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The Effect of bisphenol A and Photobiomodulation Therapy on Autophagy-Related Genes Induction in Adipose Tissue-Derived Stem Cells

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Abstract
Introduction: As adipose tissue-derived stem cells (ADSCs) can divide rapidly and be prepared non-invasively, they have extensively been used in regenerative medicine. On the other hand, a new method of therapy, known as photobiomodulation (PHT), has been used to treat many diseases, such as inflammatory conditions, wound healing and pain. Besides, exposure to chemical substances such as bisphenol A (BPA), at low levels, can lead to autophagy. This study investigated the effects of BPA and PHT on the expression of autophagy-related genes, including LC3, NRF2, P62, in rat ADSCs as a model.
Methods: ADSCs isolation and purification were confirmed by immunocytochemistry (ICC). The cells were then treated with different concentrations of BPA and also subjected to PHT. Reverse transcription polymerase chain reaction (RT-PCR) was used for the evaluation of LC3, NRF2 and P62 gene expressions. Oil red O staining was used for adipogenic vacuole formation.
Result: ICC showed that the isolated cells were CD 49-positive but CD 31 and CD 34-negative. The viability test indicated that the number of live cells after 24 hours in the BPA groups at concentrations of 0, 1, 50, 100 and 200 µM was 100%, 93%, 81%, 72%, and 43% respectively. The difference in cell viability between groups 50, 100 and 200 µM was significant as compared with the control groups ($P<0.05$). Moreover, in the group with 1 µM concentration of BPA, the expressions of LC3, NRF2 and P62 genes were upregulated. However, in the treatment group at the concentration of 200 µM of BPA, the LC3 gene was expressed, but NRF2 and P62 genes were downregulated.
Conclusion: BPA and PHT induce autophagy and adiposeness in ADSCs in a dose-dependent manner.
Keywords: Adipose tissue-derived stem cells; Bisphenol A; Photobiomodulation; NRF2 gene; P62 gene.

Introduction

Recent studies have shown that autophagy can act as an inducer or an inhibitor in the cell differentiation processes. Some data have shown a positive role of autophagy in the process of differentiation of adipose tissue-derived stem cells (ADSCs) into neuronal cells.[1] ADSCs showed more neuron-like morphology after drug interactions than the control group cells; in addition, they significantly increased the level of proteins responsible for autophagy.[2] One of the genes involved in autophagy is Nrf2 (Protein (p62/sequestosome 1, also known as SQSTM1). It acts as a receptor for the degradation of autophagy target proteins which have been ubiquitinated. In the cell, Nrf2 is sequestered and inhibited by the Keap-1 protein. p62 binds directly to Keap-1 inhibiting the interaction between Keap-1 and Nrf2, leading to Nrf2 release and entering the nucleus and activating the target genes. Indeed, p62 orchestrates a positive response to Nrf2 target genes in response to oxidative stress. It has been shown that oxidative stress plays an important role in autophagy induction and enhancement of osteoblastic differentiation of ADSCs. However, these effects increase with inhibiting the Nrf2 pathway, indicating a negative association between the Nrf2 pathway and autophagy which mitigates oxidative stress in the osteoblastic differentiation of fat stem cells.[3] The occurrence of a series

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of events including mitochondrial autophagy dysfunction leads to increased oxidative stress, mitochondrial fragmentation and apoptosis as occurs in hippocampal nerve stem cells. Bisphenol A (BPA), regardless of the dose, increases the proliferation of bone marrow stem cells (BMSCs). Increased proliferation, changes in differentiation and self-renewal capacity, and increased adipogenic differentiation following BPA exposure imply that BPA alters the BMSCs ability for proliferation and differentiation.[4] Oxidative stress has been proven as a major mechanism of BPA toxicity in animal models for years through its potential for oxidative stress induction by overproduction of hydrogen peroxide in rat organs and increasing oxidized mean glutathione (GSH) levels by generating hydroxyl radicals.[5] In general, BPA in a dose-dependent manner reduces the viability and induction of apoptosis in BMSCs. One of the important effects of BPA on BMSCs is increasing their differentiation through epigenetic modifications.[6] Recently, the use of photobiomodulation (PHT) method in inducing autophagy and treating many diseases has increased; however, its concomitant use with BPA on the induction of autophagy has not been evaluated yet. In this study, the effect of BPA and PHT treatments on autophagy induction and autophagy-related genes, including LC3, NRF2, P62 expression, was investigated in rat ADSCs.

Materials and Methods

Isolation and Culture of ADSCs

All procedures were approved by Qazvin University of Medical Sciences to isolate and culture ADSCs from the adult male Dawley-Sprague rats with an average age of 6 to 8 weeks and a weight of 200 to 300 g. Briefly, adipose tissue samples were isolated and then cut into smaller pieces. Then, DMEM (Dulbecco's Modified Eagle Medium) culture medium-dissolved type I collagenase enzyme with a concentration of 075.0% was added 3 times the volume of the isolated adipose tissue to each sample and placed in an incubator at 37°C for 30 minutes. The cell precipitate obtained was pipetted with 1 mL of the DMEM medium containing 10% bovine serum albumin, and the suspension was drained into two 25 cm flasks and incubated with the DMEM medium containing 10% bovine serum albumin in an incubator at 37°C. The temperature and CO_2 content were 5%, and the humidity conditions were 98% for 24 hours, and the next day the medium was changed. From the beginning, the cells were examined daily for morphological appearance and general condition by an inverted microscope. After four to six days, the cells were passaged.

Immunocytochemistry Assay

In order to evaluate the purity of the isolated ADSCs adhering to the bottom of the flask and to confirm their mesenchymal origin, they were evaluated by the immunocytochemistry (ICC) method. 10 000 cells were evenly dispersed for each well of a 6-well culture plate. After adherence of the cells to the bottom of the wells, ICC steps were performed as recommended by a recent study. Briefly, after washing the cells with phosphate buffer saline (PBS), they were incubated with the primary antibodies (CD49 antibody (marker of ADSCs), CD34 (marker of hematopoietic cells), CD31 (marker of vascular endothelial cells)) for 24 hours at 4°C. The cells were washed with PBS and incubated with the FITC conjugated-secondary antibody (1: 100; Chemicon) at room temperature for 2 hours. Ethidium bromide dye was used to fluorescently stain the nuclei of the cells orange/red, and the cells with positive immune reactions were counted under a fluorescent microscope.

Viability Test

To investigate the toxic effects of BPA on ADSCs, the cells were exposed to BPA for 24 hours at concentrations of 0, 1, 50, 100 and 200 µM, and the number of living and dead cells was counted using the trypan blue method. In this study, at passage 3, the percentage of living and dead cells was determined. One volume of the cell suspension and an equal volume of trypan blue dye were mixed, and cell counts were performed using a Neobar slide under an inverted microscope. The groups include control, ADSC + BPA, and ADSC + BPA + PHT.

The cells were divided into $1*10^6$ cells in a 96-well plate. The PHT therapy procedure was done by the Ga–Al–As (gallium-aluminum-arsenide) laser and the following protocol: wavelength: 660 + 10, output power: 35, energy density (J/cm ^{2}): 0.28, for 12 min.[6,7]

Evaluation of Gene Expression Using Reverse Transcription Polymerase Chain Reaction

To perform reverse transcription polymerase chain reaction (RT-PCR) reactions for the aforementioned

Table 1. The Sequence of Primers Used In RT-PCR Reactions

Gene	Primer (5'-->3')	Sequence Size (bp)	Annealing Temperature (°C)	Primer Length
LC3	F: TGTTAGGCTTGCTCTTTTGG R: GCAGAGGAAATGACCACAGAT	219	60	20
Nrf2	F: ATTCCCGAGTTACAGTGTCT R: CGTGGAGAGGATGCTACT	204	59	20
P62	F: TCCTACAGACCAAGAATTATGAC R: TTCTCATGCACTTTCCTACTG	232	62	22
GAPDG	F: ATCTGACATGCCGCCTGGAG R: AAGGTGGAAGAATGGGAGTTGC	154	58	20

genes, the primer pairs were designed using Gene Runner software version 6. Total RNA was extracted from the ADSCs of each group using an RNA extraction kit (Roche biochemical, Germany). In order to remove unwanted DNA, DNase I (Thermo scientific company, Lithuania) treatment was performed for each extracted total RNA. The RNA was then transformed into complementary DNA (cDNA) using a cDNA synthesis kit (Thermo scientific company, Lithuania) as recommended by the manufacturer's instructions. The resulting cDNA was then amplified by PCR amplification at the optimal annealing temperature. The GAPDH (glyceraldehyde 3-phosphate dehydrogenase) gene was used as an internal control. (Table 1)

Oil Red O Staining

To investigate the adipogenic effects of BPA (BPA group) and PHT (PHT group), the optimal dose of this substance (a dose that caused less than 50% mortality in the viability test) was added to the fat differentiation medium (ADM) of ADSCs. DMSO 3% was used as a BPA solvent. The zero concentration of BPA and DMSO were considered as controls (CO group). After 14 days, the ADSCs released fat into the fat induction culture medium, and the fat-containing vesicles turned red using oil red staining.

Statistical Analysis

All values are given in mean ± SEM (standard error of the mean). The data obtained from cell viability and cell count were compared by the student *t* test and one-way analysis of variance (ANOVA) and Tukey test. The significance level was considered $P < 0.05$.

Result

Culture and Purification of ADSCs

ADSCs had a rounded appearance after separation but adhered to the bottom of the flask after 72 hours of incubation, and after rinsing with PBS, the non-adherent floating cells were washed. In adipose tissue, there are large populations of different stem cells such as vascular endothelial stem cells and blood stem cells which are usually washed after separation and rinsed with PBS after incubation time, except for ADSCs. However, to ensure the purity of the cultured cells, after the third passage, ADSCs were evaluated for stemness and mesenchymal origin by ICC analysis of the specialized cell surface markers. Fluorescence light was associated with the FITC-conjugated secondary antibody, which appeared green. Ethidium bromide dye was used to count the cells, and the nuclei emitted red light. ADSCs were negative for CD31 protein, which is a marker of endothelial cells, and CD34, which is a marker of blood stem cells, but the cells were positive for the CD49 protein (a marker of ADSCs). ADSCs did not express the endothelial stemness marker on their cell membrane in the culture medium (Figures 1 and 2).

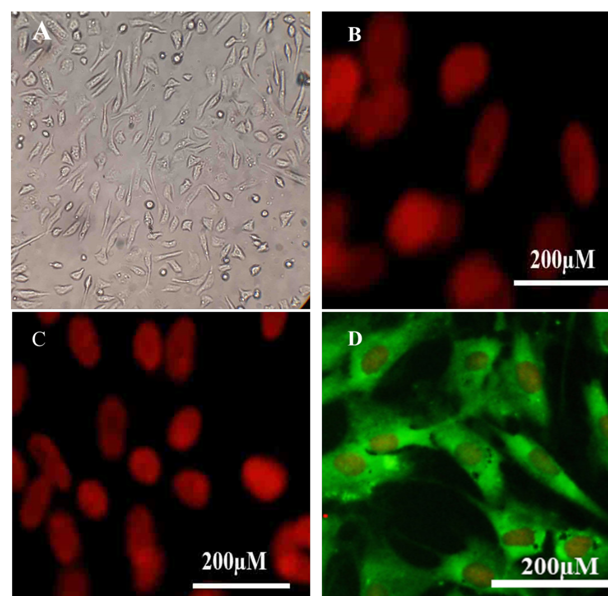

Figure 1. Immunocytochemistry result showed that ADSCs expressed CD49 but no other CD markers known as CD 31, CD34. ADSCs were negative for CD31 protein, which is a marker of endothelial cells, and CD34, which is a marker of blood stem cells, but the cells were positive for the CD49 protein (a marker of ADSCs). ADSCs did not express the endothelial stemness marker on their cell membrane in the culture medium. (A) Phase contrast image of ADSCs and (B) CD31, (C) CD34 and (D) CD49 immunostaining.

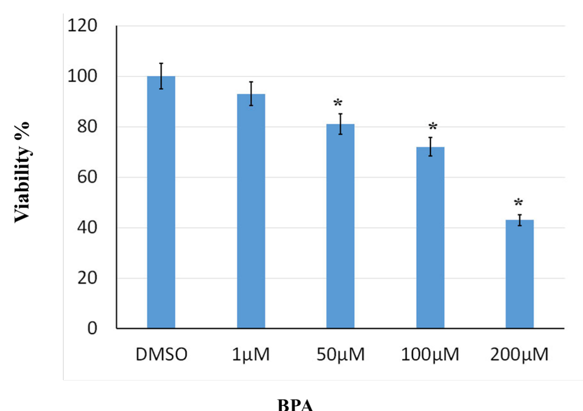

Figure 2. The Effect of BPA Concentrations on ADSCs Mortality. Dose response by BPA showed that the cell mortality increased as the BPA dose increased (* a significant difference between the groups; $P < 0.05$).

RT-PCR Result

RT-PCR results showed that after 24 hours of incubation in the control group (0 μM BPA + DMSO 3%) and BPA group with a concentration of 1 μM, the LC3, NRF2 and P62 genes were expressed. Meanwhile, in the treatment group with a concentration of 200 μM BPA, the LC3 gene was expressed, but NRF2 and P62 genes were not expressed in contrast (Figures 3 and 4).

Fat Droplet Formation

Oil red staining was applied to evaluate the formation of adipogenic vacuoles after the treatment of ADSCs by BPA and PHT. The rate of adipogenesis in ADSCs after culturing in the medium containing BPA at a concentration of 1μM

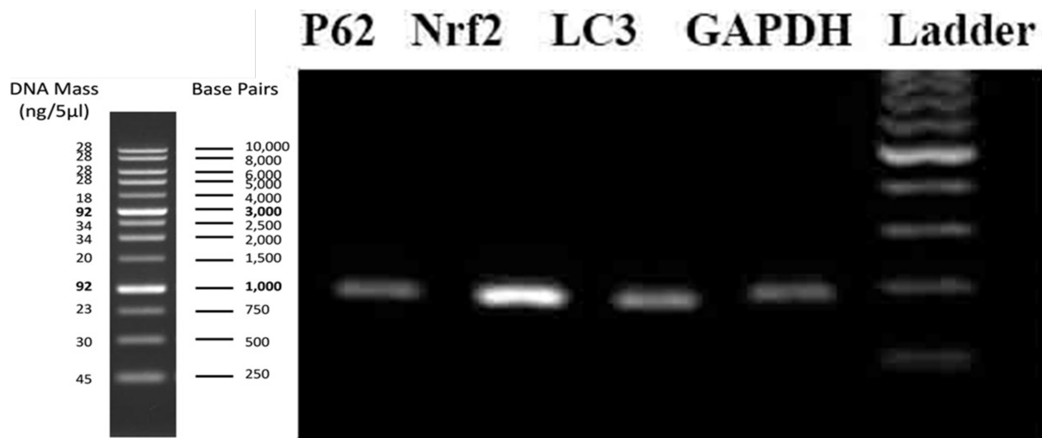

Figure 3. RT-PCR results showed that after 24 hours of incubation with only DMSO 3% in the control group and the BPA group (with a concentration of 1 μM of BPA), the LC3, NRF2 and P62 genes were expressed.

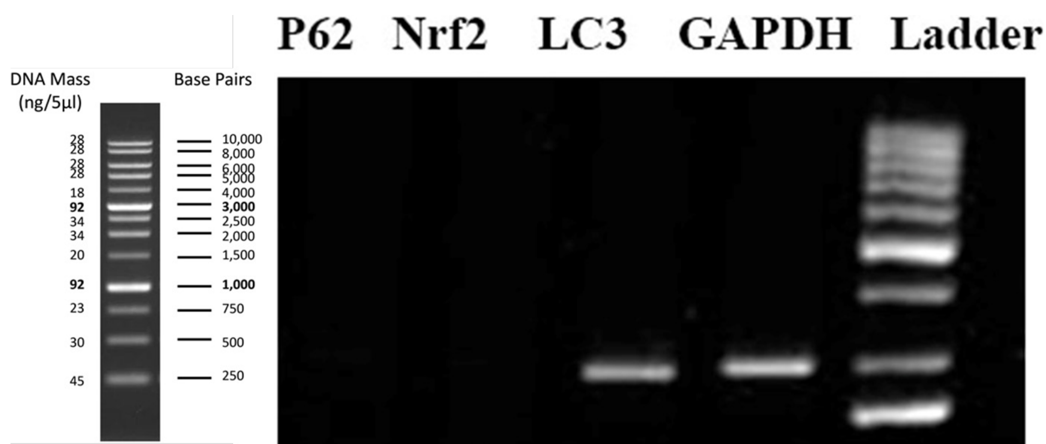

Figure 4. RT-PCR results showed that after 24 hours of treatment in the group with a concentration of 200 μM of BPA, the LC3 gene was expressed; however, the NRF2 and P62 genes were not expressed.

(A) and with PHT was assessed (Figure 5). BPA and PHT increased adipogenesis in ADSCs, which was highlighted in red in the image of fat vacuoles. After oil red staining and imaging of the cells and analysis of fat content in ADSCs, it was observed that the rate of adipogenesis in the control group (0 μM BPA + DMSO 3%), BPA group at concentrations of 1 μM and BPA + PHT group was 51%, 88% and 90% after 14 days of culture incubation respectively (*a significant difference between the control group and the other groups) ($P < 0.05$; Figure 6).

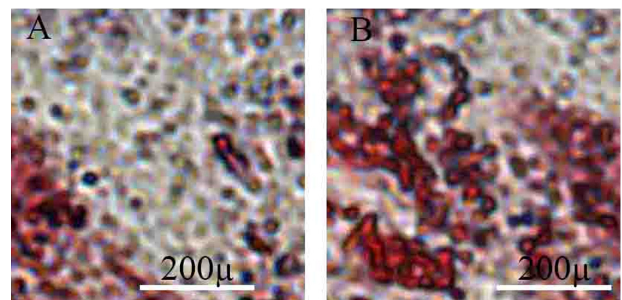

Figure 5. Oil Red Staining to Evaluate the Adipogenic Formation After Treatment by BPA and PHT in ADSCs. The rate of adipogenesis in ADSCs cells after exposure to the ADM culture medium and BPA at a concentration of 1µM (A) and with PHT (B). BPA and PHT increased adipogenesis in ADSCs, which are seen in red in the image showing fat vacuoles.

Discussion

In this study, we showed that low concentrations of BPA and PHT increased the expression of P62, Nrf2 and LC3 genes in ADSCs, but high concentrations of BPA only induced LC3 expression, and BPA and PHT increased fat droplet formation in ADSCs.

These results indicate that autophagy is induced at low concentrations of BPA in ADSCs, and there was a direct correlation between its dose of BPA and cell death. BPA also increases mortality and lipid production in a dose-dependent manner in ADSCs. Some researchers have reported that BPA increases nitric oxide (NO) levels, and some have shown an increase in ROS in the liver cancer cell lines (Hep3B) and uridine diphosphate-glucuronosyltransferase (UGT). The incubation of Hep3B cells with BPA increases NO levels and activates Nrf2 by Keap1 nitrification to induce heme oxygenase-1 and UGT 2 family, polypeptide B1 (UGT2B1) mRNAs.[8] Moreover, the exposure of hippocampal-derived neurons to PHT

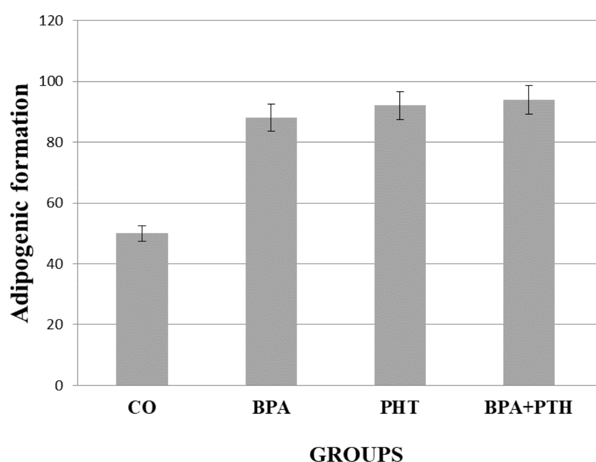

Figure 6. Fat Induction Production in BPA and PHT on ADSCs. After oil red staining and imaging of the cells and analysis of fat content in ADSCs, it was observed that after 14 days in the control group (0 μM BPA+DMSO 3%), the adipogenesis rate was 51%, but in the BPA group, at the concentration of 1 μM, and PHT group, the rate of adipogenesis was 88% and 90% respectively (* a significant difference between the control group and the other groups; $P<0.05$).

increased the expression level of autophagy-related genes and proteins. The activation of autophagy against BPA and BHT activates intracellular energy sensors of Adenosine monophosphate-activated protein kinase (AMPK), increases the phosphorylation of reptiles and acetyl-coenzyme A carboxylase, decreases the phosphorylation of ULK1 and shuts down the AMPK pathway.[8] The effects of BPA on the induction of autophagy in goat Sertoli cells (gSCs) as well as the association between autophagy and apoptosis were investigated. The results showed that BPA exposure dose-dependently reduced cell viability. Apoptosis was induced by increasing the BCL2-associated X (Bax)/B-cell lymphoma 2 (Bcl-2) ratio and increasing the autophagy rate, including autophagosome formation and increased expression of autophagy-related markers in gSCs.[9] BPA activated the ATM serine/threonine kinase -P53 signal transduction pathway which, in turn, inhibited the cell cycle progression. In stage G1, premature aging, autophagy and reduced cell proliferation occurred in the HFLF cell line. BPA exposure caused autophagosome accumulation in HepG2 cells. These changes were associated with increased expression of rapamycin, phosphorylated-p70S6 kinase, phosphorylated- Unc-51 like autophagy activating kinase (ULK1) and decreased expression of autophagy-related gene 5 (Atg5). Cell exposure to the disruption of autophagy degradation due to the increased levels of P62 expression was further observed in BPA-treated HepG2 cells. Overall, BPA treatment induced fat accumulation in the liver of male CD1 mice, which was related to protein changes associated with hepatic autophagy. Long-term treatment with BPA disrupts the autophagy/ mitophagy pathway and increases apoptosis in the hippocampus of mice.[10] The effects of autophagy degradation may be associated with increased apoptotic cell death and neuronal cell degradation with increased ROS levels and decreased antioxidant levels. However, a decrease in neural stem cell proliferation and neuronal differentiation and an increase in nerve damage by BPA were observed. Chronic induction of BPA reduced the levels of autophagy proteins such as microtubule-associated proteins 1A/1B light chain 3B (LC3B), lysosome-associated membrane protein 2 (Lamp-2), and antioxidant enzymes such as catalase. P62 could selectively absorb and aid in the accumulation of cytosolic proteins. These results suggest that long-term treatment of BPA is associated with impaired autophagy and antioxidants and that apoptosis increases in response to high doses of BPA.[11] In this study, BPA and PHT increased fat droplet formation and BPA increased aspartate aminotransferase and alanine aminotransferase activities in female and male mice respectively. Increased fat mass accumulation was observed in both male and female mice. BPA also decreased Nrf2/Keap1 protein in male mice.[12] In addition to its effects on autophagy and the expression of autophagy-related genes and proteins, PHT has other important effects on the differentiation of stem cells into other cell types. Mesenchymal stem cells (MSCs) exposed to PHT form smaller colonies, indicating a reduction in the potential for self-renewal. PHT enhances both the differentiation of adipogenesis and osteogenesis by increasing mRNA expression from glucose transfer 4, lipoprotein lipase, Peroxisome proliferator- activated receptor gamma, leptin, osteonectin, and cell-binding factor 1. These features can also be seen in BPA, which was consistent with our results. The expression of these factors in BPA-treated cells is similar to that expressed in estrogen-treated MSCs and indicates that BPA acts through the estrogen receptor pathway.[13] Numerous studies have shown that BPA has an effect on obesity in living organisms. Adult adipocytes exert most adipose tissue biochemical activities, such as their ability to metabolize glucose, store and release fatty acids, and secretion of adipokines.[14] Many studies have been performed on the effects of BPA on adult rodents. The researchers found that mice treated with either high-dose or low-dose of BPA gave birth to heavier offspring than the control group, and this phenotype persisted into adult mice.[15] BPA has previously been shown to increase fat formation. As in our experiment, the rate of fat accumulation in the cells of a group of ADSCs that received 1 μM of BPA for 2 weeks was much higher than the control group (containing 3% DMSO). There are other experiments similar to what we proved. In their study, Masuno et al concluded that BPA induces differentiation of fat cells as well as marker genes involved in lipid biosynthesis in 3T3-L1 fat progenitor cells over a period of 6 days.[16] BPA is induced by apoptosis of stem cells, altering the functional abilities of these cells and possibly causing genetic disorders in them.[17-19] Finally,

researchers have shown that PHT has positive effects on inducing proliferation and differentiation in different stem cells.[20] The results of this study showed that PHT can increase the induction of autophagy in the ADSC and also cause the formation of more lipid droplets, which was consistent with the results of other studies.[21]

Conclusion

Both BPA and PHT induce autophagy and increase cell death and lipid production in ADSCs. Also, low concentrations of BPA and PHT increase the expression of P62, Nrf2 and LC3 genes in ADSCs, but high concentrations of BPA just induce LC3 expression.

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Conflict of interests

None.

Ethical Considerations

This article does not contain any studies with human participants or animals performed by any of the authors.

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