Genotoxic and cytotoxic potential of Bisphenol A in the early developing stages of chick embryo (*Gallus gallus domesticus*) evidenced by HET-MN and Comet assay

Langthasa P.[1], Barhoi D.[1], Devi S.H.[2], Giri A.[3] and Giri S.[1*]
1. Molecular and Cell Biology Laboratory, Department of Life Science and Bioinformatics, Assam University, Silchar 788 011, INDIA
2. Department of Zoology, Manipur University, Canchipur-795003, Imphal West, Manipur, INDIA
3. Environmental and Human Toxicology Laboratory, Department of Life Science and Bioinformatics, Assam University, Silchar-788011, INDIA
*girisarbani@gmail.com

Abstract
Bisphenol-A (BPA) is ubiquitously present in the environment and consumer products and is a crucial environmental contaminant known to cause significant health risks to various organisms. The present study aims to evaluate the genotoxicity effect of BPA in developing chick embryos. Fertile chicken eggs were treated with increasing concentrations of BPA (1, 10, 20, 30, 40 and 50 mM/egg) on embryonic days (ED) 8, 9 and 10. On ED11, the number of viable embryos was recorded and LD_{50} values were calculated for 24 h (ED 10), 48 h (ED 9) and 72 h (ED 8). HET-MN (Hens egg test for micronucleus assay) and comet assay were performed on the 11^{th}-day to evaluate the cytotoxicity and genotoxicity potential of BPA. Experiments were performed in three different time points i.e. 24 h, 48 h and 72 h of exposure. Results showed an increased mortality rate in a dose and time-dependent manner. Significant alterations of cellular dynamics erythropoiesis and PCE/NCE ratio were observed.

Also, significant induction of total MN and other nuclear abnormalities was observed. A decrease in head DNA content, increase in tail DNA, olive tail moment and damage indexes were detected upon exposure. Thus, the results confirmed the genotoxic and cytotoxic potential of Bisphenol-A that caused genetic alterations extensively during the early development of embryos.

Keywords: Hen's egg test for micronucleus (HET-MN), chick embryo, Bisphenol A, Comet assay, DNA damage.

Introduction

Bisphenol-A (BPA; 2, 2-bis-(4-hydroxyphenyl)-propane) is a synthetic organic compound which is ubiquitously present in consumer products and the environment. It is widely used as a monomer in the preparation of polycarbonate polymers and epoxy resins[3]. Besides, it is extensively used in fabricating personal care products, dental sealants, toys, plastic bottles, paper, water pipes and protective coatings in metal cans of foods, beverages and several consumer products[8,20,25]. It is the most potent and highly produced chemical globally[1] and humans are ubiquitously exposed to it. It can leach into the food and beverages from the internal coatings of epoxy resins and also from other consumer products that account for the majority of daily human exposure[5,38].

BPA mimics estrogen, binds to estrogen receptors and acts as an endocrine disruptor[10,34]. It was found that BPA can cross the blood-brain barrier[17], placental barrier[31] and can bio-accumulate in different parts of the body. Previous studies reported its detection in the amniotic fluid and fetus, semen, breast milk and saliva[16,19]. Besides, its exposure leads to adverse health effects such as testicular cancer, precocious puberty, low sperm count, hypospadias and cryptorchidism etc.[22,42] Studies revealed that BPA causes an imbalance in the prooxidant and antioxidant status causing damage to the liver, kidneys, brain and other vital organs[4,6,7,15].

The possible genotoxic effects of BPA have been studied extensively in many biological systems but the results remain controversial. Studies have reported that BPA could induce DNA damage and micronuclei formation in both HEp-2 and MRC-5 cell lines[29], chromosome aberrations and formation of DNA adducts in Syrian hamster embryonic (SHE) cells[37]. Earlier studies also reported that BPA exposure causes structural changes (achromatic gaps), aneuploidy and DNA adduct formation in mice system[26, 36]. On the other hand, different studies showed that BPA does not induce chromosomal abberations[14] and micronucleus formation[26] as well as hyperploidy, polyploidy and micronucleus in mice model[28]. Considering these reports, it remains unclear whether BPA exposure promotes genotoxicity *in vitro* and *in vivo*. Therefore, the present study aimed to assess the genotoxic potential of BPA during the early embryonic development of chick embryos *in ovo* by employing HET-MN (Hens egg test for micronucleus) assay.

HET-MN assay provides a simple and rapid indirect measure for genotoxicity[41]. The occurrence of micronuclei (MN) represents an integrated response to chromosome instability phenotypes and altered cellular viability, induced by genetic defects and/or exogenous exposures to genotoxic agents[12]. In chick embryos, most of the erythroid cells are formed in the yolk sac and appear quickly in the peripheral blood with well-distinguished stages of mature erythrocytes. Additionally, it has been reported that a high rate of erythropoiesis occurs in the developing chick embryos showing all stages of erythrocytes during the embryonic day

(ED) 11[40].

Therefore, MN scoring is considered as a sensitive assay method for genotoxicity determination[40]. Single-cell gel electrophoresis (SCGE) or comet assay is also another sensitive and rapid method for DNA strand break detection upon exposure to any physical or chemical agent in different systems[23,27,30].

Previous studies conducted to study BPA-induced genotoxicity are inconclusive. Moreover, the BPA-led genotoxicity during the early embryonic development in chick embryos has not been investigated to date. Therefore, the present study was designed to study the genotoxic and cytotoxic potentials of BPA during the early embryonic development of chick embryos using HET-MN and SCGE assay.

Material and Methods

Chemicals and reagents: Bisphenol-A (BPA) (CAS No. 80-05-7) was obtained from Sigma-Aldrich Laborchemikalien, Germany. Giemsa stains, May Grunwald solution, sodium chloride (NaCl), tris HCl, ethylenediaminetetraacetic acid (EDTA), triton-X 100, dimethyl sulphoxide (DMSO), sodium hydroxide (NaOH), potassium chloride (KCl), tris buffer, methanol, hydrochloric acid (HCl) and Dulbecco's phosphate-buffered saline (DPBS) were procured from HiMedia Laboratories Pvt. Ltd., India. Normal melting point agarose (NMA), low melting point agarose (LMPA) and ethidium bromide (EtBr) were procured from Sisco Research Laboratories, India. Formaldehyde and DPX mounting media were purchased from Fisher Scientific. All the reagents and buffers were freshly prepared in distilled water before experiment.

Source of fertilized egg and incubation conditions: Fresh fertilized white leghorn chicken eggs (*Gallus gallus domesticus*) were obtained from M/S Reba Hatcheries, Barik Nagar, Assam India. The eggs were incubated in an automatically rotating incubator (3-h intervals) at 37.5°C and relative humidity of 62-68%. Eggs were examined for fertility by candling and maintaining proper hygienic conditions. The study was approved by the Institutional Ethics Committee (IEC) of Assam University, Silchar.

Determination of lethal concentrations of BPA in the chick embryo: The incubated eggs were candled to examine fertility and were windowed on the embryonic day (ED) 8, 9 and 10. BPA stock solution was prepared in DMSO. The fertile eggs were treated with increasing concentrations of BPA (1, 10, 20, 30, 40 and 50 mM/egg) in the air sac. One control (unopened) group and vehicle control group were also included in the study where the eggs in the vehicle control group were exposed to an equal volume of DMSO. After the administrations of the test agents, the windows were closed by a piece of sterilized adhesive tape to restore the vitality of the embryo until the termination of the experiment. The incubation was terminated on embryonic day 11 and the embryos were removed from the shell to check the viability. The number of viable embryos per treatment level was recorded and LD_{50} values were calculated using probit analysis for 24 h (exposed at ED 10), 48 h (exposed at ED 9) and 72 h (exposed at ED 8) exposure. The detailed study designed to determine the lethal concentrations of BPA is shown in table 1.

Dose and treatment of BPA for genotoxicity study: To study the genotoxic potential of BPA during the early development of chick embryo, fertile eggs (weighing 58-66 g) were randomly divided into three groups: control (unopened), DMSO (vehicle control) and BPA treated group, each group consisting of 6 experimental eggs (n=6). The experiments were performed in three different time points i.e. 24 h, 48 h and 72 h of exposure. The different treatment groups included for evaluating genotoxicity are shown in table 2. To evaluate the genotoxic potential, the dose of BPA was selected based on the LD_{50} concentrations of BPA in chick embryos (Figure 1B) where 2 mM concentration of BPA ($1/10^{th}$ of 72 h LD_{50}) was selected. The endpoint evaluation was done on the 11^{th} day of embryonic development.

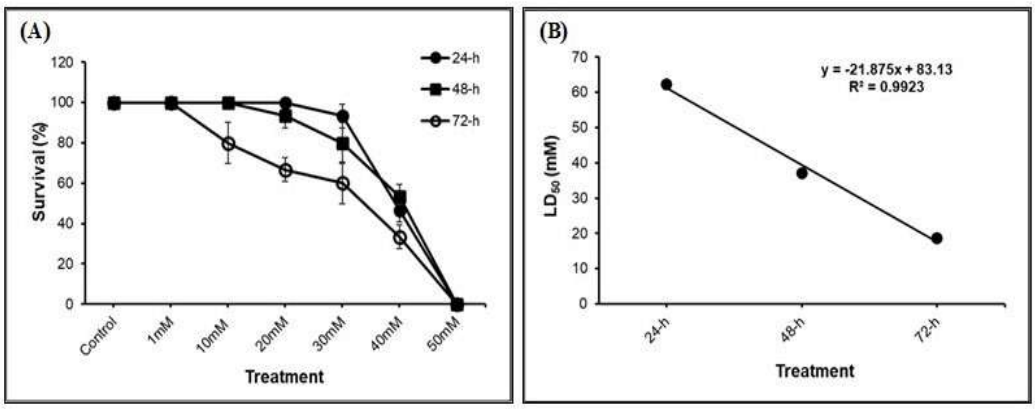

Figure 1: Effect of BPA on the survivability of chick embryos. (A) Line graph showing the viability of chick embryo at 11^{th}-day post-incubation exposed to different concentrations of BPA. (B) LD_{50} values of BPA in chick embryo at 24 h, 48 h and 72 h exposure

Table 1
Study design for determining lethal concentrations of BPA and studying its genotoxic potential on the 11th –day old chick embryo

Exposure time	Study groups	Concentrations for determining LD_{50} (per egg)	Concentration for studying genotoxicity (per egg)
24 h (Exposed at ED 10)	Control (unopened)	-	-
	DMSO (vehicle)	125 µl	125 µl
	BPA	1, 10, 20, 30, 40, 50 mM	2 mM
48 h (Exposed at ED 9)	Control (unopened)	-	-
	DMSO (vehicle)	125 µl	125 µl
	BPA	1, 10, 20, 30, 40, 50 mM	2 mM
72 h (Exposed at ED 8)	Control (unopened)	-	-
	DMSO (vehicle)	125 µl	125 µl
	BPA	1, 10, 20, 30, 40, 50 mM	2 mM

ED; Embryonic day, *LD_{50};* Lethal dose 50%, *DMSO;* Dimethyl sulphoxide, *BPA;* Bisphenol A

Hen's egg test for micronucleus (HET-MN): The HET-MN test was done following the method developed by Wolf et al.[41] Briefly, peripheral blood was collected by incising the chorioallantoic blood vessel with a heparinized capillary. This was followed by preparations of blood smears on a clean grease-free slide. The slides were air-dried followed by fixing in absolute methanol for 5-10 minutes. On the following day, slides were stained with May-Grunwald stain (2 mL) for 3 min followed by staining with 10% Giemsa for 15 min. The slides were rinsed with distilled water thoroughly to wash off the excessive stains and were marked properly. For each slide, at least 1000 erythrocytes were studied under the light microscope (Leica DMLS, Leica, Wetzer, Germany) at 100x magnification in oil immersion for the presence of micronuclei.

The numbers of cells with MN were recorded to evaluate the frequency of micro-nucleated cells to the proliferating cell population. HET-MN provides a simple and rapid indirect technique to measure the extent of genotoxicity of any agent. Besides, the presence of any other nuclear abnormalities like nuclear bud, nuclear bridge, binucleated cells and poikilocytes was also studied and reported as other nuclear abnormalities (ONA) for simplification.

The cytotoxic potential of BPA was also determined by studying the ratio of polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE) in the peripheral blood erythrocytes of 11th- day old chick embryo. The identification of PCE and NCE was similar to the criteria described by Wolf and Luepke [40].

Comet assay: Alkaline comet assay was carried out using the standard protocol by Singh et al[33] with minor modifications. Briefly, blood samples from the chorioallantoic blood vessels were collected and diluted in DPBS (Mg^{2+}, Ca^{2+} free). Then, the blood samples were mixed with 0.5% LMPA in a ratio of 1:15, rapidly spread on frosted slides pre-coated with 1% NMA and kept at 4°C for rapid polymerization. After solidification, 1 % LMPA (80 µL) was added onto the slides and kept at 4°C for 2 h in cold lysis buffer (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Trizma base, 1% Triton X 100 and 10% DMSO; pH 10).

Then, the slides were removed and placed in the electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA; pH 13.5) for 20 min at 4°C followed by electrophoresis at a constant voltage (24 V) for 45 min. After electrophoresis, the slides were neutralized with neutralizing buffer (0.4 M Tris buffer, pH 7.5) and the process was repeated three times (5 min each). The cells were stained with ethidium bromide (EtBr) (20 µg/L) for 5 min at dark and washed off to remove excess stains. The scoring was done using Komet 5.5 software (Kinetic Imaging, UK) attached to a fluorescence microscope Leica, DM 2000 (Leica, Wetzlar, Germany). At least 100 cells per egg were studied to evaluate the BPA-induced genotoxicity in chick embryos.

The comet parameters i.e. head DNA, tail DNA, olive tail moment (OTM), damage index (DI) were analyzed to evaluate the BPA induced genotoxicity in chick embryos. The comet software (Komet 5.5) quantifies the comet tail length (µm), percentage of DNA in the head and tail regions, OTM etc. Head DNA content, tail DNA content and OTM were expressed as fold change compared to the control group. The DI was calculated from the tail length. For this, each data set was divided into four comet classes based on their tail length. The different comet classes were Class 0 (tail length 0-0.49 µm), class 1(tail length > 0.5-4.99 µm), class 2 (tail length >5–9.99 µm), class 3 (tail length >10–19.99 µm) and class 4 (tail length > 20 µm). Damage index (DI) was calculated following the equation mentioned below:

$$DI(mean) = \frac{1}{5}\sum_{0-4}^{X} Xn$$

where X= Class number and n= % cells in each class.

Statistical analysis: LD_{50} doses were determined by probit analysis and quantitative data were expressed as mean ± standard error. One-way analysis of variances (ANOVA) was used to test the levels of significance among the control and treated groups followed by Tukey's test for multiple comparisons. Study groups were considered significantly different at $p < 0.05$. All the analyses were performed using GraphPad Prism version 4.0 for Windows, GraphPad Software, San Diego, California, USA.

Results

Effects of BPA exposure on the survivability of chick embryos: The developing chick embryos were exposed to increasing concentrations of BPA (1, 10, 20, 30, 40 and 50 mM/egg) for 24 h, 48 h and 72 h to assess the effects of BPA on the survivability. In our study, it was observed that the mortality (%) of the chick embryos following BPA exposure was increased in a dose and time-dependent way (Table 2). At 24 h of exposure time, BPA treatment with concentrations up to 20 mM did not affect the survivability of the chick embryo and no mortality was observed (Figure 1A). However, increasing the BPA concentration from 20 mM to 50 mM affected severely the survival of the embryos. Interestingly, treatment with 50 mM BPA for 24 h showed 100% mortality (Table 2).

Similar trends of a dose-dependent increase in mortality upon BPA exposure for 48 h and 72 h were also observed (Figure 1, Table 2). The LD_{50} of BPA in chick embryo was also determined using probit analysis and it was found to be 18.62 mM (24 h), 37.15 mM (48 h) and 62.37 mM (72 h) (Figure 1B).

Cytotoxicity effects of BPA on developing chick embryos: The BPA-induced cytotoxicity was assessed by analyzing the incidence of erythroblast cells and PCE to NCE ratio. In the control group, the incidence of erythroblasts was 0.55% (±0.04), 0.53% (±0.03) and 0.52% (±0.05) for 24 h, 48 h and 72 h respectively. No significant increment in erythroblast cells in DMSO treated (vehicle control) group was observed. However, BPA treatment showed significant alterations of cellular dynamics of erythropoiesis. BPA exposure with 2 mM concentration considerably increased the incidence of erythroblast cell population and it was found to be 0.66% (±0.04), 0.83% (±0.05; $p < 0.01$ vs. control) and 1.43% (±0.01; $p < 0.001$ vs. control) for 24 h, 48 h and 72 h respectively (Figure 2A).

Table 2
Incidence of mortality in chick embryos following exposure to Bisphenol A on 11th -day of incubation

Exposure time	Treatment	Dose (mM)/egg	Number of eggs incubated (n)	Number of dead embryos (n)	Mortality (%)
24 h (exposed at ED10)	Control	0	15	0	0
	DMSO	125 µl	15	0	0
	BPA	1	15	0	0
		10	15	0	0
		20	15	0	0
		30	15	1	7
		40	15	8	53
		50	15	15	100
48 h (exposed at ED9)	Control	0	15	0	0
	DMSO	125 µl	15	0	0
	BPA	1	15	0	0
		10	15	0	0
		20	15	1	7
		30	15	3	20
		40	15	7	47
		50	15	15	100
72 h (exposed at ED8)	Control	0	15	0	0
	DMSO	125 µl	15	0	0
	BPA	1	15	0	0
		10	15	3	20
		20	15	8	53
		30	15	6	40
		40	15	10	67
		50	15	15	100

ED; Embryonic day, *UN;* Unopened control, *DMSO;* Dimethyl-sulfoxide, *BPA;* Bisphenol A. Sampling of embryos was done ED11

The PCE to NCE ratio was also significantly affected by BPA exposure. In the chick embryos of the control group, the PCE to NCE ratio was found to be 1.52 (±0.10), 1.63 (±0.06) and 1.57 (±0.05) at 24 h, 48 h and 72 h respectively (Figure 2F). Here also, BPA treatment significantly increased the PCE/NCE in a time-dependent manner. The incidence of PCE/NCE ratio following BPA exposure was found to be 1.81 (±0.05), 1.93 (±0.07; p <0.05 vs. control) and 1.96 (±0.07; p <0.001) respectively (Figure 2F). Therefore, the results suggested that BPA exposure could cause extensive embryotoxicity to the developing chick embryos as evidenced by an increased incidence of erythroblast cell population and PCE to NCE ratio.

DNA damage analysis in developing chick embryos: The DNA damaging potential of BPA was evaluated in the peripheral blood of chick embryos using HET-MN assay. The incidence (%) of MN in PCE (i.e. MnPCE) and NCE (i.e. MnNCE) following BPA exposure was significantly increased as compared to control (Figure 3B, C). The frequency of MN in both PCE and NCE was combined to quantify the total MN frequency in the peripheral blood of chick embryos. In the present study, the incidence (%) of total MN in control group embryos was found to be 0.26% (±0.09), 0.25% (±0.08) and 0.21% (±0.07) after 24 h, 48 h and 72 h of exposure respectively (Figure 2D). In the vehicle control (DMSO) group, no significant change for MN occurrence was observed. BPA exposure led to a drastic increase in the MN frequency where the total MN frequency (%) was observed to be 0.33% (±0.06), 0.52% (±0.07; p <0.01 vs. control) and 0.70% (±0.06; p <0.001 vs. control) at 24 h, 48 h and 72 h respectively.

Similarly, the incidence of ONA was also significantly increased in the BPA-treated embryos. In the control group embryos, the incidence of ONA was 0.10% (±0.02), 0.12% (±0.03) and 0.13% (±0.04) at 24 h, 48 h and 72 h respectively. Contrastingly, in the BPA exposed embryos, this was elevated to 0.37% (±0.05; p <0.01), 0.55% (±0.07; p <0.01) and 0.80% (±0.04; p <0.001) as compared to control embryos upon exposure for 24 h, 48 h and 72 h respectively.

The results of the HET-MN assay were further validated by comet assay and similar results were observed. The BPA-induced DNA damage was assessed by analyzing DNA damage parameters i.e. DNA content in the head region (Head DNA), tail region (Tail DNA), OTM and DI. The results showed a time-dependent decrease in head DNA content (fold change) in BPA-treated embryos (Figure 3A). The fold change of tail DNA and OTM was significantly increased in embryos treated with BPA as compared to control (Figure 3B, C). The DI was expressed in terms of percentage and in the control group, the DI was found to be 36.60% (±3.67), 36.00% (±3.67) and 35.90% (±3.61) at 24 h, 48 h and 72 h respectively (Figure 3D).

However, upon treatment with BPA, the DI was increased to 59.67% (±10.57), 77.60% (±0.21; p <0.01 vs. control) and 79.73% (±0.24; p <0.01 vs. control) when exposed for 24 h, 48 h and 72 h respectively. Therefore, the results indicated the potential of BPA to cause genetic alterations extensively during the early embryonic development in chick embryos.

Discussion

The present study aimed to evaluate the genotoxic and cytotoxic effects of BPA in developing chick embryos employing HET-MN and comet assay. Chick embryos have been used for years to investigate the effect of environmental chemicals. Studies reported that the early embryonic stage is the most sensitive period[39] and the exposure of mutagens/carcinogens at this period may severely influence organogenesis and cancer development in some organs. Several studies have reported that BPA has the potential to cause severe health problems and is more vulnerable to infants[18]. Atay et al[2] reported that BPA delays the development of the nervous system in chick embryos.

In the present study, a difference in the LD_{50} value with different developmental stages in chick embryos was observed. BPA exposure was introduced on ED 8, 9, 10 of the developmental stage of chick embryo and early introduction of exposure showed more toxicity with low LD_{50}. Therefore, it is possible to conclude that the BPA has maximum lethal toxicity in the early stages of embryo development. Recently, it has been reported that BPA as well as its metabolite 4-methyl-2,4-bis(4-hydroxyphenyl) pent-1-ene (MBP) interfered with reproductive organ development in the chicken embryo when exposure was made at ED4[24]. MBP-treated males showed retention of Müllerian ducts and feminization of the left testicle and the MBP-administered females displayed a diminished left ovary[24].

For analysis of the cytotoxic and genotoxic effects of agents, micronucleus assay has been extensively used. Wolf and Luepke[40] have reported that HET-MN assay in the peripheral blood is a useful technique to study the effects of environmental mutagens and pro-mutagens in the chick embryo. The micronucleus test demonstrated mainly definitive erythrocytes which are formed by embryonic hematopoietic stem cells in the yolk sac, the most metabolically active tissue[40].

Similarly, the present study depicted a significant increase in the number of micronucleated erythrocytes after treatment of BPA on ED8 of the developmental stage in comparison to control. The LD_{50} at 72 hours (ED8) was found to be lower than 48 (ED9) and 24 (ED10) hours and thereby it can be assumed that the effect of BPA exposure in terms of micronucleated erythrocytes also depends on the developmental stage. Moreover, as reported earlier, up to day 13 of incubation, the embryonic spleen does not contribute to rapid phagocytosis of damaged erythrocytes or micronucleated erythrocytes thereby accumulating the damaged cells for a longer period[41].

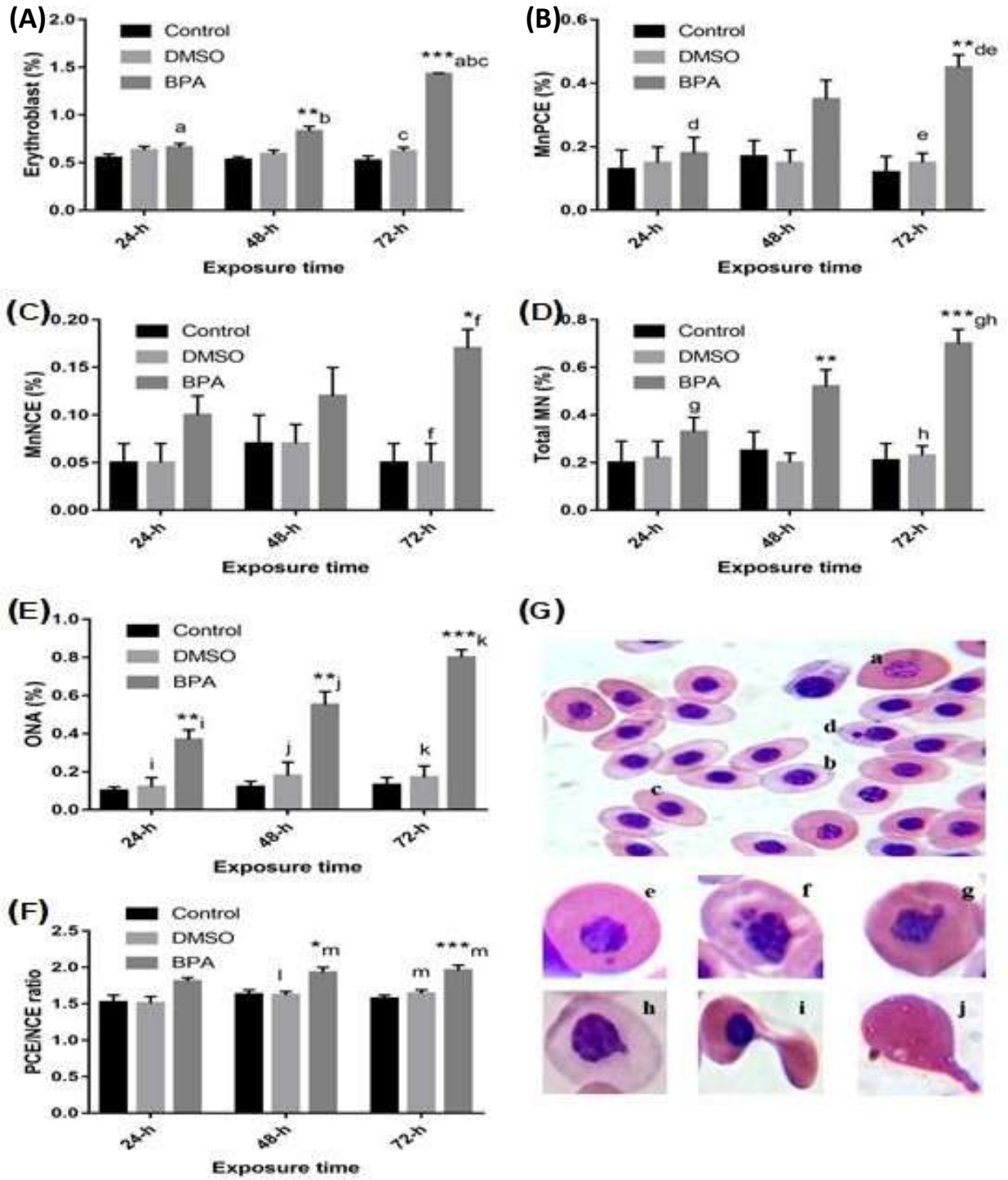

Figure 2: Incidence of micronuclei and other nuclear abnormalities in the peripheral blood erythroid cells following exposure to BPA. Histograms showed effects of BPA (A) on erythroblast cells; (B) frequency of MnPCE; (C) frequency of MnNCE; (D) incidence of total MN; (E) percentage of ONA and (F) PCE to NCE ratio. (G) Representative photomicrographs of different types of aberrant cell types in the peripheral blood of chick embryos upon exposure to BPA- (a) primitive erythrocytes, (b) PCE, (c) NCE, (d-f) erythrocytes with MN, (g,h) Nuclear bud, (i) poikilocytes and (i) cell without a nucleus. Each data point indicated mean±SE (n=6). Control (*unopened*); DMSO (*Vehicle control*) and BPA (*Bisphenol A; 2 mM/egg*). Statistical analysis: One-way analysis of variance (ANOVA). Values are significantly different from control at $p<0.05$ (*); $p<0.01$ (**); $p<0.001$ (***). PCE; *polychromatic erythrocytes*, NCE; *normochromatic erythrocytes*, MnPCE; *micronucleated polychromatic erythrocytes*, MnNCE; *micronucleated normochromatic erythrocytes*, MN; *micronucleus*, ONA; *other nuclear abnormalities*

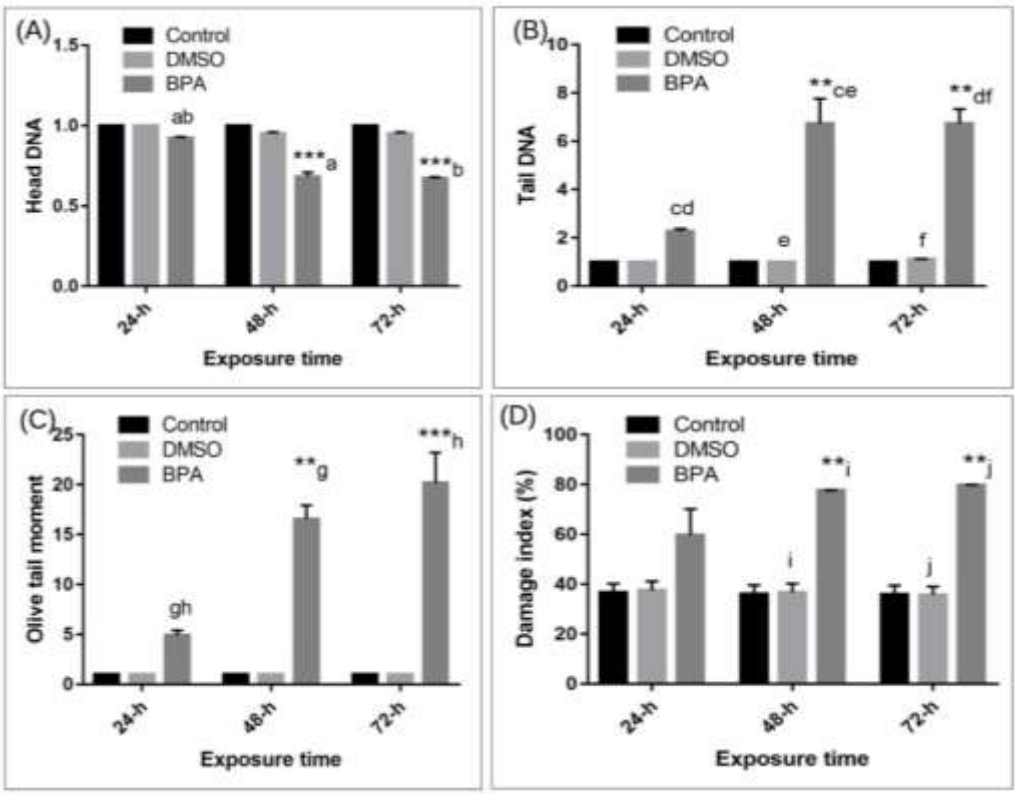

Figure 3: BPA-induced DNA damage in the peripheral blood of chick embryos as assessed by comet assay. Histograms showing the effect of BPA on (A) Fold change in head DNA, (B) Fold change in tail DNA, (C) Fold change in the olive tail moment and (D) Percentage of damage index upon exposure for 24 h, 48 h and 72 h. Each data point indicated mean±SE for six experimental eggs (n=6). Control (*unopened*); Vehicle control (*DMSO*) and BPA (*2 mM*). Statistical analysis: One-way analysis of variance (ANOVA). Values are significantly different from their respective control at $p<0.01$(**) and $p<0.001$ (***)

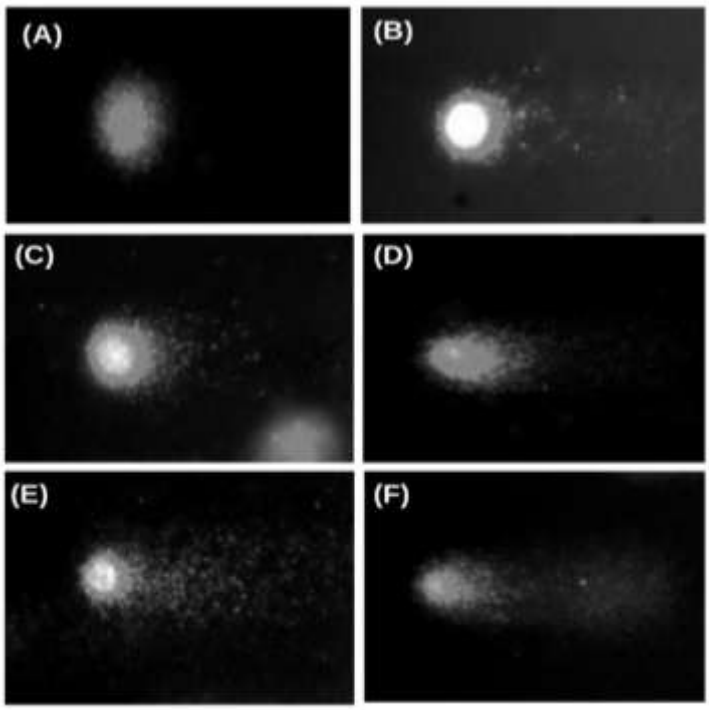

Figure 4: Photomicrographs showing comet images of different damage classes of erythrocytes observed in the peripheral blood of the 11th-day old chick embryo. (A) Normal erythrocytes (class 0), (B) low damaged erythrocytes (class 1), (C-D) moderately damaged erythrocytes (class 2 and class 3) and (E-F) highly damaged erythrocytes (class 4).

It may be due to the chemical which accumulates in the developing embryo for a longer period irrespective of dose. Research related to other cell types also has shown that BPA induces DNA damage in estrogen receptor (ER) - positive MCF-7 cells and its genotoxicity is ER-dependent. It was observed that it induces micronucleus frequencies and chromosomal aberrations in rat bone marrow and causes DNA damage in lymphocytes[9].

The ratio of PCE and NCE indicates hematopoietic cell proliferation rate and its abnormality. Apart from common erythroid cell types (early, middle and late PCE, NCE), undifferentiated cells like erythroblasts and proerythroblasts (discriminated from cells of the definite line by their greater size, lower nucleus–cytoplasm ratio and circular outline) have been considered as a parameter for hampered hematopoietic studies, whose life span is 8 days.

Therefore, the production of primitive erythrocytes ceases after day 7 of incubation and under normal conditions, these cell types are not present at the stage of ED11 (day of analysis for the present study) of embryonic development in the peripheral blood[41]. Our result reports an increase in erythroblasts in all the treated groups, which confirmed cytotoxic effects. Thus, apart from inducing aberrance in the nuclear material, BPA potentially induced cytotoxic changes which inhibited erythropoiesis and cell differentiation.

Similar observations were found by Sharaf et al[32] in broilers treated with cypermethrin where the treated birds showed micronucleated erythrocytes and various other morphological alterations like spindle- and pear-shaped erythrocytes along with nuclear segmentation. Therefore, present study suggests that the time, dose and stage of the embryos are most vital to which the chemicals are exposed to the animal.

The present study detects the level of DNA damage using an alkaline comet assay in the blood cells of the developing chick embryo after exposure to BPA. Data obtained from comet assay demonstrated a significant increase in the various parameters viz. tail DNA and damage index in peripheral blood of chick embryo exposed to BPA. Several other investigations demonstrated the genotoxic potential of various chemicals through comet assay[43, 44].

Similar damage was also found in the blood lymphocytes of rats after the treatment of BPA[36]. These findings are in agreement with the studies demonstrating DNA damage in MCF 7 cells after estradiol (E2) and BPA exposure[13] and are also consistent with the findings in the Chinese hamster ovary (CHO K1) cells[35].

The present study demonstrates a significant increase in DNA strand breaks in peripheral blood of chick embryos of all the BPA exposed groups similar to the results of Gowri and Balakrishnan[11] and Malladi et al.[21] The DNA fragmentation detected by comet assay reveals that BPA is potent enough to cause genetic damage in blood lymphocytes.

Conclusion

The results of the present study confirm the genotoxic and cytotoxic potential of BPA in the embryos of *Gallus gallus domesticus*. The developing chick embryo seemed to be highly sensitive to BPA and its exposure during the early embryonic development could induce significant damage. Thus, this study warrants further investigation of BPA-induced development anomalies during the late embryonic developmental stages. It will assist to understand the BPA-mediated toxicity clearly during embryonic development.

Acknowledgment

All the authors acknowledge the infrastructural support by the Department of Science and Technology, Government of India under the DST-FIST program; PL acknowledges the University Grants Commission, New Delhi, India, for providing National Fellowship for Higher Education (NFHE).

References

1. Alonso-Magdalena P., Ropero A.B., Soriano S., García-Arévalo M., Ripoll C., Fuentes E., Quesada I. and Nadal A., Bisphenol-A acts as a potent estrogen via non-classical estrogen triggered pathways, *Moleular and Cellular Endocrinology*, **355(2)**, 201-7 **(2012)**

2. Atay E., Ertekin A., Bozkurt E. and Aslan E., Impact of Bisphenol A on neural tube development in 48-hr chicken embryos, *Birth Defects Research*, **112(17)**, 1386-1396 **(2020)**

3. Bakker J., Hakkert B.C., Hessel E.V.S., Luit R.J., Piersma A.H., Sijm D.T.H.M., Rietveld A.G., Van Broekhuizen F.A., Van Loveren H. and Verhoeven J.K., Bisphenol A: Recommendations for risk management (part 2) (RIVM Report), The National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands, 17-62 **(2015)**

4. Bindhumol V., Chitra K.C. and Mathur P.P., Bisphenol A induces reactive oxygen species generation in the liver of male rats, *Toxicology*, **188**, 117–124 **(2003)**

5. Calafat A.M., Ye X., Wong L., Reidy J.A. and Needham L.L., Exposure of the US population to Bisphenol A and 4-tertiary-octylphenol: 2003–2004, *Environmental Health Perspect*, **116(1),** 39–44 **(2008)**

6. Chitra K.C., Latchoumycandane C. and Mathur P.P., Induction of oxidative stress by bisphenol A in the epididymal sperm of rats, *Toxicology*, **185**, 119–127 **(2003)**

7. D'Cruz S.C., Jubendradass R. and Mathur P.P., Bisphenol A Induces Oxidative Stress and Decreases Levels of Insulin Receptor Substrate 2 and Glucose Transporter 8 in Rat Testis, *Reproductive Sciences*, **19(2),** 163–172 **(2011)**

8. Ehrlich S., Calafat A.M., Humblet O., Smith T. and Hauser R., Handling of thermal receipts as a source of exposure to bisphenol A, *JAMA*, **311**, 859–60 **(2014)**

9. Fic A., Žegura B., Sollner Dolenc M., Filipič M. and Peterlin Mašič L., Mutagenicity and DNA damage of bisphenol A and its structural analogues in HepG2 cells, *Archieves of Industrial Hygiene and Toxicology*, **64**, 189-200 **(2013)**

10. Gao H., Yang B.J., Li N., Feng L.M., Shi X.Y., Zhao W.H. and Liu S.J., Bisphenol A and hormone-associated cancers: current progress and perspectives, *Medicine (Baltimore)*, **94(1),** e211 **(2015)**

11. Gowri K.U. and Balakrishnan S., Genotoxic effects of two different classes of insecticide in developing chick embryos, *Toxicology and Environmental Chemistry*, **95(6),** 992-1005 **(2013)**

12. Iarmarcovai G., Bonassi S., Botta A., Baan R.A. and Orsière T., Genetic polymorphisms and micronucleus formation: a review of the literature, *Mutation Research*, **658**, 215-233 **(2008)**

13. Iso T., Watanabe T., Iwamoto T., Shimamoto A. and Furuichi Y., DNA damage caused by bisphenol A and estradiol through estrogenic activity, *Biol. Pharm. Bull*., **29**, 206–210 **(2006)**

14. Ivett J.L., Brown B.M., Rodgers C. anderson B.E., Resnick M.A. and Zeiger E., Chromosomal aberrations and sister chromatid exchange tests in Chinese hamster ovary cells in vitro. IV. Results with 15 chemicals, *Environmental and Molecular Mutagenesis*, **14**, 165–187 **(1989)**

15. Kabuto H., Hasuike S., Minagawa N. and Shishibori T., Effects of bisphenol A on the metabolisms of active oxygen species in mouse tissues, *Environmental Research*, **93(1),** 31–35 **(2003)**

16. Kang J.H., Kondo F. and Katayama Y., Human exposure to Bisphenol A, *Toxicology*, **226(2–3)**, 79–89 **(2006)**

17. Kim C.S., Sapienza P.P., Ross I.A., Johnson W., Luu H.M. and Hutter J.C., Distribution of Bisphenol A in the neuroendocrine organs of female rats, *Toxicology and Industrial Health*, **20(1–5)**, 41–50 **(2004)**

18. Konieczna A., Rutkowska A. and Rachoń D., Health risk of exposure to Bisphenol A (BPA), *Annals of the National Institute of Hygiene*, **66(1),** 5-11 **(2015)**

19. Li D.K., Zhou Z., Miao M., He Y., Wang J., Ferber J., Herrinton L.J., Gao Er S. and Yuan W., Urine Bisphenol-A (BPA) level in relation to semen quality, *Fertility and Sterility*, **95(2),** 625–30, e1-4 **(2011)**

20. Lin C.Y., Fang Shen F.Y., Lian G.W., Chien K.L., Sung F.C., Chen P.C. and Su T.C., Association between levels of serum bisphenol A, a potentially harmful chemical in plastic containers and carotid artery intima-media thickness in adolescents and young adults, *Atherosclerosis*, **241(2),** 657–663 (**2015**)

21. Malladi S.M., Bhilwade H.N., Khan M.Z. and Chaubey R.C., Gamma ray induced genetic changes in different organs of chick embryo using peripheral blood micronucleus test and comet assay, *Mutation Research /Genetic Toxicology and Environmental Mutagenesis*, **630(1)**, 20-27 **(2007)**

22. Manikkam M., Tracey R., Guerrero-Bosagna C. and Skinner M.K., Plastics derived endocrine disruptors (BPA, DEHP and DBP) induce epigenetic transgenerational inheritance of obesity, reproductive disease and sperm epimutations, *PLoS One*, **8(1),** 55387 **(2013)**

23. Martins M. and Costa P., The comet assay in Environmental Risk Assessment of marine pollutants: Applications, assets and handicaps of surveying genotoxicity in non-model organisms, *Mutagenesis*, **30**, 89-106 **(2015)**

24. Mentor A., Bornehag C.G., Jönsson M. and Mattsson A., A suggested bisphenol A metabolite (MBP) interfered with reproductive organ development in the chicken embryo while a human-relevant mixture of phthalate monoesters had no such effects, *Journal of Toxicology and Environmental Health*, **83(2)**, 66-81 **(2020)**

25. Michalowicz J., Bisphenol A—sources, toxicity and biotransformation, *Environmental Toxicology and Pharmacology*, **37**, 738–758 **(2014)**

26. Naik P. and Vijayalaxmi K.K., Cytogenetic evaluation for genotoxicity of bisphenol-A in bone marrow cells of Swiss albino mice, *Mutation Research*, **676**, 106–112 **(2009)**

27. Olive P. and Banáth J., The comet assay: a method to measure DNA damage in individual cells, *Nature Protocols*, **1**, 23–29 **(2006)**

28. Pacchierotti F., Ranaldi R., Eichenlaub-Ritter U., Attia S. and Adler I.D., Evaluation of aneugenic effects of bisphenol A in somatic and germ cells of the mouse, *Mutation Research*, **651**, 64–70 **(2008)**

29. Ramos C., Ladeira C., Zeferino S., Dias A., Faria I., Cristovam E., Gomes M. and Ribeiro E., Cytotoxic and genotoxic effects of environmental relevant concentrations of bisphenol A and interactions with doxorubicin, *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, **838**, 28–36 **(2019)**

30. Russo C., Acito M., Fatigoni C., Villarini M. and Moretti M.B., Comet Assay (Comet Assay on Buccal Cells) for the evaluation of Primary DNA Damage in Human Biomonitoring Studies, *International Journal of Environment Research and Public Health*, **17**, 9234 **(2020)**

31. Schönfelder G., Wittfoht W., Hopp H., Talsness C.E., Paul M. and Chahoud I., Parent bisphenol A accumulation in the human maternal-fetal-placental unit, *Environmental Health Perspect*, **110(11),** A703-7 **(2002)**

32. Sharaf F.S., Khan A., Khan M.Z., Aslam F., Saleem M.K. and Mahmood F., Clinico Hematological and Micronuclear Changes Induced by Cypermethrin in Broiler Chicks: Their Attenuation with Vitamin E and Selenium, *Experimental and Toxicologic Pathology*, **62**, 441–450 **(2010)**

33. Singh N.P., McCoy M.T., Tice R.R. and Schneider E.L., A Simple Technique for Low Levels of DNA Damage in Individual Cells, *Experimental Cell Research*, **175**, 184–191 **(1988)**

34. Staples C.A., Dome P.B., Klecka G.M., O'Block S.T. and Harris L.R., A review of the environmental fate, effects and exposures of bisphenol A, *Chemosphere*, **36(10),** 2149-73 **(1998)**

35. Tayama S., Nakagawa Y. and Tayama K., Genotoxic effects of environmental estrogen-like compounds in CHO-K1 cells,

Mutation Research, **649**, 114–125 **(2008)**

36. Tiwari D., Kamble J., Chilgunde S., Patil P., Maru G., Kawle D., Bhartiya U., Joseph L. and Vanage G., Clastogenic and mutagenic effects of bisphenol A: an endocrine disruptor, *Mutation Research*, **743**, 83–90 **(2012)**

37. Tsutsui T., Tamura Y., Yagi E., Hasegawa K., Takahashi M., Maizumi N., Yamaguchi F. and Barrett J.C., Bisphenol-A induces cellular transformation, aneuploidy and DNA adduct formation in cultured Syrian hamster embryo cells, *International Journal of Cancer*, **75**, 290–294 **(1998)**

38. Vandenberg L.N., Chahoud I., Heindel J.J., Padmanabhan V., Paumgartten F.J.R. and Schoenfelder G., Urinary, Circulating and Tissue Biomonitoring Studies Indicate Widespread Exposure to Bisphenol A, *Environmental Health Perspect*, **118(8),** 1055–1070 **(2010)**

39. vom Saal F.S., Akingbemi B.T., Belcher S.M., Birnbaum L.S., Crain D.A., Eriksen M., Farabollini F., Guillette Jr. L.J., Hauser R., Heindel J.J., Ho S.M., Hunt P.A., Iguchi T., Jobling S., Kanno J., Keri R.A., Knudsen K.E., Laufer H., LeBlanc G.A., Marcus M., McLachlan J.A., Myers J.P., Nadal A., Newbold R.R., Olea N., Prins G.S., Richter C.A., Rubin B.S., Sonnenschein C., Soto A.M., Talsness C.E., Vandenbergh J.G., Vandenberg L.N., Walser-Kuntz D.R., Watson C.S., Welshons W.V., Wetherill Y. and Zoeller R.T., Chapel Hill Bisphenol A Expert Panel Consensus Statement: integration of mechanisms, effects in animals and potential to impact human health at current levels of exposure, *Reproductive Toxicology*, **24(2),** 131-138 **(2007)**

40. Wolf T.H. and Luepke N.P., Formation of micronuclei in incubated hen's eggs as a measure of genotoxicity, *Mutation Research*, **394,** 163–175 **(1997)**

41. Wolf T.H., Rolf C.N. and Luepke N.P., Some new methodological aspects of the hen's egg test for micronucleus induction (HET-MN), *Mutation Research*, **514**, 59–76 **(2002)**

42. Wolstenholme J.T., Edwards M., Shetty S.R., Gatewood J.D., Taylor J.A., Rissman E.F. and Connelly J.J., Gestational exposure to bisphenol a produces transgenerational changes in behaviors and gene expression, *Endocrinology*, **153(8),** 3828–3838 **(2012)**

43. Zani C., Ceretti E., Zerbini I., Viola G., Donato F., Gelatti U. and Feretti D., Comet Test in Saliva Leukocytes of Pre-School Children Exposed to Air Pollution in North Italy: The Respira Study, *International Journal of Environmental Research Public Health*, **17(9),** 3276 **(2020)**

44. Zhang H., Wang Z., Meng L., Kuang H., Liu J., Lv X., Pang Q. and Fan R., Maternal exposure to environmental bisphenol A impairs the neurons in hippocampus across generations, *Toxicology*, **432**, 152393 **(2020)**.

(Received 06th September 2021, accepted 08th October 2021)