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Propyl gallate induces cell death and inhibits invasion of human trophoblasts by blocking the AKT and mitogen-activated protein kinase pathways

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ABSTRACT

Propyl gallate (PG) is an antioxidant widely used in food additives, cosmetics, adhesives, and lubricants. PG protects the oils in food products from reacting with hydrogen peroxide and oxygen free radicals, thus preventing spoilage. It is known to have both protective and cytotoxic effects on various cells, but its effects on human trophoblasts remain unclear. Therefore, we investigated the effects of PG on proliferation, apoptosis, and invasiveness of human trophoblasts using an immortalized HTR8/SVneo cell line. We found that PG significantly reduced proliferation of and induced apoptosis in HTR8/SVneo cells. In addition, the invasiveness of HTR8/SVneo cells was attenuated in response to PG. Signaling pathways including the PI3K/AKT and MAPK pathways involved in the proliferation and invasiveness of human trophoblasts were regulated by PG treatment in HTR8/SVneo cells. We confirmed that mitochondrial membrane disruption and Ca^{2+} overload were markedly elevated in response to PG treatment, suggesting that PG-induced apoptosis is closely related to mitochondrial dysfunction and further PG-induced apoptosis in HTR8/SVneo cells is related to endoplasmic reticulum (ER) stress. Collectively, these results indicate that PG exerts harmful effects on human trophoblasts; therefore, exposure to PG in early pregnancy is predicted to cause abnormal implantation and placental development.

1. Introduction

Propyl gallate (PG, propyl 3,4,5-trihydroxybenzoate) is an antioxidant preservative used in artificial food additives, cosmetics, adhesives, and lubricants. PG is a synthetic ester of gallic acid, a naturally occurring antioxidant, and protects against rancidity and spoilage of fats. PG-related allergic reactions have been reported, as it is commonly used in skin cosmetics such as lipstick and sunscreen (Gamboni et al., 2013). Recently, computational studies regarding the electrochemical properties of gallate esters in aqueous and lipid solvents have been performed to explore the effective antioxidant conditions in preservatives (Medina et al., 2013). The hydrophobic ester group allows PG to pass through the cell membrane easily, and the polyphenolic structure that enters the cytoplasm plays a role in scavenging hydroxyl radicals.

The modulating effects of PG on oxidative conditions affect cell sensitivity to toxicity, and are related to both chemo-preventive and cytotoxic effects in different cell types. The antioxidant effect of PG protects against the toxicity of oxyradicals in hepatocytes (Wu et al., 1994). PG also reduces proliferation in glomerular endothelial cells, and protects against diabetic nephropathy (Tian et al., 2012). In contrast, the cytotoxicity of PG has been described in several cell types as a disturbance of the redox cycle and induction of DNA strand breaks (Hamishehkar et al., 2014; Nakagawa and Tayama, 1995). Excessive DNA damage induced by PG has anti-cancer effects in several types of cancer cells. In human leukemia cells, PG induces apoptosis via activation of the mitogen-activated protein kinase (MAPK) pathway and expression of pro-apoptotic proteins (Chen et al., 2011). PG also induces cell death in cervical adenocarcinoma cells, and enhances mitochondrial oxyradical levels as well as disrupts mitochondrial membrane potentials (Han and Park, 2009).

Several studies using mouse models have reported that exposure to endocrine disrupting chemicals (EDCs) during the gestational period induces adverse effects on the female reproductive system. For instance,

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exposure to phthalate, which is used in plasticizers, causes pregnancy complications in generations exposed as germ cells and fetuses (Zhou et al., 2017). Moreover, EDCs are implicated in various female reproductive disorders including female infertility, polycystic ovary syndrome (PCOS), and endometriosis accompanied by morphological alterations of the female reproductive organs (Palioura and Diamanti-Kandarakis, 2015). PG binds to estrogen receptor α and exerts antagonistic effects, blocking 17β-estradiol activity (Amadasi et al., 2009). However, it is uncertain whether the biological activities of PG influence the development of female reproductive disorders or pregnancy failure.

In the present study, we used the immortalized human trophoblast cell line HTR8/SVneo to investigate the effects of PG on human trophoblasts. We first investigated PG's effects on proliferation and apoptosis in HTR8/SVneo cells, as well as on the regulation of the invasive properties of these cells. Finally, we confirmed whether cell signaling pathways were mediated by PG and studied its effects on mitochondrial dysfunction in HTR8/SVneo cells.

2. Materials and methods

2.1. Chemicals

The PG was purchased from Sigma-Aldrich, Inc (St. Louis, MO, USA). Antibodies against phosphorylated AKT (v-Akt Murine Thymoma Viral Oncogene) (Ser^{473}), P70S6 kinase (P70S6K, Thr^{421}/Ser^{424}), ribosomal protein 6 (S6, Ser^{235}/Ser^{236}), extracellular signal-regulated protein kinase 1 and 2 (ERK1/2, Thr^{202}/Tyr^{204}), P90 ribosomal S6 kinase (P90RSK, Thr^{573}), and c-Jun N-terminal kinase (JNK, Thr^{183}/Tyr^{185}), and total AKT, P70S6K, S6, ERK1/2, P90RSK, JNK, inositol-requiring enzyme 1 alpha (IRE1α), and Caspase-9 were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against glucose-regulated protein 78 kDa (GRP78) and α-tubulin (TUBA) were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA).

2.2. Cell culture

HTR8/SVneo cells were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were maintained in RPMI-1640 supplemented with 2.05 mM L-glutamine (Cat No: SH30027.01, HyClone, Carlsbad, CA, USA) and 5% fetal bovine serum (FBS) at 37 °C in a CO_2 incubator. For experiments, monolayer cultures of HTR8/SVneo cells were grown in culture medium to 70% confluence in 100-mm tissue culture dishes. Cells were serum starved for 24 h, and then treated with PG. In each assay, dimethyl sulfoxide (DMSO) was used as a vehicle.

2.3. Proliferation assay

Proliferation assays were conducted using a Cell Proliferation ELISA, BrdU kit (Cat No: 11647229001, Roche, Indianapolis, IN, USA) according to the manufacturer's recommendations as described previously (Lim et al., 2017). HTR8/SVneo cells were treated in triplicate in 96-well culture dishes with the following concentrations of PG for 48 h: 0, 5, 10, 20, 50, and 100 μM. Absorbance of the reaction product was quantified by measuring values at 370 nm and 492 nm using an ELISA reader.

2.4. Immunofluorescence microscopy

The effects of propyl gallate on the expression of proliferating cell nuclear antigen (PCNA) in HTR8/SVneo cells were determined via immunofluorescence microscopy as described previously (Lim et al., 2017). Cells were treated with PG (20 μM) for 24 h. Images were captured using a LSM710 (Carl Zeiss, Thornwood, NY, USA) confocal microscope fitted with a digital microscope Axio-Cam camera with Zen2009 software. Relative fluorescence intensity was measured via the green/blue ratio using MetaMorph software (Molecular Devices, Sunnyvale, CA, USA). Data are representative of three different experiments done in triplicate.

2.5. Determination of apoptosis via annexin V and propidium iodide (PI) staining

Apoptosis of HTR8/SVneo cells induced by propyl gallate was analyzed using a fluorescein isothiocyanate (FITC) Annexin V apoptosis detection kit I (BD Biosciences, Franklin Lakes, NJ, USA) as described previously (Lim et al., 2017). Cells were treated with PG at the following concentrations for 48 h: 0, 5, 10, and 20 μM. Fluorescence intensity was analyzed using a flow cytometer (BD Biosciences). Data are representatives of three independent experiments.

2.6. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

A TUNEL assay was performed on HTR8/SVneo cells treated with propyl gallate as described previously (Lim et al., 2017). Cells were treated with PG (20 μM) for 48 h, and were then subjected to TUNEL staining using an *in situ* Cell Death Detection Kit, TMR red (Roche). Images were captured using a LSM710 confocal microscope fitted with a digital microscope Axio-Cam camera with Zen2009 software. Relative fluorescence intensity was measured via green/blue ratio using MetaMorph software (Molecular Devices). Data are representative of three different experiments done in triplicate.

2.7. Matrigel invasion assay

The cell invasion assay was performed in 8-μm pore Transwell inserts (Cat No: 3422, Corning, Inc., Corning, NY, USA) coated with matrigel over 2 h at 37 °C. HTR8/SVneo cells (1×10^5 cells per 200 μL) suspended in serum-free medium containing PG (20 μM) were plated onto the upper chamber, and medium containing 5% FBS was added to the lower wells. After 24-h incubation at 37 °C in a CO_2 incubator, non-invasive cells were removed with a cotton swab. To evaluate the number of cells that invaded the lower surface, inserts were fixed in methanol for 10 min. The Transwell membranes were then air dried and stained using hematoxylin (Sigma) for 30 min. After washing gently with tap water, the cells on the upper side of the inserts were removed with a cotton swab. The Transwell membranes were removed and placed on a glass slide with the side containing cells facing upward, and the slide was covered with Permount solution. Invasive cells were counted in five non-overlapping locations covering approximately 70% of the insert membrane growth area using a DM3000 (Leica, Wetzlar, Germany) microscope.

2.8. Quantitative real-time polymerase chain reaction (RT-PCR) analysis

Gene expression was determined using SYBR® Green (Sigma) and a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) as described previously (Lim et al., 2016). The *GAPDH* gene was analyzed simultaneously as a control and for normalization of data due to variation in loading. All genes were analyzed in triplicate. For *GAPDH*, the sense primer (5′-GGC TCT CCA GAA CAT CAT CC-3′) and antisense primer (5′-TTT CTA GAC GGC AGG TCA GG-3′) were used. The following primers were used: *matrix metalloproteinase 2 (MMP2)* sense (5′-GTG GAT GAT GCC TTT GCT CG-3′) and antisense (5′-CCA TCG GCG TTC CCA TAC TT-3′); *MMP9* sense (5′-TTG ACA GCG ACA AGA AGT GG-3′) and antisense (5′-ACA TTG GCC TTG ATC TCA GC-3′); *plasminogen activator, urokinase* (*PLAU)* sense (5′-CAA CTG CCC AAA GAA ATT CG-3′) and antisense (5′-AAG GAC AGT GGC AGA GTT CC-3′); and *forked box protein M1* (*FOXM1)* sense (5′-GGG TTT TCT CCT TTG CTT CC-3′) and antisense (5′-ATG GGT CTC GCT AAG TGT GG-3′).