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Non-cytotoxic nanomolar concentrations of bisphenol A induce human mesenchymal stem cell adipogenesis and osteogenesis

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ABSTRACT

Bisphenol A (BPA) is a typical endocrine disrupting chemical with extensive applications, and has been correlated with various hazardous health effects, including obesity and other metabolic-related diseases. Human mesenchymal stem cells (hMSCs), due to their abilities to differentiate into adipocytes and osteoblasts, can be a good *in vitro* model to assess chemical-dependent toxicity on adipogenesis or osteogenesis. Here, we employed hMSCs as an evaluation system to assess BPA-related effects on cell viability, oxidative stress induction, self-renewal, and differentiation. Our results revealed that low concentrations (1 and 10 nM) of BPA did not impair cell proliferation nor self-renewal capacity, but stimulated adipogenesis and osteogenesis. Our findings support the concern of BPA contributing to the epidemic of obesity, and also reveal its underlying toxicity on osteogenesis.

1. Introduction

Exposure to environmental pollutants may contribute to many different adverse health effects. Nevertheless, comprehensive toxicological methods to assess chemical-derived toxicity, are still imperfect. We and others, have proposed that stem cells could be a powerful tool to assess pollution impacts on human health, as alternatives to animal tests or traditional *in vitro* assays (Faiola et al., 2015; Liu et al., 2017; Jennings, 2015). Among different adult/somatic stem cells, human mesenchymal stem cells (hMSCs), because of their capability to undergo self-renewal and differentiate into adipose, bone, and cartilage tissues, can be applied to detect chemical-derived homeostatic imbalance, especially in bone and adipose tissues (Yao et al., 2016; Strong et al., 2015).

Bisphenol A (BPA), as the raw material in the manufacture of polycarbonate plastics and epoxy resins, is widely utilized in consumer products, including food and beverage containers, which make human exposure to BPA ubiquitous and persistent among general populations, regardless of its short half-life (Vandenberg et al., 2007; Staples et al., 1998). Previous studies have demonstrated that BPA acts as a typical environmental endocrine disrupting chemical (EDC), with effects on reproduction, development, *etc.* (Rochester, 2013). Recently, increasing evidence has shown that BPA exposure might be also associated with obesity and other metabolic-related diseases (Vom Saal et al., 2012). For instance, some reports have documented that BPA exposure induced terminal adipogenic differentiation and abnormal functioning of adipocytes in rodents and human models (Atlas et al., 2014; Boucher et al., 2014a; Masuno et al., 2005; Ohlstein et al., 2014; Wada et al., 2007; Somm et al., 2009), which might lead to obesity. On the contrary, others have observed inhibitory effects of BPA on adipogenesis and adipocytes' functions in human *in vitro* models (Hugo et al., 2008; Linehan et al., 2012). Therefore, this controversy needs to be further addressed and solved. Moreover, although some EDCs, *e.g.* DDT, MEHP and DEHP, are linked to osteogenic toxicity (Bateman et al., 2017; Strong et al., 2015), the impacts of BPA on osteogenesis remain to be determined.

In this study, by using a hMSC model, we examined the influences of BPA on cell proliferation, oxidative stress, self-renewal, and differentiation. We showed that at non-cytotoxic concentrations, BPA significantly disrupted hMSC adipogenesis and osteogenesis, suggesting BPA may induce homeostatic imbalance in adipose and bone tissues.

2. Materials and methods

2.1. Chemicals

BPA (> 99.0% purity) was purchased from TCI (Shanghai, China),

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and dissolved in dimethyl sulfoxide (DMSO) to prepare 500 mM stock solutions. For the indicated working concentrations, it was further diluted in cell culture media, with a DMSO final concentration of 0.01%.

2.2. Cell culture and differentiation

Otherwise specified, all cell culture reagents and consumables were purchased from Gibco (Thermo Fisher Scientific, NY, USA) and Nunc (Thermo Fisher Scientific, USA). hMSCs were isolated, as previously reported (Li et al., 2010), from adipose tissues of patients undergoing liposuction upon informed verbal consent. Cells were maintained at 37 °C in humidified air and 5% CO_2, in KnockOut DMEM/F12 supplemented with 2% fetal bovine serum (FBS), 5 ng/mL aFGF (Invitrogen, USA), 5 ng/mL bFGF (Invitrogen, USA), 5 ng/mL EGF (Invitrogen, USA), 4.7 μg/mL Linoleic acid (LA, SIGMA Aldrich, MO, USA), 1% GlutaMax, 1% ITS, and 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). Culture medium was replaced every other day.

For adipogenesis, sub-confluent hMSCs were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 1 μM Dexamethasone (Dex, Sigma-Aldrich, MO, USA), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich, MO, USA) and 100 μM L-ascorbic acid 2-phosphate sesquimagnesium (VC-2p, Sigma-Aldrich, MO, USA). Media were replaced every other day, and samples were collected at induction days 7, 14 and 21, for RNA isolation and qRT-PCR analyses. For osteogenesis, sub-confluent hMSCs were cultured in DMEM supplemented with 10% FBS, 50 μM VC-2p and 10 mM β-Glycerophosphate disodium (β-Gly, Sigma-Aldrich, MO, USA). Media were replaced every other day and samples collected at day 7, for RNA isolation and qRT-PCR analyses. For toxicity assays, cells were pretreated with various concentrations of BPA (or DMSO vehicle control) for eight days, prior to the differentiation, as well as all along the differentiation procedures.

2.3. Cell viability assay and IC_{50} calculation

Cell viability was assessed by the Alamar Blue (AB, Sigma-Aldrich, MO, USA) fluorescence assay. Cells were seeded in 96-well plates for 24 h, and then incubated with culture media containing various concentrations of BPA (or DMSO vehicle as control) for a minimal of 72 h and up to eight days. Culture media were replaced every other day. After incubation with AB at 37 °C for 2 h, fluorescence was measured with an excitation wavelength of 530 nm and an emission wavelength of 590 nm, in a Varioskan LUX microplate reader (Thermo Fisher Scientific, Vantaa, Finland).

The minimum 50% inhibiting concentration (IC_{50}) was determined by Probit regression with the SPSS software, ver. 16.0 (SPSS Inc., USA). IC_{50} values are presented as means ± standard deviation (SD) of three independent experiments.

2.4. Evaluation of oxidative stress

2.4.1. Assessment of intracellular ROS generation

Accumulation of reactive oxygen species (ROS) was measured with the fluorescence probe DCFH-DA method (Sigma-Aldrich, USA). hMSCs were loaded with DCFH-DA (100 μM) for 30 min at 37 °C and 5% CO_2, and then treated with 1 nM to 400 μM BPA (and 1 mM H_2O_2 as a positive control) for 2 h. Fluorescence was measured with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

2.4.2. Assessment of intracellular calcium flow

Intracellular calcium ion levels were determined by measuring the F340/F380 ratio using the Fura-2/AM probe (5 μM, Sigma-Aldrich, USA). hMSCs were loaded with the probe in Hank's balance salt solution (HBSS, Solarbio Biotechnology, Beijing, China) for 30 min, before BPA exposure. Fluorescence signals were measured with 340 and 380 nm excitation wavelengths and 510 nm emission wavelength every 30 s, for up to 20 min. The F340/F380 fluorescent ratio is proportional to the levels of intracellular calcium ions.

2.5. Flow cytometry

Cells were treated with several BPA concentrations for eight days, and then harvested and re-suspended in 100 μL DPBS containing 2% FBS, at a density of 1×10^6 cells/mL. Subsequently, samples were incubated at room temperature for 30 min, with the following phycoerythrin-conjugated antibodies: CD34 (Invitrogen, MA, USA), CD44 (BD Biosciences, San Jose, CA), CD45 (Cell Signaling Technology, MA, USA), CD73 (BD Biosciences), CD79 (BD Biosciences) and CD105 (Invitrogen). Measurements were performed with an LSR II flow cytometer (BD Biosciences).

2.6. RNA extraction and qRT-PCR analysis

Total RNA was extracted with Trizol Reagent (Life Technologies, NY, USA), and then reverse transcribed to cDNA with a PrimeScript™ RT Master Mix (TAKARA, Japan) according to the manufacturers' instructions. qPCR was performed with an initial denaturation at 95 °C for 30 s, followed by 50 cycles of denaturation at 95 °C for 3 s and annealing/amplification at 60 °C for 30 s. The mRNA expression levels were calculated with the comparative C_t method and normalized to the reference gene *GAPDH*. The primers employed are listed in Table S1.

2.7. Alizarin Red, Oil Red O and Nile Red staining assays

Calcium deposit during osteogenic differentiation was visualized by Alizarin Red staining. Differentiated cells were washes twice with DPBS, before being fixed with ethanol (95%, v/v, Sinopharm Chemical, Beijing, China) for 10 min. Then, calcium deposits were stained with Alizarin Red (pH 4.2, 1%, Solarbio Biotechnology, Beijing, China) for 5 min at room temperature. Pictures were taken with an EVOS microscope (Thermo Fisher Scientific, USA) after extensive ddH_2O washes.

Intracellular lipid droplet accumulation during adipogenesis was visualized by Oil Red O and Nile Red staining. Differentiated cells were washed twice with DPBS, before being fixed with paraformaldehyde (PFA, 4%, v/v, Solarbio Biotechnology, Beijing, China) for 10 min. Then lipid droplets were stained by Oil Red O (Sigma, USA) or Nile Red (0.2 mg/mL, Sigma, USA) working solution for 10 min at room temperature. Pictures were taken with an EVOS microscope after extensive ddH_2O washes. The fluorescent intensity of the Nile Red staining was quantified by Image J.

2.8. Statistical analysis

Results are presented as the mean ± SD of independent experiments. The statistical analyses were performed with the one-way ANOVA, with non-parametric unpaired test or followed by multiple correction as indicated in the figure legends, using the GraphPad Prism software, Ver. 6.01 (GraphPad Inc., USA).

3. Results

3.1. BPA showed no cytotoxicity and did not induce oxidative stress at concentrations up to 10 μM

To test the effects of BPA on hMSC survival and proliferation, we measured cell viability after BPA treatment at concentrations ranging from 1 nM to 400 μM, for 72 h. There was a statistically significant dose-dependent decline of cell viability for BPA concentrations over 50 μM. However, no detectable cell proliferation changes were observed at concentrations lower than 10 μM (Fig. 1A). The calculated IC_{50} for 72 h BPA exposure was 83 ± 6 μM.