

# Potential Toxic Effect of Bisphenol A on the Cardiac Muscle of Adult Rat and the Possible Protective Effect of Omega-3: A Histological and Immunohistochemical Study

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## Abstract

Bisphenol A (BPA) is intensely used in the production of polycarbonate plastics and epoxy resins. Recently, BPA has been receiving increased attention due to its link to various health problems that develop after direct or indirect human exposure. Previous studies have shown the harmful effect of high doses of BPA; however, the effect of small doses of BPA on disease development is controversial. The aim of this study was to investigate the effect of a low dose of BPA on the rat myocardium and to explore the outcome of coadministration of Omega-3 fatty acid (FA). Thirty adult male rats were divided equally into control group, BPA-treated group (1.2 mg/kg/day, intraperitoneally for 3 weeks), and BPA and Omega-3-treated group (received BPA as before plus Omega-3 at a daily dose of 300 mg/kg/day orally) for 3 weeks. Exposure to BPA resulted in structural anomalies in the rat myocardium in the form of disarrangement of myofibers, hypertrophy of myocytes, myocardial fibrosis, and dilatation of intramyocardial arterioles. On the other hand, mast cell density and media-to-lumen area ratio were not significantly altered. Interestingly, concomitant administration of Omega-3 FAs with BPA significantly reduced BPA-induced changes and provided a protective effect to the myocardium. In conclusion, exposure to a low dose of BPA could potentially lead to pathological alterations in the myocardium, which could be prevented by administration of Omega-3 FA.

**Keywords:** Bisphenol A, histopathology, myocardium, Omega-3

## INTRODUCTION

Bisphenol A (BPA) is a key monomer in the manufacture of very essential polycarbonate plastics products such as water bottles, sports equipment, CDs, and DVDs. Importantly, BPA is also used to line water pipes, food cans, and in making thermal paper such as that used in sales receipts.<sup>[1]</sup> The health hazard of BPA is mainly due to the incomplete polymerization reaction that leaves some unbound monomer BPA molecules in the products. These unbound monomers can be released into food or beverage over time, especially under heat, acidic, or basic environmental conditions.<sup>[2,3]</sup> It was previously thought that human contact to BPA is mainly dietary; however, there is evidence that human exposure to BPA hazard can also occur through inhalation of household dust or particles generated during the industrial synthesis process.<sup>[4,5]</sup> Moreover, BPA exposure was also reported after skin contact with BPA-containing thermal printer paper and medical services such as dental sealants.<sup>[6,7]</sup>

BPA is considered as a risk factor for the development of several human disorders such as diabetes, obesity, cancer, and reproductive disorders.<sup>[8,9]</sup> It has been also shown that acute or chronic exposure to BPA led to cardiovascular, coronary, and peripheral artery disorders.<sup>[10,11]</sup> However, most of these reports studied the biochemical and the functional disturbances in the cardiovascular system after prolonged exposure to high doses of BPA.<sup>[12-14]</sup> In experimental animals, exposure to a high dose of BPA (50 mg BPA orally for 8 weeks) resulted in morphological and structural changes in the rat myocardium in the form of vacuolated myocytes, focal loss of myofibrils,

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and distorted mitochondria and tubular system,<sup>[15]</sup> however, the effect of exposure to low doses of BPA on the rat myocardium still elusive.

The toxic effect of BPA is mediated through its effect on estrogen receptor rapid signaling, alteration of cardiac Ca<sup>2+</sup>-handling protein expressions, ion channel inhibition/activation, oxidative stress and free radical formation, and genome/transcriptome modifications.<sup>[16-18]</sup>

Antioxidant such as Omega-3 fatty acid (FA) and others have been shown to produce protective effects against several environmental toxins such as N-nitroso compounds,<sup>[19]</sup> tributyltin,<sup>[20]</sup> and polybrominated diphenyl ether.<sup>[21]</sup> However, Omega-3 FAs cannot be synthesized by the body and it must be obtained from food or taken as supplements. Food rich in Omega-3 includes fish oils, salmon, mackerel, tuna fish, canola oil, flax seed oil, and walnuts.<sup>[22]</sup> Omega-3 FAs are pivotal not only for brain function and normal growth and development<sup>[23]</sup> but also they have many beneficial effects on several health problems including cardiac diseases.<sup>[24,25]</sup> Therefore, the aim of this study was to examine the effect of exposure to BPA on the adult rat myocardium and to test the effect of concomitant administration of Omega-3 FAs.

## MATERIALS AND METHODS

### Animals and experimental design

All animal work was conducted by the guidelines for the use of animals in research that was established by Sohag University and Tanta University, Egypt. These guidelines comply with the international guidelines set by National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

Thirty adult male Wistar rats (150–200 g each) were included in this study. All the animals were housed in conventional plastic cages with free access to water and chow diet with 12-h light/12-h dark photoperiod. Animals were acclimatized to the laboratory environment for 1 week before the start of the experiment. Animals then were randomly divided into three equal groups: Group 1 ( $n = 10$ ) – rats were injected intraperitoneally with BPA, (purity 99%, CAT # 80-05-7, Sigma, USA) at a dose of 1.2 mg/kg body weight/day dissolved in 1.5 ml of corn oil (Sigma, USA, CAT # 8001-30-7) for 3 weeks.<sup>[26]</sup> Group 2 ( $n = 10$ ) – rats were injected with BPA as in the first group and were given Omega-3 FA (Kirkland Signature, US) orally through gastric tube at a dose of 300 mg/kg body weight/day for 3 weeks.<sup>[27]</sup> Group 3 (control,  $n = 10$ ) – this group was injected intraperitoneally with 1.5 ml of corn oil each day for 3 weeks.

### Specimens processing and staining

At the end of the experiment, all animals were humanely culled by cervical dislocation. The hearts were rapidly dissected and immediately fixed in 10% neutral-buffered formalin for 24 h. The left ventricular specimens were rinsed with 0.1 M phosphate buffer solution then were dehydrated

through ascending grades of alcohol. The specimens were embedded in paraplast. Myocardial sections (5  $\mu$ m thick) were deparaffinized through two changes of histoclear for 15 min and rehydrated through descending grades of alcohol (100%, 90%, and 70%). The sections were stained with hematoxylin and eosin (H and E) to look at the general morphology, picosirius red to measure the collagen fibers content, and toluidine blue for quantitative analysis of mast cells by metachromasia.

For H and E staining, the sections were stained with Mayer's hematoxylin for 8 min and rinsed in running tap water for 1 min. To stain the nuclei blue, sections were incubated in Scott's solution for another 1 min then were rinsed by water. Finally, the sections were stained with 1% eosin for 2 min, washed, dehydrated through ascending grades of alcohol and histoclear, and finally, were mounted with DPX.

To measure the collagen contents, the myocardial sections were stained with picosirius red for 1 h then were washed twice by acidified water. The sections were then dehydrated before mounting.

To label the mast cells, myocardial sections were stained with 1% toluidine blue for 3 min then were washed three times by distilled water. The sections were then dehydrated quickly through 95% and two changes of 100% alcohol (10 dips each). The sections were cleared in two changes (3 min each) of histoclear.

The bright field images have been captured from nonoverlapping fields of the entire section using AxioCam HRc (Zeiss, Germany) mounted on light microscope (Zeiss, Germany).

### Immunohistochemistry

To immunolabeling the tissue section with anti-alpha-smooth muscle actin, myocardial paraffin sections were deparaffinized and rehydrated in descending grades of alcohol and distilled water. After that, the sections were incubated in preheated 0.1M sodium citrate buffer (pH 6.0) for (2  $\times$  5 min) in a microwave for antigen retrieval. Sections were rinsed with water and 0.3 M of phosphate-buffered saline with Triton X (PBST). To prevent nonspecific binding, sections were incubated with 5% normal goat serum in 0.3M PBST for 1 h at room temperature. Sections were then incubated with polyclonal rabbit anti-alpha-smooth muscle actin, 1:100 (abcam-5694) overnight in a humidity chamber at 4°C. Primary antibody was removed by washing the sections three times with 0.3M PBST followed by incubation with Alexa Fluor<sup>®</sup> 488 goat anti-rabbit IgG, 1:500 (Life Technologies, A11008) for 1 h at room temperature. The sections were finally washed with 0.3M PBST (3  $\times$  15 min) and were mounted with VECTASHIELD Mounting Medium. The fluorescence images were captured using a fluorescence microscope (Zeiss, Germany).

### Quantitative assessment

The quantitative analysis was performed using ImageJ versus 1.48 software for measuring the following parameters using five nonoverlapping fields from five different sections from each animal.<sup>[28]</sup>

### Myocyte cross-sectional area

It was measured using H and E-stained transverse sections of LV at  $\times 400$  magnification by manually drawing a line around the circumference of cardiomyocytes presented with visible central nuclei. The average cross-sectional areas obtained for each group were used as an indicator of cell size.<sup>[29,30]</sup>

### Area percentage of the interstitial collagen fibers

It was measured from picosirius red-stained longitudinal myocardial sections at  $\times 400$  magnification.<sup>[31]</sup> To quantify the connective tissue levels, images were converted to an RGB stack, and the green channel was selected because of its high contrast. Red-stained collagen was identified by applying the thresholding of grayscale to measure the percentage of collagen about the total field area.

### Mast cell density (number/mm<sup>2</sup>)

It was measured from toluidine blue-stained myocardial sections at  $\times 200$  magnification.<sup>[32]</sup>

### The total vessel area, lumen area, and media-to-lumen area ratio

They were measured from anti-alpha-smooth muscle immune-stained myocardial sections at  $\times 400$  magnification. ImageJ was used to measure the total cross-sectional area as well as the inner area. The vessels wall area was then determined by subtracting the inner area from the total cross-sectional area.<sup>[33]</sup>

### Statistical analysis

Tests for differences between experimental groups were carried out in GraphPad Prism, version 5 (San Diego, California, USA) using one-way ANOVA with Tukey's *post hoc* multiple comparison tests;  $P < 0.05$  was used to define statistical significance.

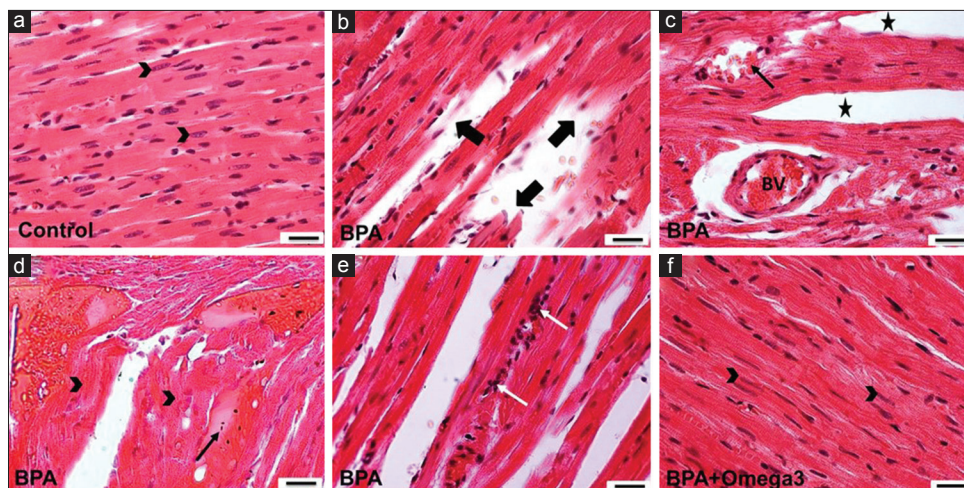
## RESULTS

### Bisphenol A administration induced severe histopathological changes in the rat myocardium that ameliorated by Omega-3

To study the effect of BPA on the structure of rat myocardium, sections were stained with H and E and were examined under the light microscope. Unlike the sections that have been taken from control rat that showed normal histological architecture of the myocardium with longitudinally striated branching and anastomosing muscle fibers with centrally located oval vesicular nuclei [Figure 1a], sections taken from BPA-injected rats showed several pathological findings. These findings include disorganization and discontinuation of cardiac muscle fibers [Figure 1b], increased intracellular spaces between myocardial fibers, congested blood vessels (BVs), and extravasation of red blood cells [Figure 1c]. Myocardial sections from the BPA-treated group also showed areas of pale homogeneous acidophilic cytoplasm with either absence of nuclei or presence of deeply stained pyknotic nuclei [Figure 1d]. In addition, mononuclear cellular infiltration in-between the muscle fibers were also obvious in the myocardium of BPA-treated rats [Figure 1e]. On the other hand, sections taken from rats that were given Omega-3 and BPA showed a near-normal structure of the myocardium presented with branching and anastomosing longitudinal muscle fibers with oval vesicular central nuclei [Figure 1f].

### Omega-3 rescued altered myocyte cross-sectional area induced by bisphenol A

To assess the effect of BPA administration on the individual myocyte, we measured cross-sectional area of cardiomyocyte in in H and E stained myocardial transverse sections. Our data show that, while BPA-treated rats developed significant



**Figure 1:** Omega-3 ameliorates cardiotoxic effect of bisphenol A in adult rat myocardium. Photomicrographs of longitudinal sections of the left ventricular myocardium of (a) control group showing branching and anastomosing cardiac muscle fibers with an acidophilic sarcoplasm and centrally located oval vesicular nuclei (arrowhead). While bisphenol A-treated group showing (b) destruction and discontinuation of cardiac muscle fibers (arrow), (c) wide intercellular space (star), congested blood vessels with extravasated red blood cells in between the muscle fibers (arrow) (d) focal areas of pale homogeneous acidophilic sarcoplasm with either absence of nuclei (arrowhead) or presence of deeply stained pyknotic nuclei (arrow) in addition to (e) mononuclear cellular infiltration (white arrow). On the other hand, (f) coadministration of Omega-3 with bisphenol A showed a near normal structure of cardiac muscle fibers with central oval vesicular nuclei. H and E, scale bar = 20  $\mu\text{m}$

increase in the cardiomyocyte cross-sectional area compared to the control group ( $205.2 \pm 2.97 \mu\text{m}^2$  and  $120.9 \pm 2.97 \mu\text{m}^2$ , respectively mean  $\pm$  standard error of the mean [SEM],  $P < 0.001$ ), concomitant administration of Omega-3 significantly ameliorated this phenotype compared to the group received BPA alone ( $155.4 \pm 6.06 \mu\text{m}^2$  and  $205.2 \pm 2.97 \mu\text{m}^2$ , respectively, mean  $\pm$  SEM,  $P < 0.001$ ). However, cross-sectional area of myocytes in Omega-3-treated rat remained significantly higher than that of the control group [Figure 2a-d].

### Bisphenol A injection resulted in increased fibrous tissue content in the myocardium

The increased intracellular space between the cardiac fibers seen in H and E stained sections could potentially result from the loss of the contractile fibers and its replacement by fibrous tissues. To validate this hypothesis, we stained longitudinal sections of the left ventricle with picosirius red (specific stain for collagen content). BPA injection resulted in significant increase in the area percentage of collagen contents compared to the control group ( $6.86\% \pm 0.21\%$  and  $1.53\% \pm 0.58\%$ , respectively, mean  $\pm$  SEM,  $P < 0.001$ ). On the other hand, coadministration of Omega-3 and BPA resulted in significant decrease in the collagen deposition in-between the muscle fibers compared to BSA-treated group ( $2.56\% \pm 0.24\%$  and  $6.86\% \pm 0.21\%$ , respectively, mean  $\pm$  SEM,  $P < 0.001$ ) [Figure 3a-d].

### Number of mast cells in the myocardium is not altered with bisphenol A

To investigate the potential effect of BPA on cardiac mast cells; left ventricular sections from different groups were stained with

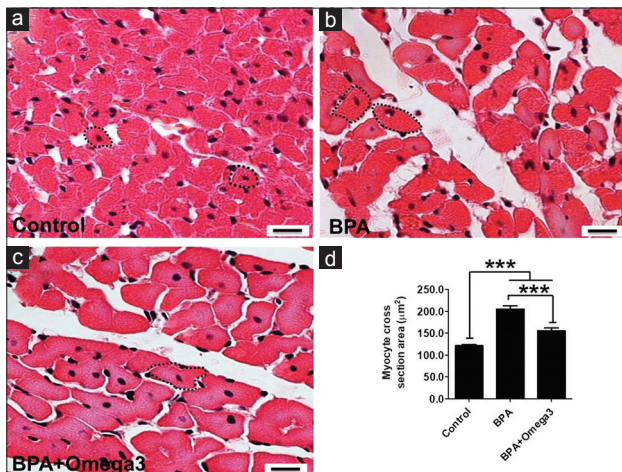
Toluidine blue. Large mast cells were detected (stained violet against blue background due to the metachromatic property of their cytoplasmic granules) in myocardial sections from all groups [Figure 4a-c]. Nevertheless, there was no significant difference in the number of mast cells per  $\text{mm}^2$  among the experimental groups (control;  $8.08 \pm 0.88$ , BPA;  $8.38 \pm 1.27$ , BPA + Omega-3;  $7.92 \pm 0.96$ , mean  $\pm$  SEM) [Figure 4d].

### Bisphenol A exposure led to dilatation of intramyocardial blood vessels

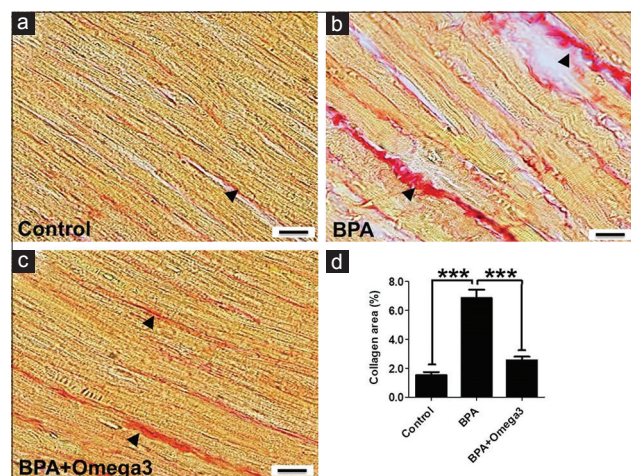
To examine potential changes in the size of intramyocardial arterioles, sections were immunolabeled with alpha-smooth muscle actin antibody as a specific marker for smooth muscle cells in the media of intramyocardial arterioles [Figure 5a-c]. Different morphometric measurements have been taken including lumen and total vessel area as well as media-to-lumen ratio as an indicator for medial thickness. BPA exposure resulted in significant increase ( $P \leq 05$ ) in the total vessel area ( $1007 \pm 200.5 \mu\text{m}^2$ , mean  $\pm$  SEM) and luminal area ( $545.4 \pm 122.1 \mu\text{m}^2$ , mean  $\pm$  SEM) compared to the control rats ( $509.7 \pm 86.47 \mu\text{m}^2$  and  $232.6 \pm 33.81 \mu\text{m}^2$ , mean  $\pm$  SEM, respectively) and BPA and Omega-3-treated rats ( $492.3 \pm 66.96$  and  $247.0 \pm 33.36 \mu\text{m}^2$ , mean  $\pm$  SEM, respectively) [Figure 5d and e]. On the other hand, there was no significant difference in the media-to-lumen area ratio between the experimental groups (control;  $0.94 \pm 0.06$ , BPA;  $1.12 \pm 0.10$ , BPA + Omega-3;  $0.95 \pm 0.06$ ) [Figure 5f].

## DISCUSSION

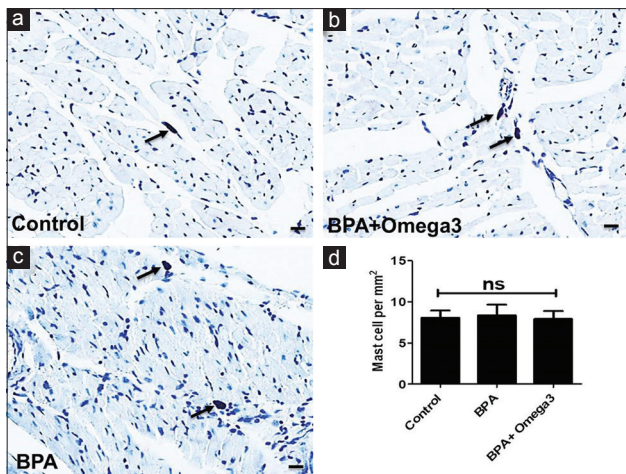
Environmental pollution is a global major concern and constitutes a foremost foundation for health risk. BPA is



**Figure 2:** Bisphenol A-induced hypertrophy of cardiomyocyte. Representative micrographs of the transverse sections of the left ventricular myocardium of (a) control group, (b) bisphenol A-treated group showing increased cardiomyocyte cross-sectional area. (c) Concomitant administration of Omega-3 significantly decreased myocytes cross-sectional area compared to bisphenol A-treated group. H and E, dashed area, representative example for cross-sectional area measurement, scale bar =  $20 \mu\text{m}$ . (d) Plot showing quantitative measurements of myocytes cross-sectional area among the experimental groups. Data are presented as mean  $\pm$  standard error of mean,  $***P < 0.0001$



**Figure 3:** Omega-3 attenuated bisphenol A-induced interstitial fibrosis. Representative micrographs of the longitudinal sections of left ventricular myocardium showing (a) few and fine collagen fibers (arrowhead) between the cardiac muscle fibers of control group. (b) Apparent increase in collagen fibers (arrowhead) between the cardiac muscle fibers of bisphenol A-treated group. (c) Apparent decrease in the interstitial collagen fibers (arrowhead) with Omega-3 administration. Picosirius red staining, scale bar =  $20 \mu\text{m}$ . (d) Graph showing the quantitative analysis of interstitial collagen fibers area percentage of the experimental groups. Data are presented as mean  $\pm$  standard error of mean  $***P < 0.0001$



**Figure 4:** Mast cells density in the myocardium was not altered with bisphenol A. Representative micrographs of the left ventricle of (a) control group, (b) bisphenol A-treated, and (c) bisphenol A and Omega-3-treated groups showing the violet metachromatic staining of mast cells (arrows) against blue background. Toluidine blue, scale bar = 20  $\mu\text{m}$ . (d) Plot showing quantitative measurements of mast cells density (number/ $\text{mm}^2$ ) among all groups. Data are presented as mean  $\pm$  standard error of the mean, ns: Nonsignificant

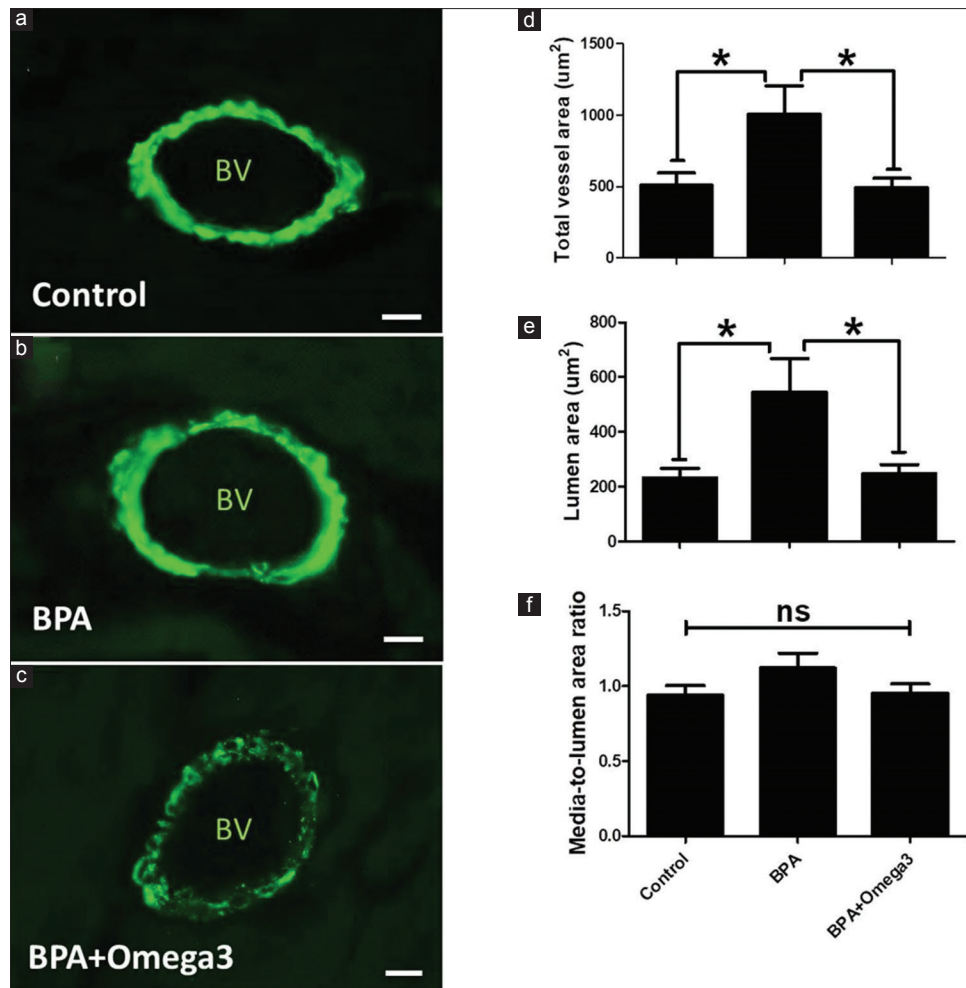
an environmental pollutant that has been shown to produce varieties of health problems depending on the dosage and the duration of exposure. In the current study, exposure to BPA led to histopathological changes in the adult rat myocardium which was consistent with previous animal studies that described similar changes in the structure of myocardium after BPA exposure either with high doses of BPA<sup>[15]</sup> or for long lifetime exposure.<sup>[34]</sup> In the current study, the increased myocardial surface area was reported after 3 weeks of BPA treatment; however, Jiang *et al.* reported similar changes only after 48 weeks of BPA exposure.<sup>[35]</sup> This discrepancy in the results could be explained by the relatively low dose (50  $\mu\text{g}/\text{kg}/\text{day}$ ) of BPA utilized, which might require more time to reveal structural changes. The mechanism behind increased cardiomyocyte surface area could potentially be a result of impaired mitochondrial function or upregulation of several genes involved in the pathogenesis of cardiac hypertrophy such as transforming growth factor  $\beta$  (TGF $\beta$ ) and Adam12-1.<sup>[18,35]</sup> Olea-Herrero *et al.* Similarly reported the expansion of the glomerular mesangial area and podocyte cytoplasmic enlargement in the kidneys of the BPA-injected animal due to upregulation of TGF- $\beta$ 1 system and the cyclin-dependent kinase inhibitor p27Kip1 and collagen IV.<sup>[36]</sup>

Beside changes in the cardiomyocytes, this study also shows increased myocardial fibrosis and increased collagen content in BPA-treated rats which is consistent with Hu *et al.* who reported similar effects on the myocardium of rats exposed to relatively higher doses of BPA.<sup>[37]</sup> Excessive myocardial fibrosis possesses detrimental effect on myocardial structure and function and is a very common feature in several cardiac disorders such as hypertension, infarction, and heart failure.<sup>[38,39]</sup> Increased myocardial fibrosis after BPA exposure

could be a consequence of the increased proliferation of cardiac fibroblasts and enhanced collagen production<sup>[37]</sup> or as a result of increased density and/or activation of cardiac mast cells.<sup>[40,41]</sup> The latter is a key player in the regulation and induction of inflammation and fibrosis in the heart through toll-like receptor-4 signaling and increased pro-inflammatory cytokine production.<sup>[42]</sup> O'Brien *et al.* previously shown that exposure to BPA during the perinatal period increases the activity of bone marrow-derived mast cells in adulthood with subsequent increase in the production of pro-inflammatory mediators associated with asthma.<sup>[43]</sup> However, the possible effect of BPA on the cardiac mast cells remains elusive. For the best of our knowledge, the current study is the first to describe the effect of BPA exposure on the cardiac mast cells. It shows that despite the increased myocardial fibrosis and increased collagen content, there is no significant change in the density of cardiac mast cells in BPA-injected rats compared to the control group; however, this does not exclude BPA as a potential mast cells activator. More experiments are required to investigate the effect of BPA on mast cells activation and to test the effect of exposure to larger doses of BPA for longer duration on mast cells density and activation.

BPA exposure has been linked to increased risk of several vascular disorders including coronary and peripheral arteries.<sup>[10,11,13]</sup> The current study showed that exposure to BPA led to dilatation of the intramyocardial BVs. This result could explain the presence of extravasated blood and the increased cellular infiltrates in between myocardial fibers in the rats exposed to BPA. These results are in agreement with Klint *et al.* who showed that exposure to BPA increases vascular endothelial growth factor and endothelial nitric oxide synthase expression that controls angiogenesis and vascular tone in cardiac tissues.<sup>[44]</sup> Increased VEGF signaling and its subsequently increased angiogenesis are involved in the development of atherosclerosis through helping plaque formation and rupture.<sup>[45,46]</sup>

The increased evidence of BPA-induced hazard to human health has encouraged scientists to search for drugs or natural substance to provide protection against BPA effect particularly to those under high risk of exposure.<sup>[47,48]</sup> The current study shows, for the first time, that Omega-3 FA provides a protective effect against BPA-induced myocardial toxicity. Similar protective rule of Omega-3 FA against environmental toxins such as diethylnitrosamine<sup>[19]</sup> and propionic acid<sup>[49]</sup> has been shown before. Several mechanisms could potentially contribute to the beneficial effects of Omega-3 against BPA-induced myocardial pathology. First, Omega-3 inhibits the inflammatory signaling pathways (nuclear factor-kappa B activity) and decreases the production of prostaglandin E2 and triglycerides.<sup>[50,51]</sup> Second, Omega-3 has strong antioxidant activities and it has been shown to enhance mitochondrial FA oxidation and antioxidant capacity in the human atrial myocardium and mitigate oxidative/inflammatory stress.<sup>[52]</sup> Third, Omega-3 downregulates sterol regulatory element binding protein-1c gene which increases FA synthesis and upregulates peroxisome



**Figure 5:** Bisphenol A exposure resulted in dilatation in the intramyocardial blood vessels. Representative micrographs of the left ventricle of (a) control group, (b) bisphenol A-treated, and (c) bisphenol A and Omega-3-treated groups showing immunoreactivity to anti-alpha-smooth muscle actin (green). Histogram presented quantitative analysis of the (d) total vessel area, (e) lumen area, and (f) media to the lumen ratio of experimental groups. Data are represented as mean  $\pm$  standard error of mean \* $P \leq 0.05$ . ns; nonsignificant, scale bar = 20  $\mu\text{m}$

proliferator-activated receptor-alpha gene which is involved in FA oxidation.<sup>[53,54]</sup> Finally, it has antithrombotic and antiarrhythmic effects and can modulate cardiac ion channels.<sup>[25]</sup>

In the current study, we used the intraperitoneal route of administration to deliver BPA, to ensure accurate dose delivery. However, we do appreciate the fact that exposure to BPA in human or in previous animal studies is mainly enteral. This method of delivery could represent a limitation of the current study, nevertheless using exact similar dose and route of administration has been reported to be lower than an oral administration of 5 mg/kg, which is the no-observed-adverse-effect level after considering BPA pharmacokinetics.<sup>[26]</sup>

## CONCLUSION

This study showed that exposure to BPA resulted in pathological changes in the rat myocardium. Importantly, coadministration of Omega-3 FAs partially ameliorated these changes. Further studies are suggested to assess the effect of prolonged exposure

to different doses of BPA on the cardiovascular system. In addition, daily administration of Omega-3 FAs-containing foods is also recommended to help decrease the harmful effect of incidental exposure to BPA-contaminated products.

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Nil.

## Conflicts of interest

There are no conflicts of interest.

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