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LOW CONCENTRATION OF BISPHENOL A INDUCES PROLIFERATION OF GASTRIC CANCER CELLS, HGC-27

Noorul Izzati Hanafi[a], Siti Hamimah Sheikh Abdul Kadir[a,b]*, Maslinda Musa[c], Mohd Hafiz Dzarfan Othman[d], Roziana Kamaludin[d], Nur Amira Zulkifli[c], Normala Abd Latip[e], Zeti Rahayu A Karim[f]

[a]Institute of Molecular Medicine and Biotechnology, Faculty of Medicine, Universiti Teknologi MARA, Selangor, Malaysia
[b]Biochemistry and Molecular Medicine Discipline, Faculty of Medicine, Universiti Teknologi MARA, Selangor, Malaysia
[c]Department of Biomolecular Sciences, Faculty of Applied Sciences, Universiti Teknologi MARA, Selangor, Malaysia
[d]Advanced Membrane Technology Research Centre (AMTEC), Universiti Teknologi Malaysia, Johor, Malaysia
[e]Atta-ur-Rahman Institute for Natural Products Discovery (AuRIns), Faculty of Pharmacy, Universiti Teknologi MARA, Selangor, Malaysia
[f]Discipline of Surgery, Surgical Cluster, Universiti Teknologi MARA (UiTM), Selangor, Malaysia

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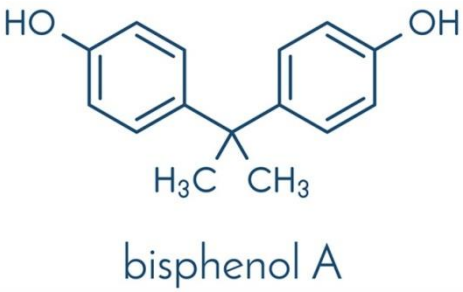
*Corresponding author
sitih587@salam.uitm.edu.my

Graphical abstract



Abstract

Bisphenol A, an endocrine disrupting compound that affects human homeostasis. Studies on BPA are focusing on the impact of BPA in reproductive function and brain development. However, the effect of BPA on gut especially gastric cells is not well explored. Gut is directly in contact with ingested BPA; therefore, we aimed to determine the effect of BPA exposure on gastric cells proliferation at safe recommended concentration. Human gastric cancer cells (HGC-27) were treated with BPA at different concentration (low: 10^{-9}M, 10^{-7}M; high10^{-5}M, 10^{-4}M) and time point (24 hr, 48 hr, 72 hr). Cell viability assays were determined using MTS assay. Cells were further stained with Alexa Fluor-635 (F-actin) and Fluorescein (Hif-1a) protein for immunocytofluorescence. Data were analysed using ANOVA ($p<0.05$, $n\geq3$). Cells treated with 10^{-9}M BPA showed significance increase of cell viability after 48 hr (Mean ±SEM; 146%±0.03, p=0.01) and 72 hr (113%±0.03, p=0.00) compared to 24 hr treatment (77%±0.11, p=0.002). Similarly, cell treated with 10^{-7}M BPA showed a significance increase after 48 hr (141%±0.03, p=0.03) and 72 hr (190%±0.03, p=0.02) compared to 24 hr cells treated with 10^{-7}M (88%±0.05, p=0.01) and untreated (100%±0.07). Lower concentration of BPA increases the condensation of F-actin in all HGC-27 cells. Meanwhile, translocation of Hif-1a protein were observed in all BPA-exposed cells. Findings of this study revealed that BPA induced proliferation and condensation of F-actin structure of gastric cancer cells at low concentration.

Keywords: BPA, cell proliferation, HGC-27, F-actin, Hif-1 alpha video

Abstrak

Bisphenol A adalah salah satu daripada sebatian endokrin yang mengganggu dan memberi kesan kepada homeostasis manusia. Kebanyakan kajian mengenai BPA memberi tumpuan kepada kesan BPA dalam fungsi pembiakan dan perkembangan otak. Walau bagaimanapun, kesan BPA pada usus terutamanya sel-sel gastrik tidak diterokai dengan baik. Perut secara langsung bersentuhan dengan BPA yang dimakan; oleh itu, kami bertujuan untuk menentukan kesan pendedahan BPA terhadap pembiakan sel gastrik pada kepekatan yang disarankan selamat. Sel kanser perut manusia dirawat dengan BPA pada berbeza kepekatan; (rendah10^{-9}M, 10^{-7}M; tinggi10^{-5}M, 10^{-4}M) dan masa (24 jam, 48 jam, 72 jam). Ujian daya maju sel ditentukan menggunakan ujian MTS. Sel-sel seterusnya ditandakan dengan protein Alexa Fluor-635 (F-actin) dan Fluorescein (Hif-1a) untuk immunocytofluorescence. Data dianalisis menggunakan ANOVA ($p < 0.05$, $n \geq 3$). Sel-sel yang dirawat dengan BPA 10^{-9}M menunjukkan peningkatan daya tahan sel selepas 48 jam (Mean ± SEM; 146% ± 0.03, p = 0.01) dan 72 jam (113% ± 0.03, p = 0.00) berbanding dengan rawatan 24 jam (77%, p=0.002). Begitu juga, sel yang dirawat dengan BPA 10^{-7}M menunjukkan peningkatan yang signifikan selepas 48 jam (141% ± 0.03, p=0.03) dan 72 jam (190% ± 0.03, p=0.02) berbanding dengan sel 24 jam yang dirawat dengan 10^{-7}M (88% 0.05, p=0.01) dan tidak dirawat (100% ± 0.07). BPA pada kepekatan yang rendah mengganggu kondensasi struktur jaluran F-actin di dalam semua sel. Sementara translokasi aktiviti Hif-1a protein dilihat dalam semua sel terdedah kepada BPA. Penemuan kajian ini mendedahkan bahawa BPA membantu percambahan dan mengganggu struktur F-actin sel kanser gastrik pada kepekatan rendah.

Kata kunci: BPA, proliferasi sel, HGC-27, F-actin, Hif-1 alpha

1.0 INTRODUCTION

Various studies have demonstrated the adverse effects of Bisphenol A (BPA; 4,4-isopropylidene diphenol) to human health as well as to the animals and environment [1]. BPA is one of the endocrine disrupting compounds which acts via estrogen receptors and may lead to changes in hormone homeostasis. Therefore, BPA is reported to mimic natural endogenous estrogen and affects the developmental, reproductive, neurological and immune system in human through estrogen binding receptors. Anteur *et al*. (2016) reported that BPA affects body weight and reproductive function likewise the gestational or lactational period of pregnant wistar rat [2]. Furthermore, BPA reported to cause dysregulation of endocrine receptor signalling and associated with development of diseases such as cancer [3]. BPA has been linked with the incidence of breast cancer [4], hepatoma cancer [5, 6], prostate and testicular cancer [7], [8] cardiovascular disease [9], diabetes and metabolic syndrome [10]. A few studies showed that exposure to BPA at low doses could affect cells responses to certain environment or stimuli such as hypoxia [11] and insulin resistance [12]. In type-2 diabetes mellitus, BPA reported to disturb insulin and glucagon regulation [12]. BPA shown to affect F-actin formation in human mammary epithelial cells [13]. Actin filaments are important to the cells as it generates force for biochemical processes and determine the cell shape. Dysregulation in F-actin signalling due to exposure to BPA may cause cell cycle delay in oocytes during meiosis [14]. A significant loss in spindles microtubules mass was discovered in BPA exposed cells (10 µm and 30 µm) during meiosis-I and resulted in small and loosely oriented spindles microtubules in meiosis-II. Spindle microtubules consist of actin filaments that permeate the microtubules during anaphase.

In hypoxia, BPA exposure is affecting cells ability to adapt to hypoxic environment as BPA decreases heart rate and tissue vascularization of zebra fish embryos [11]. Hypoxia is a condition of oxygen depletion which occurs during cellular metabolisms. Hypoxia-inducible factor (HIF)-1a is a transcription factor known to activate a target genes and protein in cells adaptation to low concentration of oxygen. Briefly, HIF-1a in active form will dimerize with HIF-1β and binds to hypoxia responsive element (HRE) in the promoter or enhancer of the target genes. HIF-1 activity can either enter apoptosis or survival pathways which are depending on cell type, severity of hypoxia and duration of hypoxia.

Globally, BPA is one of the most manufactured chemicals components in the plastics industry. BPA is ubiquitously found in almost every kinds of plasticware including food containers, healthcare equipment, dental materials, baby bottles, reusable water bottles, papers and cardboards. BPA is also widely used as inner coating material of canned foods and

beverage which is known as epoxy resins. The presence of BPA in canned foods and beverages is to prevent rusting in product packaging. Leaching of BPA residues into the environment and food product have been reported and documented. Contamination of BPA in the environment would cause a threat to aquatic organisms, wildlife and humans as BPA has longer half-lives. The traces of BPA have been reported in seafoods sold in a market such as prawn, crab, blood cockle, white clam, squid and fish at concentration ranging from 13.3 – 213.1 µg/kg wet weight. The daily tolerance intake of BPA was set from 50 µg BPA per body weight per day to 5 mg BPA per body weight per day by United State of America Environmental Protection agency (US EPA). Yet various studies reported cells exposed to lower doses of the BPA had altered morphology, proliferation and apoptosis signalling [15]. In Europe, daily uptake of BPA for babies was estimated approximately 0.2 µg/kg through breast feeding and babies of bottle-fed consumed about 11µg/kg and adults consumed about 1.5 µg/kg [12].

In human, the most common route of BPA entry is through direct ingestion from mouth and it reaches the digestive system. The effect of BPA on the gut especially gastric cells is not well explored. Study shows that low estrogen level able to stimulate estrogen receptor alpha 36 and stimulate growth of gastric cancer cells [16]. Gastric cancer is the leading cause of cancer death worldwide, however inadequate understanding of its pathogenesis leads to late presentation of this disease [17]. Therefore, this study was designed to investigate whether BPA is a able to induce proliferation in gastric cancer cells, HGC-27.

2.0 METHODOLOGY

All media for cell culture were purchased from Gibco LifeTechnologies. Human Gastric Cancer Cell lines HGC-27 were purchased from American Type Culture Collection (ATCC, USA). Bisphenol A was purchased from Sigma Aldrich (USA). HGC-27 was cultured in the complete culture medium, respectively in 5% CO_2 incubator at 37°C till 80% confluence. The cells were harvested for further tests.

For cell viability (MTS assay), CellTiter 96® AQueous One Solution reagent was used. The 96-well plates were transferred to the incubator for 1 hr incubation. After 1 hr, the plate was read for absorbance at 490 nm. Cytotoxicity level is calculated by measuring the cell viability as reported by Wang *et al*. (2011) through the conversion of yellow tetrazolium salt to purple formazan product. The formazan produces by dehydrogenase enzymes were measured by the OD values recorded at 490 nm was proportional to the number of viable HGC-27 in the culture. Scattered graph of BPA concentration against cells viability was plotted. Survivability average value was presented in percentage (%). Each standard and treatment was done in triplicates. The study was repeated for four times.

For immunocytofluorescence staining, HGC-27 were seeded in 6-well plate containing sterile coverslips (0.17 mm thickness) with the density of 300 000 cells per well with some optimization according to manufacturer suggestion (Orange Scientific, Belgium). The cells were treated with BPA at different concentrations (10^{-9} M, 10^{-7} M, 10^{-5} M, 10^{-4} M), different time-point (F-actin expression for 24 hr, 48 hr, 72 hr; Hif-1a expression for 72 hr) and untreated cells were used as control. Cells were fixed by incubating with 4% paraformaldehyde for 15 min and followed by permeabilization with 1% triton for 20 min. The cells were blocked with 10% BSA for 1 hr. After 1 hr, the cells were first incubated with HIF-1a (Abcam, UK; 1:500 dilutions) for 1 hr then followed by fluorophore-conjugated secondary antibodies, Fluorescein and Alexa-635 phaloidin for F-actin (Invitrogen, USA; 1:1000 dilutions) for 1 hr. The cells were counterstained with DAPI for 5 min. The coverslips were left to dry out and mounted after 30 min. The glass slide was viewed under fluorescence microscope. Successfully stained HGC-27 was verified as the structure of cytoskeleton; F-actin (red), Hif-1 alpha (green) and nuclei (blue). The experiment was repeated three times.

3.0 RESULTS AND DISCUSSION

3.1 The Effects of BPA at Different Concentration on HGC-27 Cells Viability

For cell viability study, the HGC-27 cells were treated with BPA at concentrations of 10^{-9} M, 10^{-7} M, 10^{-5} M, and 10^{-4} M (Figure 1) for 24 hr, 48 hr and 72 hr. In 24 hr treatment, cells treated with BPA at concentration 10^{-9}(mean, ± SEM; 77%, ± 0.11, p = 0.002), 10^{-7} (88% ± 0.05, p = 0.01), 10^{-5} (94% ± 0.03, p = 0.04) and 10^{-4}(53% ± 0.07, p = 0.001) showed significance changes compared to untreated cells (100% ± 0.07). As for 48 hr, BPA at all four concentrations showed significance differences at 10^{-9}(146%, ± 0.03, p = 0.00), 10^{-7} (141% ± 0.03, p = 0.00), 10^{-5} (124% ± 0.05, p = 0.01) and 10^{-4}(69% ± 1.02, p = 0.01) compared to untreated cells (100% ± 0.03). While for 72 hr, significance different was only observed at 10^{-9}(113%, ± 0.03, p = 0.00), 10^{-7} (109% ± 0.03, p = 0.00) and 10^{-4}(16% ± 0.01, p = 0.00) compared to untreated (100% ± 0.02). Cells treated with 10^{-9} M BPA showed a significance increase of cell viability after 48 hr (Mean ± SEM; 146% ± 0.03, p = 0.01) and 72 hr (113% ± 0.03, p = 0.00) compared to 24 hr treatment (77% ± 0.11, p = 0.002). Similarly, cell treated with 10^{-7} M BPA showed a significance increase after 48 hr (141% ± 0.03, p = 0.03) and 72 hr (190% ± 0.03, p = 0.02) compared to 24 hr cells treated with 10^{-7} M (88% ± 0.05, p = 0.01) and untreated (100% ± 0.07). In 10^{-4} BPA treatment, a significance reduction of cells viability were observed in 24 hr (53% ± 0.07, p = 0.001), 48 hr (69% ± 0.102, p = 0.01) and 72 hr (16% ± 0.00, p = 0.01).

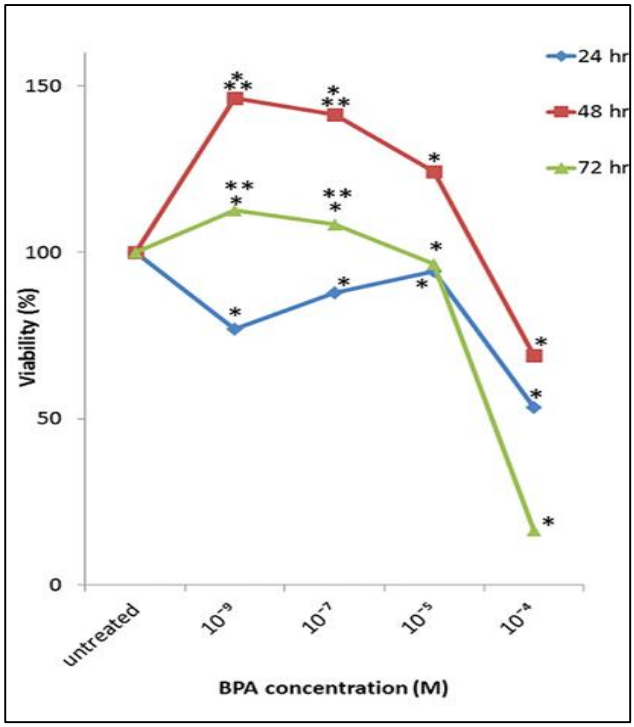


Figure 1 The effects of BPA at different concentration on HGC-27 cells viability. (Indicates statistical significance at $p < 0.05$ *; compared to untreated, ** compared to 10-9 M BPA exposed for 24 hr; using one-way ANOVA with Bon-ferroni's post hoc test, n = 6 independent observations)

Nowadays, gastric cancer cells have been reported as one of common malignancies with high mortality rate. Continuous research designed to investigate the development and progression of the gastric cancer cells to combat the disease through prevention, diagnosis and treatment. Result obtained from this present study revealed that BPA is a potential contaminant promoting the gastric cancer cells growth (Figure 1) at lower concentration (10^{-9} M and 10^{-7} M) BPA compared to high concentration (10^{-5} M and 10^{-4} M) BPA. Cells viability of HGC-27 treated with 24 hr BPA at high doses shown a decrease in cell viability. In contrast, cells exposed to 48 hr and 72 hr BPA at lower concentration, showed increase in cell proliferation. BPA is known as a compound that has similar structure to estrogen. Therefore, it is suggested that BPA exposure increased the effect of estrogen binding and interferes with uterine growth [18]. BPA has been reported as weak estrogenic compound that trigger weak carcinogenic effect [19]. However, in breast cancer cells, BPA reported to increase proliferation at lower doses via ERK 1/2 and estrogen related receptor gamma (ERRγ) [20]. Interestingly, BPA at 10^{-6} M promote highest proliferation rate in normal breast cells, HBL-100 and promotes the expression of cyclin D1 and CDK4, implicated in cells progression [21]. Moreover, in prostate cancer cells, BPA shown to activate androgen receptor and mitogenesis (8). The mechanisms of BPA in regulating cells proliferation and inhibition are reported to be cell specific and depending on the genetic and epigenetic regulation.

3.2 BPA Affects F-actin Expression in HGC-27

In the second part, the HGC-27 cells were stained with F-actin to observe the cellular effects of BPA on the actin at different concentrations (10^{-9} M, 10^{-7} M, 10^{-5} M, and 10^{-4} M) and time-point (24 hr, 48 hr and 72 hr). At 24 hr, the striated actin were observed in HGC-27, however the expression were decreased in HGC-27 treated with BPA at 10^{-5} M (Figure 2 D) and 10^{-4} M (Figure 2 E). The expression of striated actin was increased in HGC-27 treated with BPA at 10^{-9} M (Figure 2 B) and 10^{-7} M (Figure 2 C).

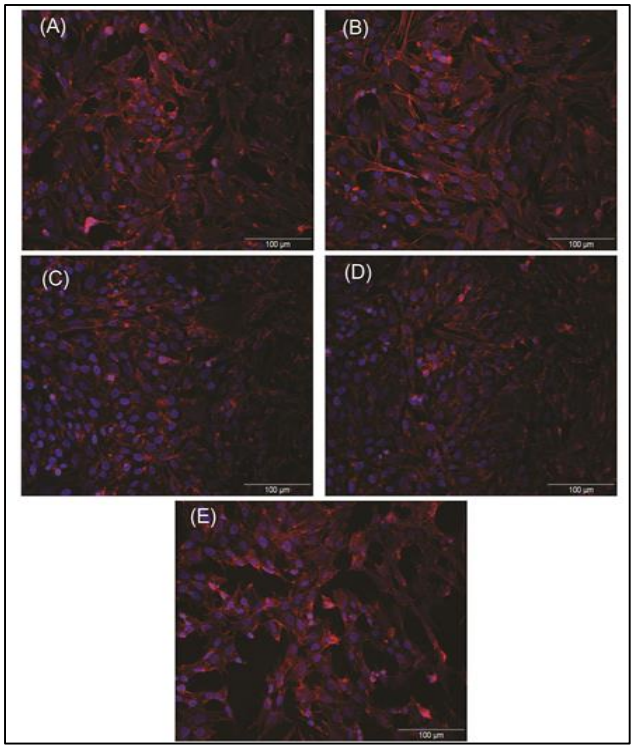


Figure 2 The HGC-27 treated -BPA stained for F-actin at 24 hr. The HGC-27 stained with DAPI for nucleus (blue) and Alexa-635 phaloidin for F-actin (red) for (A) untreated, (B) 10^{-9} M, (C) 10^{-7} M, (D) 10^{-5} M, (E) 10^{-4} M. The changes were viewed under fluorescence microscope at 20× magnification as a network of HGC-27; a representative of 3 independent observation (n = 3).

At 48 hr, the actin expression was more prominent in HGC-27 treated with BPA at 10^{-9} M (Figure 3 B), 10^{-7} M (Figure 3 C), 10^{-5} M (Figure 3 D) and 10^{-4} M (Figure 3 E). However, the filaments were observed more condensed actin expression in HGC-27 treated with BPA at concentration 10^{-9} M (Figure 3 B), 10^{-5} M (Figure 3 D) and 10^{-4} M (Figure 3 E) compared to untreated (Figure 3 A). The HGC-27 treated with BPA at concentration 10^{-7} M (Figure 3 C) was observed to have less condensed expression of actin.

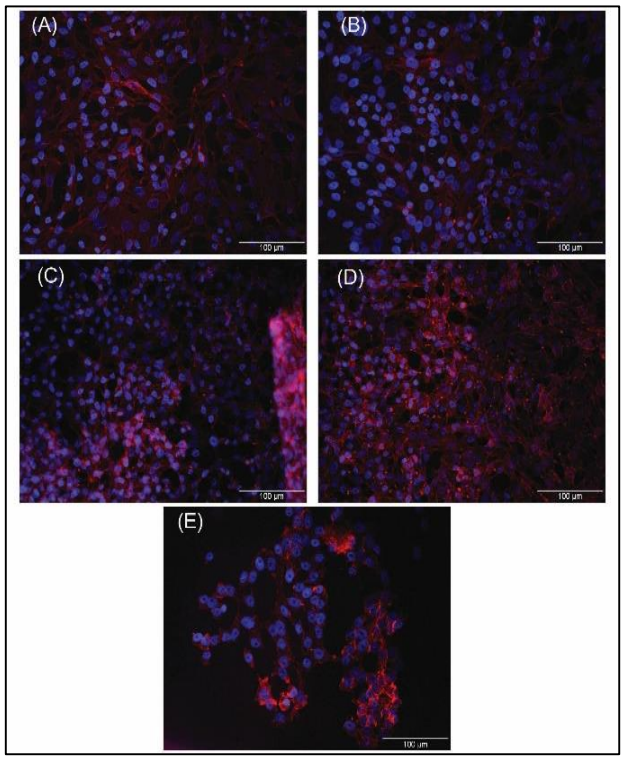


Figure 3 The HGC-27 treated -BPA stained for F-actin at 48 hr. The HGC-27 stained with DAPI for nucleus (blue) and Alexa-635 phaloidin for F-actin (red) for (A) untreated, (B) 10^{-9} M, (C) 10^{-7} M, (D) 10^{-5} M, (E) 10^{-4} M. The changes were viewed under fluorescence microscope at 20× magnification as a network of HGC-27; a representative of 3 independent observation (n = 3)

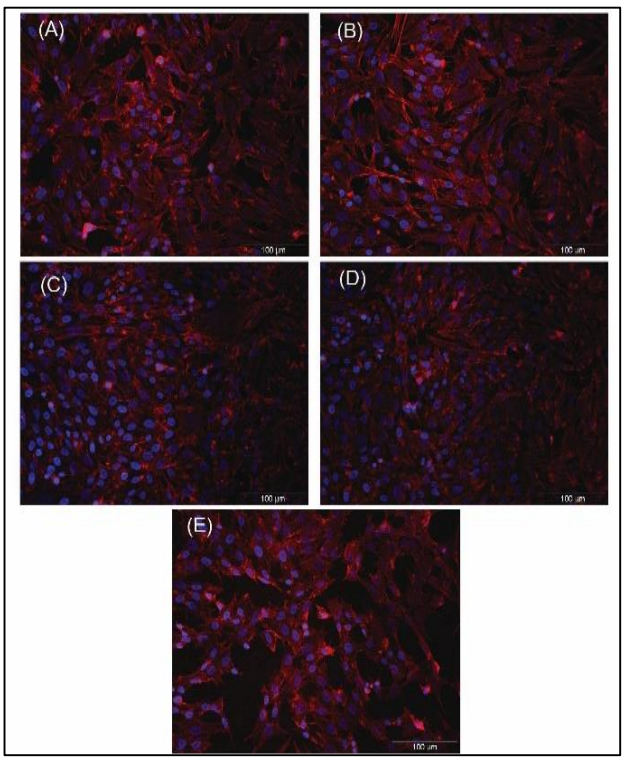


Figure 4 The HGC-27 treated -BPA stained for F-actin at 72 hr. The HGC-27 stained with DAPI for nucleus (blue) and Alexa-635 phaloidin for F-actin (red) for (A) untreated, (B) 10^{-9} M, (C) 10^{-7} M, (D) 10^{-5} M, (E) 10^{-4} M. The changes were viewed under fluorescence microscope at 20× magnification as a network of HGC-27; a representative of 3 independent observation (n = 3)

At 72 hr, the actin was more condensed in HGC-27 treated with BPA at all concentrations as compared to the untreated (Figure 4 A). The HGC-27 treated with BPA at 10^{-7} M (Figure 4 C) and 10^{-5} M (Figure 4 D) showed more condensed actin as compared to untreated. The HGC-27 treated with BPA at 10^{-9} M (Figure 4 B) show no effect on the actin structures. The result shows that the HGC-27 treated with BPA at concentration 10^{-9}M were not affecting the actin structure.

Actin is the most abundant protein in eukaryotic cells in the form of monomeric (G-protein) and filamentous (F-actin) for the maintenance of the cell shape [22]. Actin polymerization in cells is very important for cellular events such as endocytosis, adhesion, cytokinesis and membrane division. Nonetheless, BPA shown to interrupt the actin filaments elongation [23]. HGC-27 cells were polygonal or short spindle-shaped that adhered to glass surfaces as monolayer [24]. A spindle-shaped structure composed of microtubules and actin filaments, which form near the cell nucleus. Microtubules are important for the cells during cell division and organelle movement. Disruption of microtubules by BPA may disrupt mitotic spindle and induced metaphase arrest [25]. Disruption of actin filaments by BPA is not well understood.

In here, we observed the effects of low concentration of BPA on F-actin structures of HGC-27 cells. F-actin structures of HGC-27 treated with BPA at lower doses, 10^{-9} and 10^{-7} were more condensed (Figure 3) than BPA at high doses, 10^{-5} and 10^{-4} (Figure 3). BPA at high doses does not affect the F-actin striated structure and it was similar to untreated HGC-27 (Figure 4). HGC-27 shows positive actin expression in untreated and all treated cells at 24 hr BPA exposure (Figure 2). The results suggested that BPA interrupts the filamentous actin of HGC-27 and regulates gastric cancer cells proliferation. Apart from BPA, perfluorooctanesulfonate (PFOS) is another types of endocrine disrupting compound which reported in affecting the cell-cell interface in maintaining the barrier functions of sertoli cell blood-testis [26]. Disorganized actin-filament bundles results in disorganized branched network thus result in F-actin network truncation and defragmentation. PFOS is affecting actin regulatory protein, Arp3, a branched actin polymerization protein dislocation [27].

3.3 The HGC-27 Exposed to BPA Regulates Hif-1a Protein Expression at 72 hr

As BPA showed more effects on the F-actin after 72 hr treatment, the subsequent experiment was done in 72

hr treated-BPA only for Hif-1a protein expression. The association of Hif-1a proteins expression with the condensation of F-actin striated structure were observed. Figure 5 shows representative diagram of HGC-27 stained with DAPI for nucleus (blue) and fluorescein (green) for Hif-1 alpha protein at 72 hr. In untreated cells, high expressions of Hif-1 alpha protein were observed in the cytoplasm. While, low expression of Hif-1 alpha protein in the nucleus was observed in 10^{-9} M (Figure 5 B) and 10^{-7} M (Figure 5 C) BPA compared to untreated. Interestingly, increased of Hif-1 alpha protein expression in the nucleus were observed in 10^{-5} M (Figure 5 D) and 10^{-4} M (Figure 5 E).

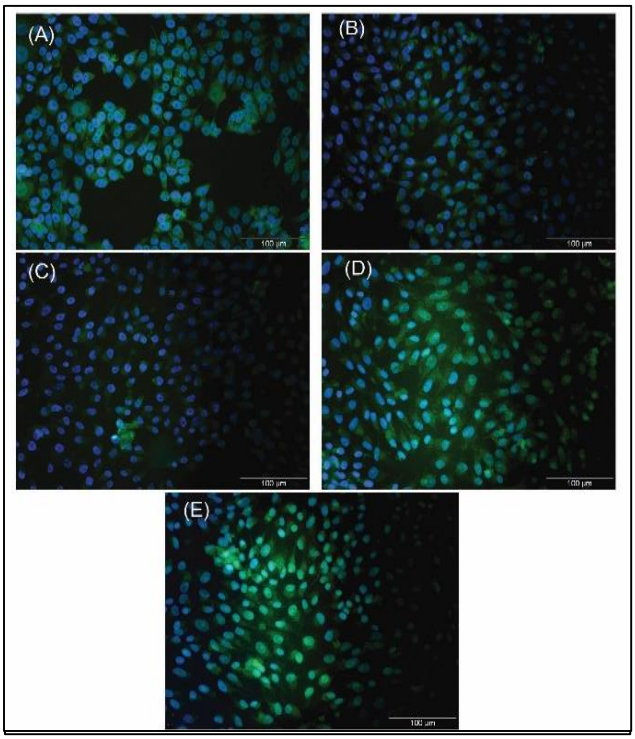


Figure 5 The expression of Hif-1 alpha protein in HGC-27 treated with BPA at 72 hr. The HGC-27 cells from (A) untreated, (B) 10^{-9} M, (C) 10^{-7} M, (D) 10^{-5} M and (E) 10^{-4} M were stained. The changes were viewed under fluorescence microscope at 20× magnification; a representative of 3 independent observation (n=3)

In solid tumor, hypoxia is known as a major disturbance towards an effective cancer therapy. Hif-1 protein is a major oxygen regulator. It is a heterodimeric transcription factor consists of two subunits known as Hif-1 alpha and Hif-1 beta. Hif-1 beta is constitutively expressed because it is oxygen dependent nuclear protein. While Hif-1 alpha is rapidly degraded in normoxic environment. Inactivation of Hif-1 alpha hydroxylation activity stabilizes the Hif-1 alpha and promotes apoptosis in cells. BPA reported to inhibits Hif-1 alpha regulation and interfere with mRNA expression, protein synthesis, protein degradation and dimerization, DNA binding and transcriptional of Hif-1 protein activity [28].

Here, our results suggest that BPA might play a role in stabilizing the Hif-1 alpha protein expression at high and lower concentration (Figure 5). Translocation of activated Hif-1 alpha into nucleus was observed in BPA exposure on HGC-27 cells. In contrast, previous study showed that BPA inhibits hypoxic response via degradation of Hif-1 alpha of human hepatoma cells. While, our result showed expression of Hif-1 alpha in both high and low concentration of BPA-treated gastric cancer cells (Figure 5) and a reduction of cell viability was observed (Figure 1). Thus, BPA at high concentration, 10^{-5} and 10^{-4} shown to upregulates Hif-1 alpha expressions and reduces growth in HGC-27 cancer cell after 72 hr. Study found that reduction in Hif-1 alpha expression regulates gastric cancer growth, angiogenesis and vessel maturation. However, in gastric cancer patients, an upregulation of Hif-1 alpha was reported by Urano *et al*. (2006) that this is due to low expression of p53 and VEGF protein expression, thus increases malignant potential [29].

4.0 CONCLUSION

In summary, the data obtained from this study suggested that BPA decreases cells viability of HGC-27 at high concentration, 10^{-5} and 10^{-4} and affecting the actin structures of the cells. At lower doses 10^{-9} and 10^{-7} concentration, BPA increases cells viability at 48 hr and 72 hr exposure in HGC-27 cells compared to 24 hr. The data also suggested that BPA upregulates activity of Hif-1 alpha protein at 72 hr exposure in all concentration of BPA.

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