal models have reported that prenatal exposure to BPA could induce birth defects [11,12].Embryotoxic and teratogenic potential of BPA have been of particular concern in recent years.

Bisphenol- A (BPA) is an organic compound with two phenol functional groups. Bisphenol A (BPA) is a widely used as industrial plasticizer with known estrogenic properties. Human exposure to bisphenol A (BPA), a high production volume chemical, is ubiquitous due to its widespread use in numerous products including polycarbonate plastics and epoxy resins such as those used to line food and beverage containers [13,14], in medical equipment, thermal paper and personal care products [15,16]. While the primary source of BPA exposure is through food, there is uncertainty with regard to the amount of exposure that can also occur dermally and through air [2,17-20]. However, recent evidence also indicates that exposure may occur through dermal contact with thermal paper, used widely in cash register receipts

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[21&22]. BPA can leach from products made with these materials [23-25]. The general human population can be exposed to BPA mainly via ingestion, inhalation and skin contact at micrograms per kilogram of body weight daily [2–26]. Heat and either acidic or basic conditions accelerate hydrolysis of the ester bond linking BPA monomers, leading to release of BPA into food and liquid and making human environmental exposure inevitable and ubiquitous [13,27]. BPA also has been detected in the amniotic fluid, maternal and fetal plasma and placental tissue of pregnant women and in breast milk of lactating mothers and adipose tissue [28-31]. Many studies indicate that the developing fetuses is more sensitive to estrogenic chemicals than the adults [13].

Studies on human populations have correlated higher BPA exposure with disorders such as cardiovascular diseases, diabetes, liver dysfunction and male sexual dysfunction [26, 32,33,34], dyslipidemia, obesity and high blood pressure [35-38,]. Laboratory studies on animals have demonstrated multiple adverse effects of BPA, including the development of the male and female reproductive tracts, obesity and other aspects of metabolic function, development of the brain and neurobehaviors and development of the mammary gland and its response to chemical carcinogens and induction of cancer [39-43]. However, it may also be argued that low doses of BPA could have adverse effects on human reproductive and developmental health [44,45].

Bisphenol-A accumulates in the fatty tissues of pregnant adult females [46]. BPA 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 of pregnant rat and women and accumulates both in the placenta and in the fetus and potentially impacting the developing fetus [47-50]. 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 [51,2]. 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 [52].

The reproductive system is a main target of endocrine disruptors. Extensive laboratory studies have revealed multiple adverse effects of BPA on the reproductive system. In the male reproductive system, effects of BPA include decreased sperm motility, impaired spermatogenesis and decreased fertility of male offspring [53,54]. In the female reproductive system, BPA may target the mammary gland, the ovary, the oviduct, the uterus and the placenta, also, BPA increased human ovarian cancer cell proliferation in a dose-dependent manner [36,55-58]. A recent study demonstrates that CD-1 mice exposed to environmentally relevant BPA levels during the perinatal period (gestation day 8 to postnatal day 16) showed decrease in reproductive capacity, although the causes of such a decrease have not been determined [59]. It was reported that BPA exposure (10.125 mg/mouse/day, ~400 mg/kg/day) during (1^{st} -4^{th}) days of gestation led to fewer implantation sites [60]. So, this study was aimed to shed more light on the effects of BPA exposure on embryonic development in pregnant rats.

MATERIALS AND METHODS

Chemicals: Bisphenol-A (BPA) was purchased from Sigma -Aldrich Chemical Co., St. Louis, USA.)Catalog number (CAS:80-05-7)) Batch (133027). Dissolved in few drops of alcohol and made as micro - crystalline suspension up to desired volume with olive oil, purchased from Sigma -Aldrich Chemical Co.

Animals, Mating and Condition: This study was carried out using sexually mature male and female *Wistar albino* rats (160-180gm), purchased from the animal house of VACSERA, Giza, Egypt. Rats were healthy and acclimated to the laboratory environment for 2 weeks before use. Temperature and relative humidity were maintained at 23±2°C and approximately 50% respectively, with a 12 hr: 12 hr light: dark photoperiod. They were housed in stainless steel cages and given a standard diet and water ad libitum throughout the study. The proestrous females cohabited in a ratio of (male / two females). On the next morning sperm-positive females and females with a vaginal plug were considered to be in Day 0 of pregnancy [61,62].

Experimental Groups: Sixty bred females were divided into six groups (10 pregnant rats /group) according to approximately equal mean body weight. Olive oil served as the vehicle for the two control groups, while the other four groups were treated with Bisphenol-A doses of 300 and 600 mg/kg body weight and sacrificed at both 15^{th} and 19^{th} day of gestation respectively [68,69].

Maternal-Foetal Investigation: Maternal-Fetal investigations were conducted according to the US Environmental Protection Agency (EPA) TSCA (Toxic Substances Control Act) Test Guidelines [63]. All animals were observed twice daily for mortality and toxicological effects. Body weight and feed consumption values were recorded during treatment. Post exposure observations were performed approximately a half hour after exposure. On (GD 15th& 19th), all females were euthanized via carbon dioxide inhalation and a cesarean section and a macroscopic postmortem examination were performed on each. The uteri of apparently non pregnant Rats were stained with 10% silver nitrate and examined for evidence of implantation sites. Fetuses were removed from uteri, weighed, sexed and examined for external malformations and measured for crown rump values. Implantation sites, live, dead, resorbed fetuses indices were calculated. After recording all measurements and parameters, fetuses were divided into two groups. The first group was fixed in Bouin's solution for morphological investigations [64], while the second group was placed in Formaldehyde 10% to dissolve the body fats, then transferred to potassium hydroxide (1%) to clear the skeletons and applying Alizarin red S to stain the ossified skeletal bones [65,66]. After staining, the fetuses were examined for skeletal anomalies.

Statistical Procedure: Data were expressed as mean ± standard error of mean (S.E). Student's t-test was e employed to determine the significance of differences between treated and control means [67].

RESULTS

Maternal Body Weights: Body weights of pregnant rats at both 15th and 19th days of gestation showed that BPA causes remarkable reduction when compared to control and these reductions were dose dependent and statistically significant as shown in Table 1.

Fetal Investigations: Numbers of implantation sites in pregnant rats treated with BPA doses at both 15th and 19th days of gestation showed decrease when compared to the control groups. This decrease reached (-13 % & -24%) and (-25 % & -44%) at 15th& 19th days of gestation respectively as shown in Table 1.

Referring to embryonic mortality, it was calculated that BPA induced significant increase in the number of dead embryos among embryos obtained from BPA treated groups. This increase reached (+ 89.06 % & + 88.62 %) and (+ 91.15 % & + 92.74 %) at both 15th day 19th day of gestation respectively. Also, increasing the mortality rate was dose and time dependent as shown in Table 1.

The fetuses were identified as living or dead by responding to prick technique using fine needle. Also, significant decrease was observed in the number of live fetuses when compared with the embryos from the control groups. This decrease in live embryos reached approximately (- 34.02 % &- 42.98 %) and (- 30.10% & - 53.76 %) at 15th and 19th day of gestation respectively. Also, significant reduction was observed in the crown-rump values of fetuses from treated groups as shown in Table 1 and Plate 1. This reduction reached (-27.16% & -54.9%) and [-21.63% & -44.44] in rat embryos at both 15th and 19th days of gestation respectively Total resorption of rat embryos from pregnant rats treated with BPA doses was significantly increased when compared to the control group. This increase reached (13.1% & 11.71%.] and [11.68% & 15.17%) at both 15th and 19th day of gestation respectively.

Assessment of Bone Calcification and Anomalies: Examining alizarin red-S stained skeletal bones of rat embryos using dissecting microscope revealed that BPA induced reduction in the ossification of the skull bones and these reductions were dose and time dependent. The most affected skeletal parts were skull bones, which manifested in incomplete ossification. Ribs abnormalities, included irregular shape ribs, missed ribs and incomplete ossification as represented in Table (2) and Plates (3&4).

Incomplete ossification, missed central discs vertebrae, delayed ossification as well as scoliosis of vertebral column and missing of some cervical and some thoracic vertebrae were recorded in some fetuses from pregnant rats treated with PBA doses at both 15th and 19th days of gestation respectively.

BPA doses induced a very harmful effect on the limbs of rat embryos at both 15th and 19th days of gestation. Examining both fore-and hind limbs of rat embryos revealed incomplete ossification of several bones as Carpals, metacarpals, fore phalanges, tarsal, metatarsal and hind phalanges. In addition, all bones of the treated fetuses showed retardation in their length and size when compared with those of control.

Table 1: Effect of Bisphenol-A doses on the pregnant Rats and their offspring at both stages

	Stage 15th Days			Stage 19th Days		
Groups	GI Control	GII 300 mg	GIII 600 mg	GIV Control	GV 300 mg	GVI 600 mg
Mother body Weight	216.1±1.1	204.2±2.8*	199.6±1.9*	230±0.8	221.3±2.3*	210.3±2.7**
Implantation Sites	10 ± 0.5	8.7± 0.3*	7.6±0.6**	9.5 ±0.61	8.56±0.4**	6.92± 0.4**
% of change	100 %	-13 %	- 24 %	100 %	- 25 %	- 44 %
Still live Embryos	9.7 ± 0.4	6.4±0.61*	5.53 ±0.67**	9.3 ± 0.5	6.5±0.46 **	4.3 ± 0.72**
% of change	100 %	- 34 %	- 42.9 %	100 %	- 30.10 %	-53.76 %
Dead Embryos	0.3 ± 0.05	1.16±0.43**	1.18±0.28**	0.2±0.02	1.06±0.04**	1.57±0.12**
% of change	100 %	+89.06%	+88.6 %	100 %	+91.15 %	+ 92.74 %
Resorbed Embryos	0.00	1.14±0.02	0.89 ±0.01	0.00	1.00 ±0.01	1.05 ±0.04
% of change	0.00	13.1 %	11.71 %	0.00	11.68 %	15.17 %
Crown rump	1.73±0.04	1.26±0.03**	0.78±0.05**	3.42 ± 0.03	2.68± 0.03**	1.9± 0.06**
% of change	100 %	-27.16 %	-54.91 %	100 %	-21.63 %	-44.4 %
Embryo body weight	0.93±0.04	0.33±0.03**	0.22± 0.01**	4.17 ±0.09	2.12±0.07**	1.51±0.02**

Data expressed as mean ± SD. ** = highly Significant. %= percent of change from control.

Table 2: Teratogenic effect of (BPA) doses on the skeletal system of the rat embryos at both stages

		Stage 15th Days		Stage 19th Days	
Groups & Doses		GII 300 mg	GIII 600 mg	GV 300 mg	GVI 600 mg
Skull	Incomplete Ossification	65.21	70.41	66.51	78.49
	Complete ossification	34.79	29.59	33.49	21.51
Ribs	Irregular Shape	23.18	32.51	20.39	25.45
	Missed	20.22	23.10	24.55	19.96
	Incomplete ossification	36.42	29.23	35.26	38.21
	Complete Ossification	20.18	15.16	19.18	16.38
Vertebral centra	Missed	64.28	72.37	40.16	61.44
	Scoliosis	5.20	9.18	29.72	18.40
	Normal	30.52	18.45	30.12	20.16
Fore limbs	Incomplete Ossification	66.88	75.38	64.77	73.56
	Complete ossification	33.12	24.62	35.23	26.44
	Incomplete Ossification	71.83	79.85	65.36	72.43
Hind limbs	Complete Ossification	28.17	20.15	34.64	27.57

The data represented as percentage (%)

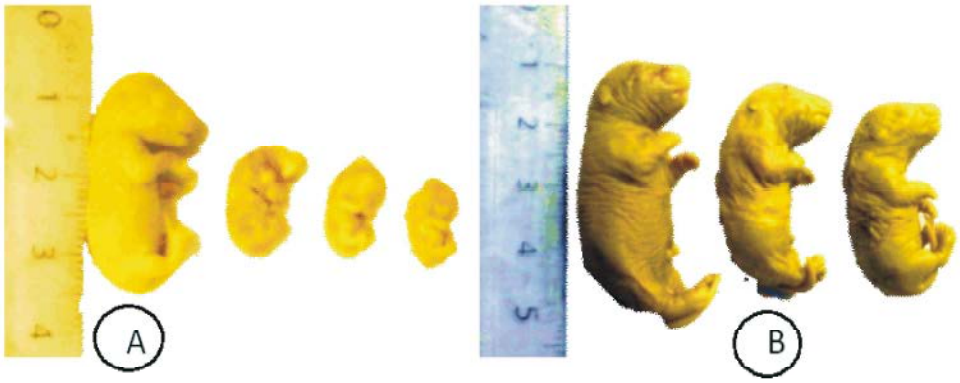

Plate 1: A&B- photograph showing a lateral view of fetus's size obtained from control and treated pregnant rat after 15th and 19th days of gestation

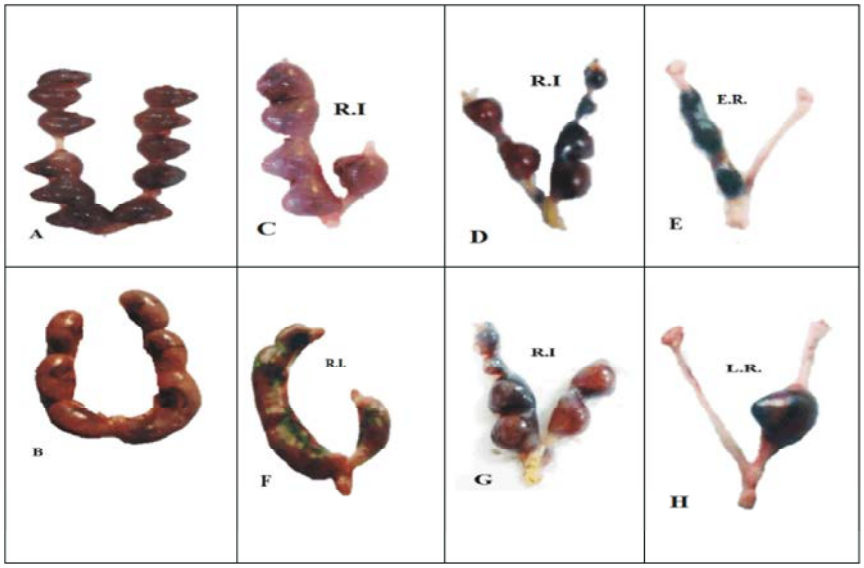

Plate 2: A&B-Photomicrograph of control uterus on both 15th and 19th day of gestation respectively, showing normal distribution of embryos.C,D& E –Uteri from treated rats from 6th up to 15th day of gestation showing (S.H.) shortness of horn, both early and late resorbed embryos (L.R&E.R). While (F,G & H) Uteri from treated rats from 6th up to 19th day of gestation showing shortness of horn, early and late resorbed (E.R.)

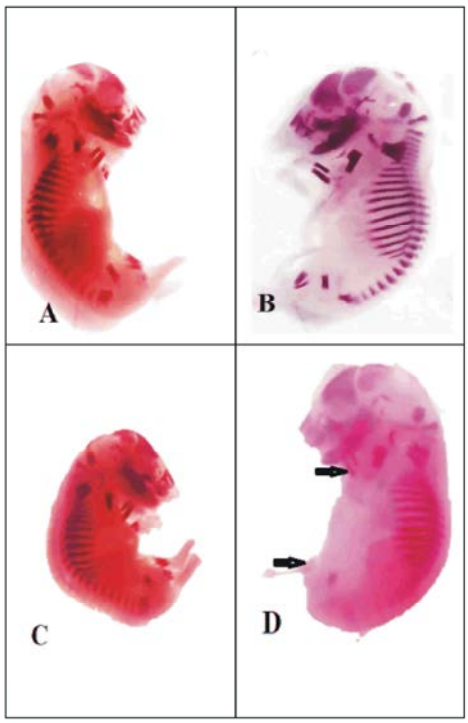

Plate 3

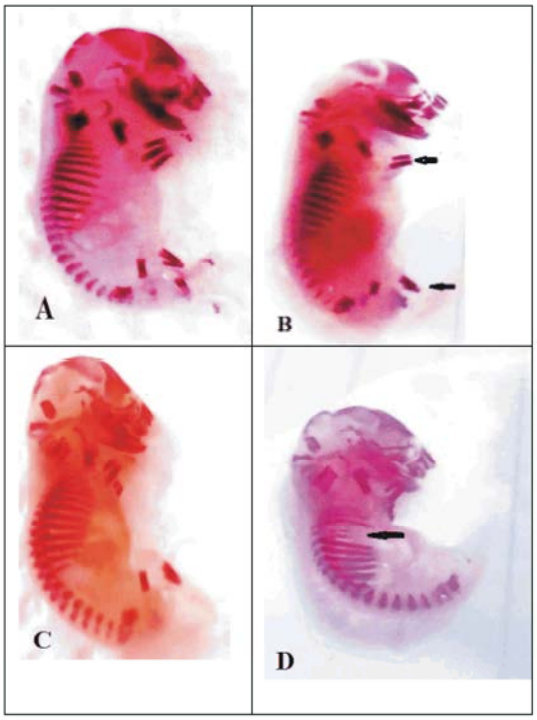

Plate 4

Plate 3: A-Photomicrograph showing a lateral view of fetus's skeleton obtained from control on 15th day of gestation, B,C&D- treated rat embryos after 15thdays of gestation, showing abnormal ossification of the skull parts, forelimbs and hind limbs (black arrows)at 15th stage

Plate 4: A-Photomicrograph showing a lateral view of fetus's skeleton obtained from control on 19th day of gestation, B,C&D treated rat embryos after 19th days of gestation, showing abnormal ossification of the skull parts, missed ribs, forelimbs and hind limbs(black arrows) at 19th stage

DISCUSSION

In the present developmental toxicity study, treatment of the pregnant rats with Bisphenpl-A from day 6 up to 15th and 19 th of gestation resulted in several deleterious effects not only on the pregnant rats but also on their offspring. These effects manifested in reduction of maternal body weight elevated mortality and resorption rates, foetal growth retardation and skeletal malformations as compared to control ones. Increase the rate of resorption is in agreement with results obtained by [68] who reported increase in foetal death and resorption in offspring of pregnant Sprague-Dawley rats administrated a high BPA level during the entire gestational period. Also, this phenomenon of decrease in number of implantation sites, resorption and teratogenicity is confirmed when pregnant rats and mice treated with BPA (600mg/kg) and (10.125 mg/mouse/day, ~400 mg/kg/day) from day 0 - 15th of gestation [69,70& 62].

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 exert a great role in the nutrition of the early embryo and it was reported that BPA administration to the pregnant rats able to impair this process and hence induce reduction in the body weight of rat embryos, which support the results of this study [71].

BPA inhibits follicle growth and decreases steroidogenesis *In vitro* follicle culture system which resulting in decrease in estradiol, estrone, testosterone androstenedione, dehydroepiandrosterone sulfate and progesterone levels produced by the follicles which impair the reproductive axis physiologically [72].

In contradiction with the results of this study some authors reported that no increase in percentage resorptions per litter whose mothers were exposed to BPA during gestation [73]. Also, they attributed this difference the amount of dose and time of administration. While, in this study the whole gestation period was covered and investigated using the dose allowed from the Environmental Protection Agency (EPA) and resulted in significant deleterious effects to rat embryos at different dose and time levels. On the other hand, several reports support our results as those whom administrated a high BPA level (300 mg/kg) during the entire gestational period to Sprague-Dawley rats resulted in reduced the weight of the fetuses.

Prenatal exposure to BPA was correlated with adverse effects on fetal growth parameters such as low birth weight (LBW) and small for gestational age [74] 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) 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 [75].

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 [76].

Some studies described how BPA induce developmental toxicity to rat embryos *In vitro* and explained that BPA directly damaging embryos by 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 [60].

Regarding skeletal malformations resulted in this study, BPA doses administrated to pregnant rats significantly increased the incidence of fetal skeletal malformations. Which supported by several authors reporting that skeletal ossification sites were decreased in fetuses exposed to1000 mg/kg BPA group, but it was suggested to be a delay in ossification rather than anomalies due to maternal toxicity [68].

Also, BPA In Vitro can influence cell growth and morphological differentiation resulting in malformed embryos with small forebrain and midbrain, small fore limb 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 Li *et al*., 2010 and Berger *et al*., 2007 [77,78].

In conclusion, this study also, sends several warnings to the mankind to exert more efforts at national and international levels to informing the world community about the deleterious effects of BPA as one of the endocrine disruptors agents not only on the pregnant dams but also on their offspring and should be make rules and precautions from the contamination and ingestion of such compounds to save ourselves and our offspring from the serious harmful effects.

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