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Histopathology

The Ameliorative Effect of Vitamin a and Stem Enhancer on Cytological Changes and Apoptosis Inducing-Activity in BPA-Treated Murine Model

Hassan BN*[1], Al beltagy RS[1], AL Azzouni AS[1], El-Garawani IM[2]
[1]Department of Zoology, Faculty of Science, Helwan University, Al Sikka Al Hadid Al Gharbeya, Al Masaken Al Iqtisadeyah, Qism Helwan, Cairo Governorate, Egypt
[2]Department of Zoology, Faculty of Science, Menoufia University, Gamal Abd El-Nasir, Qism Shebeen El-Kom, Shebeen El-Kom, Menofia Governorate, Egypt

Original Research Article

***Corresponding author**
Hassan BN

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Abstract: Bisphenol A is a plasticizer used as monomer of epoxy resins and polycarbonate plastics which found in many different applications such as adhesives and paper coatings, food related containers and water bottles; what make human is more exposed to its hazard effects especially at specific doses. The present study was performed with the purpose of evaluating the ameliorating property of stem enhance and vitamin A against BPA toxicity on liver and kidney tissues. In this experimental study, 20 female albino rats were divided into 4 groups. Rats of BPA group was administered intraperitoneally daily dose of BPA (20mg/kg bw) for 45 days; moreover oral administration of vitamin A and stemenhance was used as ameliorative agents for other 15 successive days after BPA treatment period. At the end , rats were sacrificed and their liver and kidney were proceeded for histopathological and immune-histochemical reactivity (caspase 3). Agarose gel electrophoresis was carried out to evaluate the pattern of DNA damage furthermore, morphological examination of hepatocyte was carried out by acridine orange/ ethidium bromide (AO/EB) dual fluorescent staining. BPA caused inflammation, apoptosis and vacuolization in liver and kidney. Also caspase-3 immunohistochemical reactions showed strong intensity in BPA group in both liver and kidney tissues. While in the treated groups with stemenhance and vitamin A showed amelioration in DNA fragmentation, histopathological changes and intensity of caspase-3 immunoreactivity. Vitamin A has ameliorative effect more than stemenhance on liver and kidney against BPA supplying injurious molecular effects. Vitamin A and stemenhance can be considered as a natural supplements against environmental toxicity.
Keywords: Liver; Kidney; Bisphenol A; Vitamin A; Stem enhance; DNA fragmentation, Apoptosis.

INTRODUCTION

Bisphenol A (BPA) is predominantly used as an intermediate to manufacture polycarbonate and epoxy resin [1], which are mainly polymeric materials used for different applications due to their good physical and chemical properties such as transparency, high mechanical strength low moisture absorption and good thermal stability. BPA has been found in many common food containers and packaging, and in the epoxy lining of metal food cans, from which, especially after heating, it can leach into food products [2]. Also it is widely applied in the electrical parts of automobiles, household appliances, construction glazing, sports safety equipment, tableware, reusable bottles, food storage containers, adhesives, and thermal paper [3, 4]. Many studies have examined release of BPA from PC products by two different mechanisms: 1) Diffusion controlled release of residual BPA and 2) hydrolysis/degradation of the polymer at the surface of the material followed by an increase in BPA migration and in polymer surface area [5]. BPA has no structural homology with 17β-estradiol (E2), but because it is similar to diethylstilbestrol (DES), the synthetic estrogen known to cause cancer [6]. It is a well-known endocrine disruptor and its estrogenic properties are reported since 1936 [7]. It has been demonstrated in both in vitro and in vivo assays to act as an endocrine-disrupting chemical [8]. BPA enters the environment via open disposal or recycling of products which contains BPA [9]. BPA affects cellular physiology by binding with diverse physiological receptors, such as genomic and membrane-bound estrogen receptors, androgen receptor, peroxisome receptor γ, and thyroid receptor [10]. Bisphenol A has the potential to induce aneuploidy and DNA adduct formation in Syrian hamster embryo cells [11].

Rodent studies have shown that prenatal exposure of rats to BPA is associated with an increased risk of breast cancer in adult female rats [12] and hyperplasia of prostate in male rats, resulting in greater risk of prostate cancer [13].

Moreover, BPA has been shown to form DNA adduct in both liver and mammary cells of female CD-1 mice [14]. Bisphenol A treatment has been shown to disrupt the cytoplasmic microtubular complex as well as mitotic and meiotic spindle formations [15]. It also induced aneuploidy and chromosome congression failure in oocytes of mice exposed to low concentration of BPA [16].

BPA release was associated with potential estrogenicity in a significant number of studies [17], examined as significant risk factor regarding human fertility in female [18] and male [19], and regarding diabetes [20]. It was reported a positive association between prenatal BPA exposure and symptoms of anxiety/depression, specifically in boys [21].

Vitamin A nutrition is of profound importance to human health. It has beneficial immune and antioxidant functions [22] Stem Enhance® is natural Stem Cell Enhancer which functions as stimulator for the natural release of adult stem cells from the bone marrow. Stem Enhance is a patented blend of mobilin and migratose concentrates extracted from Aphanizomenon flos-aquae. They are cyanobacteria or blue-green algae that grow worldwide. The mobilin (water or buffered saline), which contains an l-selectin ligand, supports the release of stem cells (CD34+ cells) from the bone marrow [23]. The migratose (10%-20% ethanol at 50°C), a polysaccharide-rich fraction, may support the migration of stem cells out of the blood into tissues [24]. Stem Cell Enhancers facilitates the migration of stem cells of the bone marrow to any tissue in the body needing repair [25]. It has been reported the role of stemenhance in stem cell mobilization and the improvement of diseases such as diabetes mellitus [26]

MATERIALS AND METHODS

Materials

Bisphenol A (BPA) (2, 2 Bis-4- hydroxyl phenyl propane) suspended in water and orally administered to animals. The dose of BPA was calculated according to Takahashi and Oishi, 2003 [27].

Vitamin A: Drug purchased as dietary supplements gelatinous capsules of 500 mg, Pharco company. Therapeutic dose calculated according to Goash table.

Stem enhance: Drug purchased as dietary supplements capsules of 500mg for dosing from stem tech. health science, Inc. therapeutic dose calculated according to Goash table.

The experimental design

Twenty female albino rats of Sprague Downly strain, weighing 120±10g, at the age of 6-8 weeks were purchased from Theodore Bilharz Research Institute, Giza, Egypt. They were kept under observation for about 15 days before the onset of the experiment for adaptation. The animals were fasted before sacrifices for about 12 hours

Experimental animals were divided into four groups (five/each) as follows:

Group I (Control group): Normal female rats (without any treatment) for 30 days.

Group II (Bisphinol-A group): female rats were daily intraperitoneally injected with BPA (20mg/kg b.wt) for 45 days.

Group III (Bisphinol-A + Vit. A): female rats were daily intraperitoneally BPA daily dose for 45 days and then orally supplied with vitamin A (4.5mg/100g/day) for 15 days.

Group IV (Bisphinol-A + Stemenhance): Rats intraperitoneally injected with BPA daily for 45 days and then orally supplied with stemenhance (4.9mg/100g/day) only for other15 days.

Methods

Molecular assay

Acridine orange/ ethidium bromide (AO/EB) dual fluorescent staining

Morphological examination of isolated hepatocytes of control and treated groups. They were obtained from liver tissue homogenate after washing in PBS twice. Briefly, 20 µl of hepatocytes suspension in PBS from control and treated groups were placed on a clean glass slide and they were stained with 5µl of (1:1) acridine orange (50µg/ml)/ethidium bromide (5µg/ml) solution. Five hundred cells for each slide were immediately examined (×400) under fluorescent microscope (Olympus BX41, Japan) and the representative photos were captured. Three replicates were processed.

DNA electrophoresis and apoptosis detection

Nucleic acid extraction was done according to extraction method of Aljanabi and Martinez (1997) [28] with some modifications had been introduced by El-Garawani and Hassab El-Nabi (2016) [29] in which the direct staining of DNA samples was done. Agarose gel electrophoresis was done and apoptotic bands of DNA fragmentation appeared and located at 180 bp and its multiples 360, 540 and 720 bp against thirteen bands of DNA marker (100-3000 bp, Thermo Fisher Scientific) [30] .The intensity of released DNA fragments was measured by image J software, as a mean of optical density values.

Histopathological assay

Specimens from liver and kidney were collected from all experimental groups and fixed in 10% neutral buffer formalin. Then, tissues were

processed and 5µm sections were stained with Haematoxylin and Eosin [31]. They were observed by light microscope (Olympus BX41, Japan) and the representative photos were captured.

Immunohistochemical assay

Sections of liver and kidneys (5 µm) were fixed in 10 % neutral buffered formalin fixative immunostained using anti-caspase3 primary antibody (Labvision, Neomarkers, USA) for 90 minutes. This was followed by the secondary antibody application using the immunoperoxidase technique (Vectastain ABC kit; Vector Laboratories, Burlingame, CA).

Morphometric analysis

Morphometric estimation by image J program for anti caspase3 reaction intensity in liver and kidney sections occurred through masking of caspase-3 reaction in liver and kidney cells .Ten fields from each group were used and average reading were taken [32].

Statistical analysis

The numerical data of results were expressed as mean ± standard errors (SE). Statistical analysis was carried out using the "prism version 5" statistical software. Comparison between different groups was done using one way analysis of variance (ANOVA) followed by Tukey test. The results were considered statistically significant when the (p) value was ($P < 0.05$) [33]

RESULTS

Morphological changes after acridine orange/ Ethidium bromide dual fluorescent staining for liver tissues

The (AO/EB) fluorescent staining of isolated hepatocytes revealed the presence of cytoplasmic vacuolation and nuclear fragmentation as a feature of apoptosis in BPA-treated group more than other treated groups when compared with control. Additionally, vitamin A group gave the best ameliorative effect throughout all treatments when compared with control (Figure-1) and (Table-1).

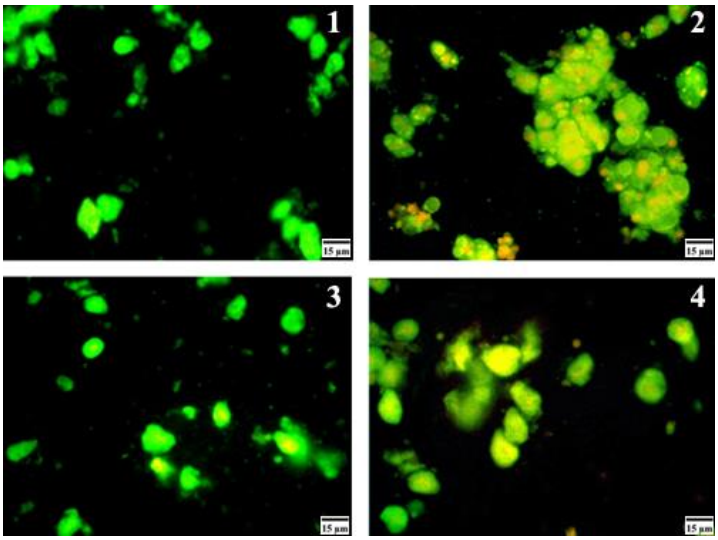

Fig-1: Significant increase (*P< 0.05*) of apoptotic features with blebbing and nuclear fragmentation of hepatocytes in BPA-treated group when compared with control and improvement after SE and Vit. A treatments (AO/EB fluorescent staining, Olympus BX41). Where 1: normal cells, 2: BPA, 3: BPA+SE and 4: BPA+ Vit. A. (n = 5)

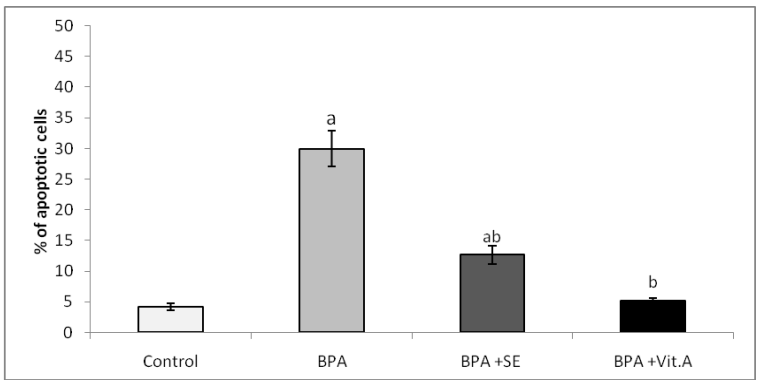

Fig-2: Percentage of apoptotic hepatocytes (AO/EB fluorescent staining) in treated and untreated groups. Data were presented as (Mean ± SEM) of three independent experiments. a: statistically significant (*P< 0.05*) with respect to control and b: statistically significant (*P< 0.05*) with respect to BPA group. (n = 5)

Effect of BPA on liver total genomic DNA damage

BPA treatment showed significant increase (*P< 0.05*) of DNA damage appeared as apoptotic laddering pattern at 180, 360, 540, 720 bp. While the treatment with SE showed an improvement with less DNA fragmentation when compared with BPA treatment group. Moreover, Vit A group showed no diagnostic DNA damage as compared with control groups revealing the excellent ameliorative potential of Vit A against BPA effect (Figure-2) and (Table-2).

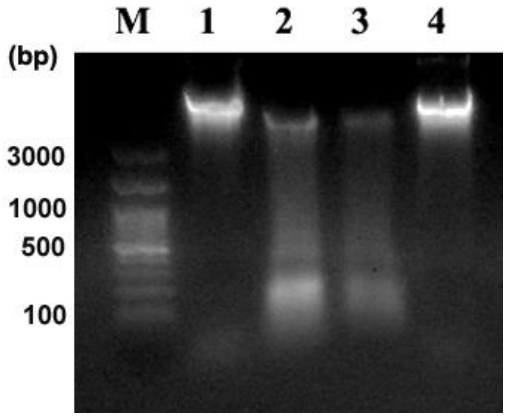

Fig-3: Digital photograph of total genomic DNA agarose gel electrophoresis (1.5 %) shows the effect of BPA on rat's liver as appeared as DNA apoptotic fragmentation with BPA treatment and significant improvement (*P< 0.05*) of DNA damage after SE and Vit A administration . Where, lane 1: control; lane 2: BPA ; lane 3: SE; lane 4: Vit. A and M: DNA marker. (n = 5).

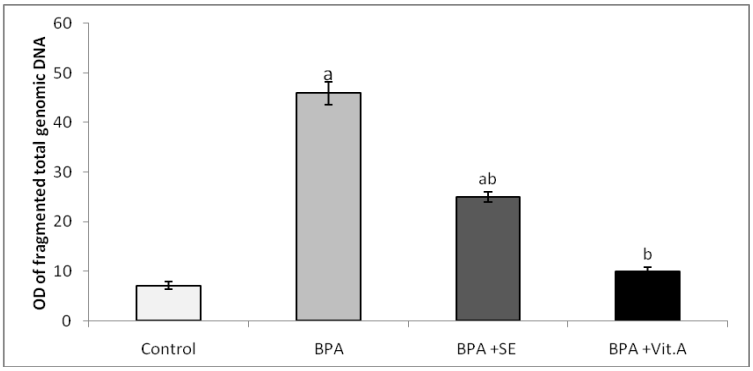

Fig-4: Total genomic DNA fragmentation in treated and untreated groups as evaluated as optical densities at the gray level (ImageJ software). Data were presented as (Mean ± SEM). a: statistically significant (*P< 0.05*) with respect to control and b: statistically significant (*P< 0.05*) with respect to BPA group. (n =5).

Histopathological result

Liver

H and E results in control liver shows normal hepatic architecture represents in closely packed hepatic cords with narrow visible sinusoids also well observed hepatocytes with intact rounded centrally located nuclei acidophilic cytoplasm with basophilic granules. A weak immune-histochemical reaction is shown for anti-caspase 3 antibody in the hepatic cytoplasm.

BPA treated liver showed disorganized hepatic cords leading to loss of lobular architecture. Also, hepatocytes with vacuolated cytoplasm were observed, blood sinusoids were obliterated and hepatic vein congestion. The nuclei of many hepatocytes were mostly disintegrated, and some others have distinct feature of pyknosis or kareolysis. A strong intensive immune-histochemical reaction was shown for anti-caspase 3 antibody in the hepatic cytoplasm .

Vitamin A treated liver showed organized hepatic cords and some cells showed cytoplasmic vaculation. Some nuclei exhibit normal shape and size while others showed pyknosis and the blood sinusoids reappear with kuppfer cells. A moderate immune-histochemical reaction was shown for anti-caspase 3 antibody in the hepatic cytoplasm.

Stem enhance treated liver showed disorganized hepatic cords and obliterated irregular blood sinusoids. Some nuclei exhibited normal shape and some others nuclei were pyknotic and showed marginated chromatin. Most of the hepatic cells still showing some vacuoles.A mild immune-histochemical reaction was shown for anti-caspase 3 antibody in the hepatic cytoplasm.

Kidney

Control kidney shows normal renal architecture. Bowman's capsule and a lobulated glomerular tuft of capillaries called the glomerulus being separated from it by the sub-capsular space well noticed .The outer layer (parietal) of Bowman capsule was noticed to be formed of simple squamous epithelium. Normal proximal convoluted tubules with epithelial lining made up of truncate columnar eosinophilic cells & D.C.T were present also with epithelial lining formed of faint acidophilic cubical cells with spherical nuclei, their lumen apparently wider than P.C.Ts. A weak immune-histochemical reaction is shown for anti-caspase 3 antibody in the tubular cell cytoplasm.

BPA treated kidney showed Abnormal Bowman's capsule with thicken parietal layer (transform from simple squamous epithelium to simple cuboidal) .The glomeruli lost the lobulation of its capillary tufts, which appeared vacuolated. Many necrotic tubules were present and vaculation in the cytoplasm of some cells of proximal convoluted tubules. A strong intensive immuno-histochemical reaction was shown for anti-caspase 3 antibody in the tubular cell cytoplasm.

Vitamin A treated kidney relatively was similar to control group as glomeruli with well-defined tuft of capillaries and filtration space. Normal proximal and distal convoluted tubules were observed. A moderate immunohistochemical reaction was shown for anti-caspase 3 antibody in the tubular cells cytoplasm .Stem enhancer treated group also showed an observed improvement clearly represented with well-defined Bowman's capsules with normal parietal layers and filtration spaces. Preserved glomerular tuft lobulation was noticed. The urinary tubules, also, improved and appeared similar to those of control ones.

A mild immune-histochemical reaction was shown for anti-caspase 3 antibody in the tubular cells cytoplasm.

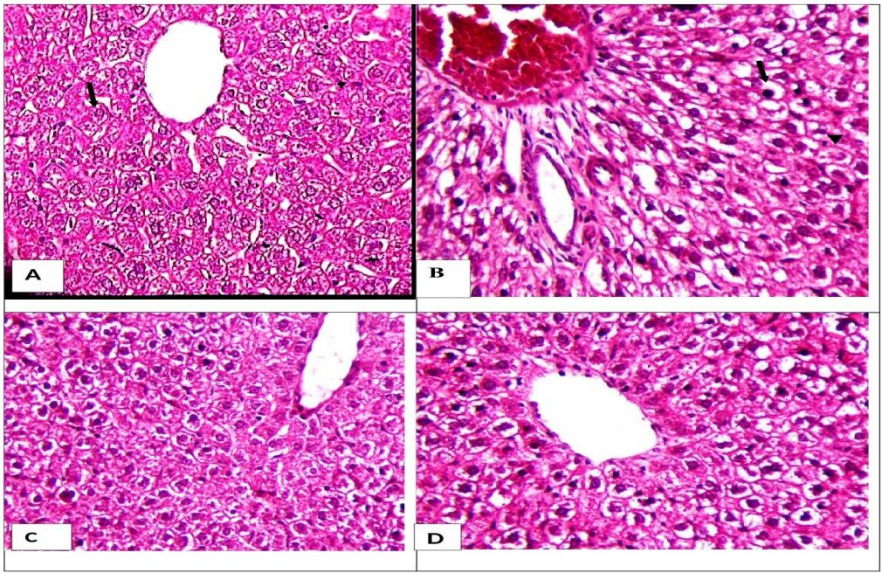

Fig-5: A photomicrograph of control liver showing normal liver architecture with clear visible hepatocytes and intact nucleus(→). B) Treated BPA liver showing loss hepatic architecture some karyohexis(arrow head),pyknosis(→), dilated sinusoids and blood congestion. C) vitamin A treated liver showing an obvious improvement in hepatic architecture and in hepatocyte. D) ST treated liver showing slight improvement in hepatic architecture H and E X40

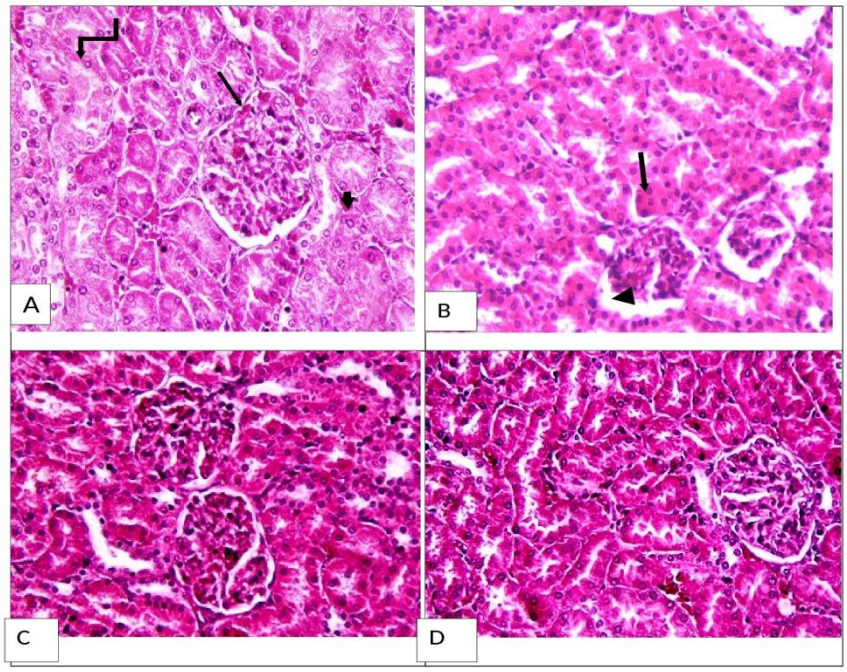

Fig-6: A photomicrograph of control kidney showing normal kidney architecture with clearly visible glomeruli(↓)proximal (arrow head) and distal tubules(elbow arrow). B) Treated BPA kidney showing loss kidney architecture some necrotic tubules(↓), apoptotic cells ,dilated tubules and shrunken glomeruli(arrow head). C) vitamin A treated kidney showing an obvious improvement in kidney architecture with well observed glomeruli .D) ST treated kidney showing a marked improvement in kidney architecture H and E X40

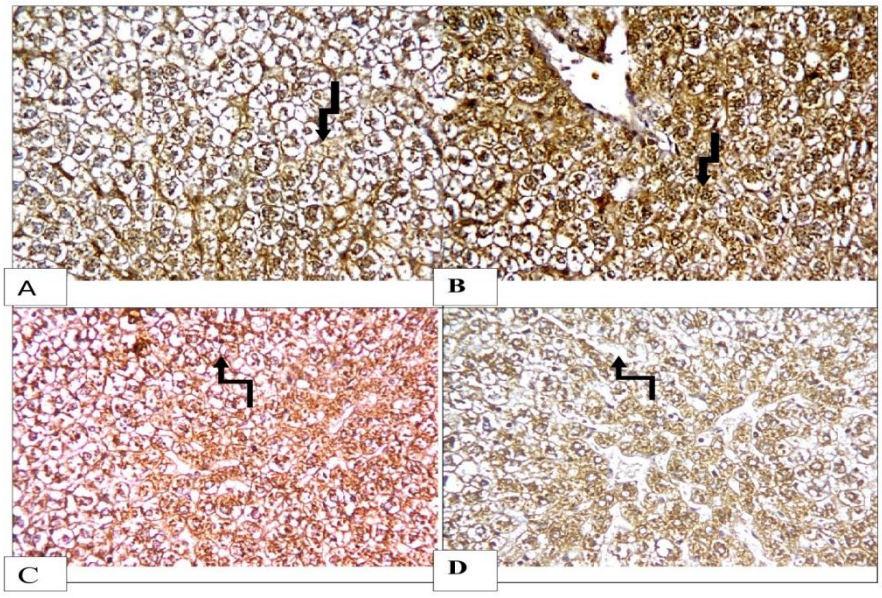

Fig-7: A photomicrograph of control liver showing weak anti caspase immunohistochemical reaction in hepatocyte cytoplasm(elbow arrow). B) Treated BPA liver showing strong intensive anti caspase immunohistochemical reaction in hepatocyte cytoplasm(elbow arrow). C) vitamin A treated liver showing moderate anti caspase immunohistochemical reaction in hepatocyte cytoplasm. D) ST treated liver showing mild anti caspase immunohistochemical reaction in hepatocyte cytoplasm (Anti caspase rx x40)

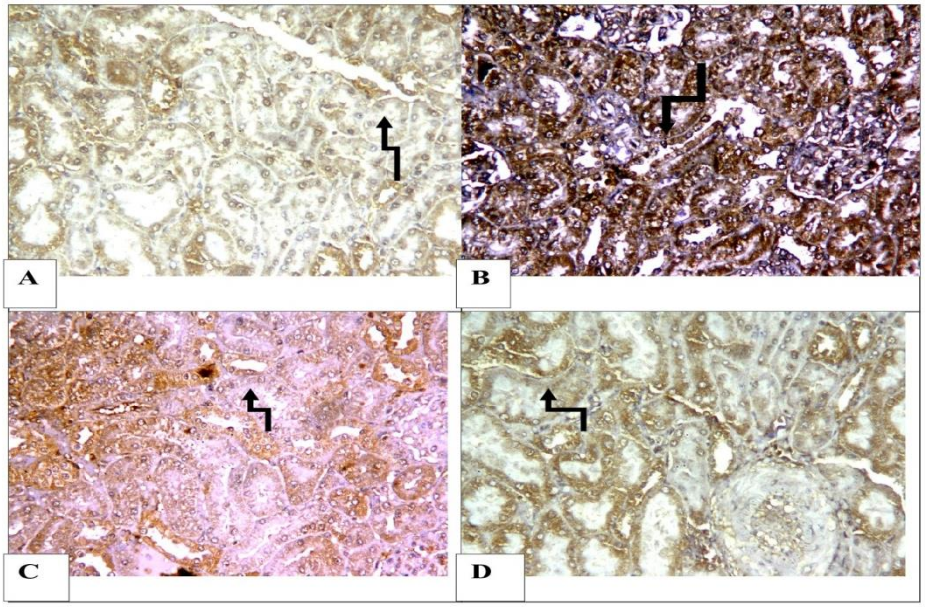

Fig-8: A photomicrograph of control kidney showing weak anti caspase immunohistochemical reaction in tubular cells cytoplasm. B) Treated BPA kidney showing strong intensive anti caspase immunohistochemical reaction in tubular cells cytoplasm(elbow arrow). C) Vitamin A treated liver showing mild anti caspase immunohistochemical reaction in tubular cells cytoplasm. D) ST treated liver showing moderate anti caspase immunohistochemical reaction in tubular cells cytoplasm Anti-caspase rx x40

Immunohistochemical morphometric results

The mean grey of intensity of the anti-caspase 3 positively immunostained cells in both hepatocytes and urinephrouse cells showed a statistically significant increase in the BPA treated group and significant decrease vit. A treated groups when compared with the untreated one ,also a significant decrease in stem enhancetreated groups when compared with the untreated one.

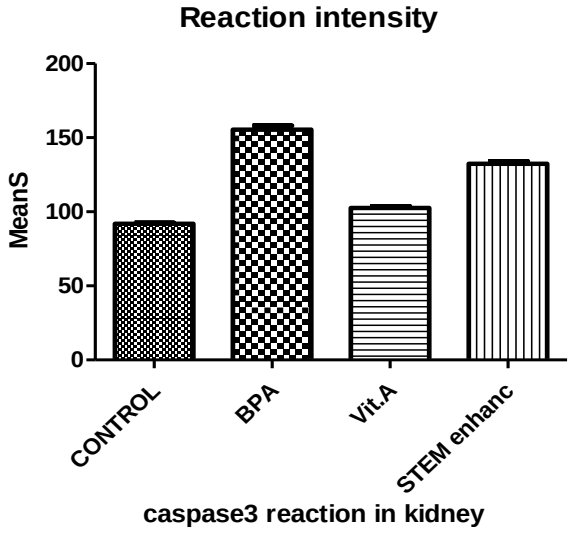

Fig-9: Histogram showing the mean grey of anti caspase3 reaction in the uriniferous tubules of different groups.

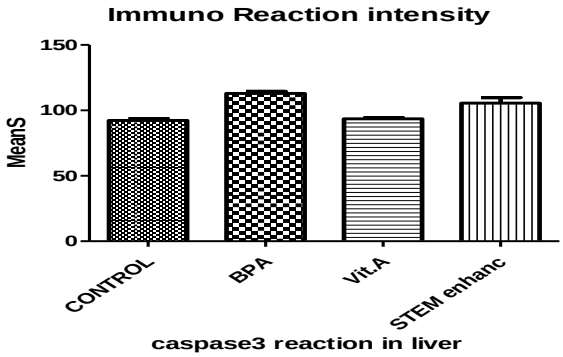

Fig-10: Histogram showing the mean grey of anti caspase3 reaction in the hepatocytes of different groups

The number of images were 5 in each group. Data are expressed as mean ± SE *****: Significant change at $p < 0.05$ with respect to corresponding control group**.**

DISCUSSIONS

Results of liver histopathology indicating karyolysis, karyohexis and pyknosis which agree with [34] who reported that BPA only at dose of 100µg/kg bw caused inflammation and vacuolization in liver tissue and increased concentration can cause damage to liver tissue. The present study showed DNA fragmentation and activation of caspase-3 in liver tissue of BPA-treated rats, suggesting the activation of apoptosis cascades.

Also there was a strong immunohistochemical reaction for caspase 3 in kidney tissue at the same dose of BPA. These result validated by the apoptotic picture which represented in the apoptotic tubular cells and necrotic tubules in the kidney tissue. A previous study has also shown that apoptosis induction by BPA is associated with caspase activation [35, 36].

The findings in the current study mostly attributed to the results of Tiwari and Vanage 2017 [37]who reported that BPA exposure induces oxidative stress, which could be one of the possible mechanisms causing reproductive and genetic toxicity. It was reported that BPA increases the generation of reactive oxygen species (ROS) [38] 39] which in turn could lead to DNA damage and mutation of tumor suppressor genes. The genetic DNA damage may be an initiation to multistep carcinogenesis later in life [40] as BPA has ability to enhance tumor susceptibility and promote tumorigenic properties in the breast and prostate glands [41] in which reactive oxygen species (ROS) are cytotoxic agents that lead to significant oxidative damage by attacking biomolecules such as membrane lipids and DNA in cells [42]. Wu et al. 2017 study revealed that low and environmentally relevant concentrations of BPA could be significantly accumulated in zebra fish and induced apoptosis with involvement of the regulation of caspase-3 and other apoptosis-related genes [43].

Vitamin A showed marked improvement at the molecular, histological as well as immunohistochemical level. Aikawa *et al*., 2004 in mice, neonatal exposure to a relatively large dose of BPA causes damage to the motility and morphology of sperm, but the BPA effect is, to some extent, inhibited by a supplement of Vitamin A, and enhanced under Vitamin A deficient condition [44] [45]. Another study indicated that vitamin A may serve as an antioxidant to protect the immune cells against oxidant stressors and thereby maintain optimum immune function [45]. Onyegeme-Okerenta & Anacletus, 2016 reported that vitamin A can alleviate the Al-mediated hapatoxicty in male Wistar rats [46].

While stem enhance results showed slight improvement in compared with BPA-treated group. These result is in consistent with Drapeau, 2010 study that hypothesized that bone marrow derived stem cells may accelerate tissue regeneration process in some animal models of injury [25]. Stemenhance® supports the natural release of adult stem cells from the bone marrow. Stem cells form the core of the body's natural renewal system. Adult stem cells are signaled by tissue and organs in need. They migrate into the tissue, reproduce and transform themselves into healthy cells for tissue injury repairing. Hassan et al., 2015 study recorded that stemenhancer treatment showed a marked improvement in ganglion cell layer and inner nuclear layer of retina in BPA female treated rats [47].

In our study, we have found that vitamin A has an obvious ameliorative effect more than stem enhance in liver and kidney tissues.

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