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# EFFECTS OF BISPHENOL A ON NEONATAL CARDIOMYOCYTES BEATING RATE AND MORPHOLOGY

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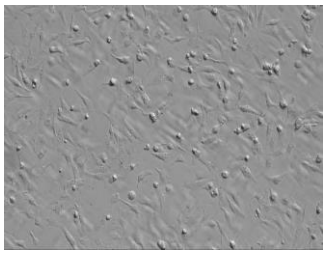
## Graphical abstract



## Abstract

Bisphenol A (BPA) has been utilised excessively at a global capacity of 2.9 billion kg/year. It is widely used in manufacturing polycarbonate polymers and epoxy resins. Hence, humans are potentially exposed to this chemical substance in their daily life. As a typical endocrine disruptor, BPA exhibits detectable hormone-like properties. Many studies have been linking BPA exposure in humans with the risk of developing cardiovascular disease, however the direct exposure of BPA on cardiomyocytes beating rates and morphology have not been entirely explored. Therefore, in this study, we aimed to investigate the effects of BPA on cells structure and function of neonatal rat cardiomyocytes culture. Cardiomyocytes were isolated from 0 to 2 days old newborn rats and treated with 0.001 to 100 µM concentration of BPA. All cardiomyocytes were subjected to immunostaining, beating frequency assessment assay, MTS assay and Scanning Electron microscopy (SEM). In immunostaining, cardiomyocytes showed positive staining for F-actin. This staining allows identification of the cells thus differentiate cardiomyocytes from other cell types. Significance effects of BPA on cardiomyocytes were observed in MTS assay ($p<0.05$) and beating rates ($p<0.01$). Significant reduction (48%-64%, ± 1.5280) was observed in beating rate of cardiomyocytes exposed to 0.1 to 100 µM of BPA. Meanwhile in MTS assay, significant reduction (54%, 0.067 ± 0.0026) in cell viability was observed in cells exposed to 0.1 µM of BPA only. Interestingly, under SEM, cardiomyocytes showed altered cell surface homogeneity after BPA exposure. Exposure of 0.1 to 100 µM BPA lead to flatten of cardiomyocytes cell surface and blurring of the cell borders. This study offers an *in vitro* evidence of BPA effects on cardiomyocytes morphology and beating rates, thus suggest the potential adverse effect of BPA exposure. However, further investigation would be required to understand how BPA effects normal cells morphology and beating rates of heart cells.

*Keywords*: Bisphenol A, cardiomyocytes, cells morphology, beating rates, cytotoxicity

**Abstrak**

Bisphenol A (BPA) telah digunakan secara berlebihan pada kapasiti global sebanyak 2.9 bilion kg / tahun. Ia digunakan secara meluas dalam pembuatan polimer polikarbonat dan resin epoksi. Oleh itu, manusia berpotensi untuk terdedah kepada kimia ini dalam kehidupan seharian mereka. BPA dilihat sebagai penggangu kepada fungsi endokrin kerana telah mempamerkan sifat-sifat seperti hormon. Banyak kajian telah menghubungkan pendedahan BPA kepada manusia dengan risiko penyakit jantung, namun kesan pendedahan langsung BPA ke atas fungsi serta morfologu kardiomiosit (sel jantung) belum dikaji sepenuhnya. Oleh itu, dalam kajian ini kami melihat kesan BPA terhadap struktur sel dan fungsi kardiomiosit tikus tersebut. Kardiomiosit telah diasingkan dari tikus baru lahir 0 hingga 2 hari dan dirawat dengan kepekatan BPA sebanyak 0.001 hingga 100 µM. Semua kardiomiosit diuji melalui eksperimen immunostaining, ujian kadar denyutan, ujian MTS dan mikroskop Scanning Electron (SEM). Di dalam immunostaining, kardiomiosit menunjukkan pewarnaan positif untuk F-actin. Pewarnaan ini membolehkan pengenalpastian sel-sel itu serta membezakan kardiomiosit daripada jenis sel yang lain. Kesan penting BPA pada kardiomiosit diperhatikan dalam ujian MTS ($p < 0.05$) dan kadar denyutan ($p < 0.01$). Pengurangan yang ketara (48%-64%, ± 1.5280) diperhatikan dalam kadar denyutan kardiomiosit yang terdedah kepada pendedahan BPA 0.1 hingga 100 µM BPA. Sementara itu dalam ujian MTS, pengurangan ketara (54%, 0.067 ± 0.0026) dalam daya tahan sel diperhatikan dalam sel-sel yang terdedah kepada 0.1 µM BPA sahaja. Menariknya, di bawah SEM, kardiomiosit menunjukkan perubahan kepada homogen permukaan selepas pendedahan BPA. Pendedahan 0.1 hingga 100 µM BPA menyebabkan permukaan sel kardiomiosit menjadi rata dan kabur. Kajian ini menawarkan bukti in vitro kesan BPA terhadap morfologi kardiomiosit dan kadar denyutan, dengan itu mencadangkan kesan buruk potensi pendedahan BPA. Walau bagaimanapun, siasatan lanjut diperlukan untuk memahami bagaimana BPA memberi kesan kepada morfologi sel normal dan kadar pemantauan sel-sel jantung.

*Kata kunci*: Bisphenol A, sel kardiomiosit, morfologi sel, kadar denyutan, keracunan

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## 1.0 INTRODUCTION

Bisphenol A (BPA; 4, 4'-dihydroxy-2, 2-diphenylpropane) is a monomer that is widely used in the production of polycarbonate, epoxy resins and as non-polymer additive in plastics manufacturing. BPA is the most highly produced chemicals worldwide as it is being used in many consumer products, such as baby feeding bottles, plastic food containers and tableware, material in food packaging, toys, eyeglass lenses, dental and medical equipment, electronics, food and beverage cans and water pipes' material [1]. Researchers showed that many consumer products contain and release BPA. Therefore, traces of BPA could be detected in environmental samples and landfill leachates [2].

BPA is firstly developed in 1890s and known as a synthetic oestrogen [3]. It works by imitating the natural hormone 17β-estradiol which is important in oestrogenic function. Researchers believed BPA would disrupt nuclear receptor function thus give adverse effect on hormone function. BPA half-life would be less than 6 hours where 90% of BPA is converted to its conjugate, BPA-glucuronide and the remaining will be converted to BPA-sulfate [4]. Based on United State Environmental Protection Agency (US EPA), the safe dose of BPA exposure is 50 µg BPA/kg bodyweight/day.

Over recent years, there has been an explosive growth of interest in the effect of BPA on human health. Few epidemiological studies have been conducted to show the effects of BPA exposure to human health [2, 5-7]. Recently, studies had investigated the link between BPA and occupational exposure that affect human health. Direct contact of BPA with skin may induce allergy symptoms and lead to dermatitis [5]. In addition, there is significant risk of sexual dysfunction and reduced in sexual desires among BPA-exposed workers who involved with production and packaging of epoxy resins [7]. The result has also been supported by other study where BPA exposure is correlated with the decreased in testosterone levels [6].

It is crucial to observe the exposure of BPA in foetuses and newborns as BPA biotransformation in them are a lot slower than adult. Furthermore, BPA exposure during early life may contribute to adult health as certain exposure during early life may programme for adult diseases. There was a study conducted to estimate the prenatal exposure of BPA through the diet intake of pregnant women and the findings found that after the exposure, there was sustained basal BPA concentration due to slow biotransformation of BPA or lack of BPA metabolic activity in foetus [8]. Similarly, another study showed that foetuses exposed to BPA during had higher accumulation of BPA in their adipose tissue [9].

BPA was shown to induce hypermethylation of oestrogen receptor promoter regions [10]. The effects of BPA are reported to dose-dependent manner [11]. Low doses of BPA inhibit adiponectin secretion in human adipocytes and stimulate the secretion of inflammatory adipokines, suggesting the involvement of BPA in the development of obesity, metabolic syndrome and insulin resistance [12-14].

Most of the studies agreed that BPA exposure alter developmental pathways and cell metabolism [15]. High levels of BPA have recently been correlated with obesity, diabetes, cardiovascular diseases (CVD), polycystic ovarian disease or low sperm count [16]. Recently, a cross-sectional study has correlated the level of urinary BPA (uBPA) with the incidence of obesity in adults aged between 18 to 74 years old. This finding revealed the risk of obesity increases with the increment of uBPA concentration [17]. Ranciere *et al.* (2015) has extensively reviewed studies reported from 2008 until 2014 [18]. They found positive correlation between BPA and few health problems such as diabetes, prediabetes, hyperglycaemia, overweight, CVD and hypertension. In cancer, BPA reported to has the capability to induce genotoxic damage to liver and breast of mice [19]. BPA was reported to upregulate the oestrogen receptor of transcriptional activity in MCF-7 breast cancer cell.

However, the investigation on relationship of BPA and CVD are few. BPA is reported as an important predictive risk of coronary artery disease in those men and women exposed to it. Shankar *et al.* (2012) reported a significant association of high uBPA with peripheral arterial disease (PAD), which also an independent risk factor for CVD, thus explained the correlation of BPA exposure to human health specifically in CVD [20]. Interestingly, there is a correlation found between high levels of uBPA in human adults and increased incidence of CVD [21]. Based on the review, four out of five cross-sectional studies reported a positive correlation between uBPA and CVD [18].

The primary objective of this study was to investigate whether BPA exposure could alter the basic function and morphology of cardiomyocytes. We hypothesized that BPA exposure effects the cardiomyocytes beating frequencies pattern and alter cardiomyocytes normal morphology.

## 2.0 METHODOLOGY

### 2.1 Animals

All experimental procedures were conducted according to the Guiding Principle of Care and Use of Laboratory Animals approved by Universiti Teknologi MARA (UiTM) Committee of Animal Research and Ethics (Approval Number UiTM CARE: 222/7/2017 (8/12/2017)).

Sprague-Dawley rats were purchased at aged between 150-180 days. Female rats were mated with male rats. Gestational day (GD) 1 was noted when sperm was observed in vaginal smears. Pregnant rats were housed individually under standard conditions (22°C, 12-hr light–dark cycle), with free access to food and water. Water bottles and cages made of BPA-free components were used in this study to avoid potential contamination from sources other than administered drinking water. Cardiomyocytes (CMs) were isolated once the rats delivered.

### 2.2 Chemicals

Bisphenol A(BPA) was purchased from Sigma Aldrich. BPA was dissolved methanol prepared according to relevant concentrations.

### 2.3 Isolation of Cardiomyocytes

Cardiomyocytes were isolated from litters rats' heart as described [22]. In brief, the left ventricle was minced and subjected to serial digestion with 0.03% collagenase and 0.03% trypsin. Following digestion, the cells were repeatedly washed and pre-plated to remove the contaminating non-myocytes. The myocytes cells then cultured in gelatine coated culture dishes in DMEM medium with 10% FBS. Isolated cells were divided into 5 groups (each group n=4 litters);

Group 1: Untreated
Group 2: Treatment with 100µM BPA
Group 3: Treatment with 10µM BPA
Group 4: Treatment with 0.1µM BPA
Group 5: Treatment with 0.001µM BPA

### 2.4 Treatment

The isolated cardiomyocytes were treated with different BPA concentration accordingly. BPA was prepared in 0.001 to 100 µM to treat isolated cardiomyocytes.

### 2.5 MTS Assay

Isolated cells were counted for seeding by using haemocytometer. Haemocytometer was used to calculate the number of viable cardiomyocytes (CMs) in the suspension. The cells suspension (10µl) was mixed with trypan blue dyes (10µl) and loaded on a haemocytometer chambered slide (10µl). Then, the cells were cultured in 96 well plate and incubated for 24 hours. CMs were treated with different concentration of BPA (0.001, 0.1, 10, 100 µM) for 24 hours. After that, the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (20µl) were added to each well and incubated in the dark for 1 hour in 5% $CO_2$ atmosphere at 37°C. The plates were analysed using a Plate Reader (Perkin Elmer, USA) at 490 nm.

### 2.6 Beating Frequencies

Beating frequencies of each cardiomyocytes were visually recorded. CMs were pre-incubated at 37°C humidified with 5% $CO_2$ for 2 hours. CMs beating rates were counted and incubated at 37°C for 24 hours after being treated with different BPA concentration. CMs with single cell activity (consist of 3-5 network of cells) were chosen to record the beating frequencies. The frequencies were calculated within one minute for three times.

### 2.7 Scanning Electron Microscopy

Cells were cultured onto the coverslip. After being treated, media was discarded and cells were rinsed twice with 1X PBS. Then, the cells were fixed with 4X glutaraldehyde in 1X PBS and incubated overnight at 4°C. Glutaraldehyde was discarded and cells were fixed with 1% osmiumoxide and 0.1M sodium cacodylate for an hour at 4°C. Fixation solution was discarded and cells were washed thrice with 0.1 M PBS before being dehydrated with series of ethanol concentration. After dehydration methods, cells were coated using sputter coated machine and cell morphology were examined using SEM.

### 2.8 Immunofluorescence

In this experiment, CMs were seeded on chambered slides at a density of 2.5 x $10^5$ cells/chamber. The cells were treated with different concentration of BPA as mentioned in method 2.5. After such treatments, the media were discarded and the attached cells were rinsed with 1x PBS. Next, the cells were fixed with 4% paraformaldehyde (1 mL) for 15 min at room temperature (RT) and blocked with 10% BSA (1 mL) for 30 min. Cells were incubated with 1 mL Alexa Fluor 635-Phalloidin (1:500) to stain a F-actin respectively for 1 hour at RT. After an hour, the antibodies were discarded and the cells were washed again with 1x PBS (1 mL) for three times. The cells were then mounted using Prolong Gold and viewed under LSCM (Leica, Germany). The ratio of positive F-actin labeled cells versus total cells was used to calculate cell purity.

### 2.9 Statistical Analysis

All analyses were performed with SPSS software version 20. A statistical analysis was performed by using one-way analysis of variance (ANOVA), Post Hoc and Bon-ferroni analysis. In all cases, probability (*P*) values below 0.05 or 0.001 were considered significant.

## 3.0 RESULTS AND DISCUSSION

There are number of risk factors in developing heart disease. However, the contribution from environmental contaminants such as BPA is not widely explored. Thus, the main focus of this study is to determine the detrimental effects of BPA on the structure and function of CMs cells. Morphology of CMs can be differentiated from another type of cell based on their cytoskeletal feature (Figure 1). The present of actin in striated orientation indicated sarcomere structures of muscle cells especially in contractile cells such as CMs. The sarcomeres are arranged together with myosin, scaffolding and regulatory protein. Actin is presented in two forms; G-actin and F-actin. G-actin is a monomeric globular molecule which has the ability to polymerise and create the double stranded filamentous polymer known as F-actin. Meanwhile, in non-myocytes cells such as fibroblast, the actin presents as one continuous line. For this study, cardiomyocytes cultures that showed more than 70% of positive expression for F-actin were used. The small clusters (a network of 3-5 cells) of CMs were observed under fluorescence microscope (Figure 1(A)). Out of thirteen cells, twelve of them are CMs (>90% positive for F-actin), thus showed the purity of our cells. CMs culture is normally contaminated with fibroblast cells (non-muscle cells) which could interfere with the function of CMs if the fibroblast cells are more than 40%. Thus, cells were stained with F-actin, Alexa Fluor 635-Phalloidin (yellow arrow, red striated cells; Figure 1(B)) to differentiate CMs from fibroblast. Furthermore, the figure also confirmed that those cells are cardiomyocytes based on the striated actin shown by yellow arrows. CMs consists of a network of red staining striated actin (Figure 1 (B), arrow) which could not be found in fibroblast. Figure 1 shows the consistent ratio of CMs to fibroblast in all cultures, thus ensure the homogeneity of cell culture.

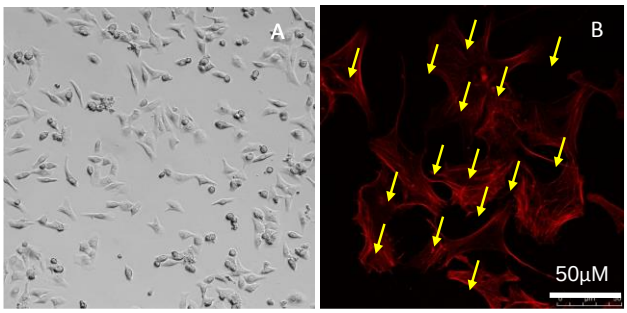


**Figure 1** The Culture of Neonatal Rat Cardiomyocytes. CMs were observed under (A) light microscope (100X) and stained with (B) TRITC for F-actin (arrow); ZF: 1.66

In beating rate experiment, we observed significantly reduction of frequency in 0.1 to 100 µM ($p<0.05$) BPA (Figure 2). Sixty four percent reduction of beating rate frequency was observed in CMs exposed to 0.1 µM of BPA. The similar patterns were observed in CMs exposed 10 and 100 µM. However, at BPA concentration of 0.001 µM, it did not show any significant reduction. This result is contradicted with previous study which found that the most effective

dose of BPA exposure is in range of 0.001 to 0.0001 µM [23]. Another study has found that exposure of CMs-derived from embryonic stem cell to 8 µM BPA affects the beating frequencies, thus supports our findings where the rate of beating significantly affected in a concentration ranging from 0.001 to 10 µM (Figure 2). The study has been conducted to determine interaction effect of three typical endocrine-disrupting chemicals which were BPA, perfluorooctane sulphonate (PFOS) and perfluorooctanaic acid (PFOA) [24]. In addition, they also reported cytotoxic effect of individual and combined effects of BPA, PFOS and PFOA, and found that BPA had a greater toxicity compared to PFOS and PFOA.

In another study, it determined the cardiac electrical conduction of female and infants heart after BPA exposure ranging from 0.1 to 100 µM. They observed significant reduction in maximum heart rate of female rats after BPA exposure. Their results reported reduction in ventricular beats which leads to complete heart block after in 100 µM BPA exposure in female rat [25]. Interestingly, they observed the changes in cardiac conduction of female and infant rats exposed to BPA even at low concentration, 0.1 µM. BPA metabolism is reported poor in infants, neonates and foetuses. At 0.1 µM concentration, BPA is already harmful to the infants, neonates and foetuses compared to adult. Recently, Ramadan *et al.* (2018) showed that the BPA-treated cardiomyocytes had slower spontaneous beating rate (SBR) compared to the non-treated [26]. This result is in line with our data. All those findings further supported our result which showed exposure to 0.1 µM affects the cardiomyocytes beating frequencies.

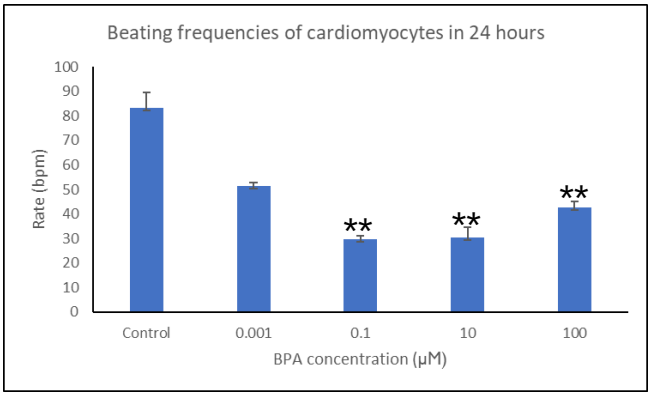


**Figure 2** The frequencies of beating for untreated and BPA-treated cells in 24 hours treatment. BPA-treated cells at concentration of 0.001 µM show no significant reduction in rates. (** denotes $p<0.01$ on the treated cells versus before treatment, n=4)

Meanwhile, in MTS assay, BPA-treated CMs showed significant reduction (54%, 0.067 ± 0.0026) in cell viability only in cells exposed to 0.1 µM of BPA with p-value at 0.047 (Figure 3). In other concentration (0.001, 10 and 100 µM) no significant changes in cell viability were observed (56%, 0.800 ± 0.0093), (84%, 0.893 ± 0.0189) and (62%, 0.667 ± 0.0020), respectively. This results contradicted with recent study by Ramadan *et al.* (2018) which observed no significant difference in cell viability of BPA-exposed cardiomyocytes [26]. This might be due to the duration of BPA exposure to the cells, Ramadan et al. (2018) study was an acute exposure and ours were chronic exposure.

Our results are aligned with results obtained by other research groups as they observed the significant increase in cell viability in a concentration dependent manner [27]. Findings from previous study has shown that exposure to BPA may contribute to reduction in cardiac function by observing the increased interstitial fibrosis. However, Bolli *et al.* (2008) [28] observed a significant reduction in human cervix adenocarcinoma (HeLa) cell viability which were 75% and 96% for 100 and 1000 µM BPA, respectively. They also found only 40% reduction of cell viability in 1000 µM BPA which is similar in our findings revealed that further increment of BPA levels does not affect cell viability.

In hepatocytes, only BPA at concentration 1000 µM and above could affects cell viability [29]. Thus, this suggested that BPA could alters cells structure and function but not affecting the cell viability. In addition, there was a study conducted to differentiate metabolic activity between pregnant rats and foetuses. The study concluded that that BPA metabolic activity in foetuses were slower than pregnant mothers even at lower exposure scenario. Therefore, BPA is more detrimental to foetuses compared to mothers [8].

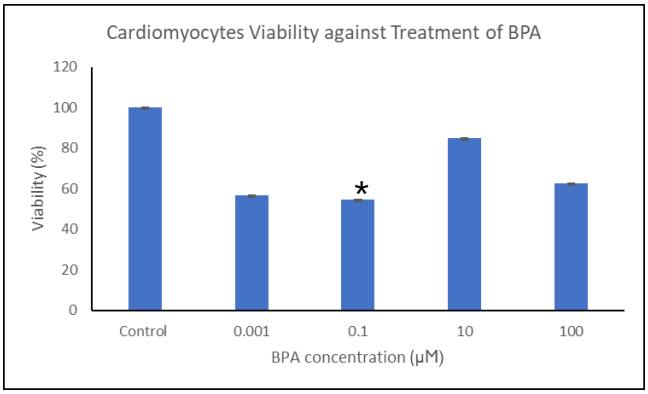


**Figure 3** The effect of different concentration of BPA exposure on cardiomyocytes viability. BPA-treated cells at concentration of 0.1 µM show significant reduction in cell viability. (* denotes $p<0.05$ on the treated cells versus before treatment, n=4)

We further observed the changes in CMs structure based on Figure 4. The figures show scanning electron microphotographs (SEM) result that highlighted morphology of CMs for untreated and BPA-treated CMs. Exposure to BPA flattened CMs

surface and reduced cells size (as shown in arrow). Cells were shrink after BPA exposure. The confident signs of cell shrinking were evident after exposure to 10 and 100 µM BPA. Interestingly, altered cell integrity was clearly visible in 10 µM. The result is supported by other findings where they observed BPA causes changes in adipocytes cell functions which are gonadal and renal fat pads. However, the changes were at very low concentration which are between 1 pM and 1 nM [30].

Similarly, Clement *et al*. (2017) observed reduction of cell surface expression of human mammary epithelium stem cells after being exposed to low concentration of BPA, 0.0001 µM [31]. From those findings, we can suggest that BPA exposure even at very low concentration is harmful to human health generally, and specifically on cardiac function.

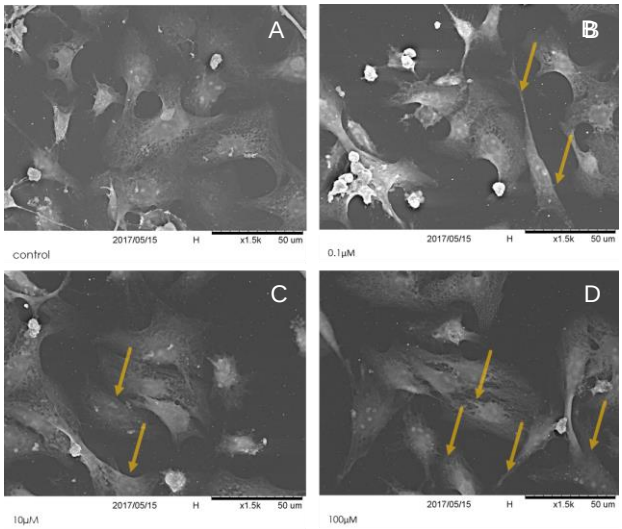


**Figure 4** Scanning electron microphotographs of neonatal rat cardiomyocytes (NRCM) exposed to bisphenol A (BPA). Untreated cardiomyocytes (A) were compared to cardiomyocytes treated with 0.1µM BPA(B), 10µM BPA (C) and 100µM BPA (D), BPA-treated cardiomyocytes show altered cell surface homogeneity after BPA exposure. The signs of flattening cardiomyocytes cell surface, reduction of the size, and blurring of the cell borders were observed after exposure of cell cultures to BPA (B-D, yellow arrow)

## 4.0 CONCLUSION

In this study, we provide an *in vitro* evidence of the potential adverse effects of BPA on cardiomyocytes morphology and function. BPA-treated cardiomyocytes showed significant reduction in beating frequencies and alteration in homogeneity of cells structure. However, further investigation is required to understand the mechanism of BPA effects on developing heart cells.

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