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Avobenzone suppresses proliferative activity of human trophoblast cells and induces apoptosis mediated by mitochondrial disruption

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ABSTRACT

Avobenzone is widely used in various personal care products, is present in swimming pools, and is toxic to aquatic organisms. However, it is unclear how avobenzone affects human trophoblast cells. Results of the present study demonstrated that avobenzone inhibited the proliferation of HTR8/SVneo cells, the immortalized human trophoblast cell line, and inhibited the expression of PCNA. In addition, avobenzone increased the activity of AKT and ERK1/2 in HTR8/SVneo cells. When LY294002 (AKT inhibitor) and U0126 (ERK1/2 inhibitor) were treated with avobenzone, the anti-proliferative effect of avobenzone was alleviated. Moreover, avobenzone promoted Ca^{2+} overload into the mitochondria and induced depolarization of the mitochondrial membrane. Expression of *IFI27*, which is located in the mitochondria, was elevated by avobenzone via inhibition of expression through siRNA transfection against *IFI27*, but did not alter cell properties. This study suggests that avobenzone induces mitochondrial dysfunction-mediated apoptosis leading to abnormal placentation during early pregnancy.

1. Introduction

Avobenzone (butyl methoxydibenzoylmethane) is an endocrine disruptor that directly binds to estrogen receptor β and acts as an estrogen agonist [1–3]. According to a survey in Switzerland, approximately 71% of personal care products contain avobenzone, which is the highest proportion among the various compositions [4]. The amount of avobenzone in personal care products is limited to 3% by The Food and Drug Administration (FDA) and 5% by Cosmetics Directive of the European Union, and it is toxic if products used frequently have concentrations of avobenzone greater than 5%. Avobenzone acts as an agonist or antagonist by reacting with various hormone receptors [5]. Additionally, avobenzone is found in underwater environments such as seawater swimming pools, making this potential toxin easily accessible to the human body [6]. In particular, under UV-irradiation and chlorination conditions, avobenzone produces a wide variety of eco-toxicant products which are much more reactive than avobenzone [7]. It is known to be toxic to aquatic organisms even at low concentrations [8]. Moreover, exposure of human immune cells to avobenzone promotes the release of inflammatory cytokines and reduces the viability of macrophages and monocytes at concentrations of 10 μg/mL (32 μM) [9]. However, little is known about the physiological activity of avobenzone in other types of human cells.

Human trophoblast cells form the placenta through a precisely controlled differentiation process during the early pregnancy of women. Routine exposure to various environmental factors is known to affect the viability, proliferation, and invasiveness of human trophoblast cells. The endocrine disruptor, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), induces apoptosis through mitochondrial dysfunction and reactive oxygen species (ROS) production in human trophoblast cells [10]. In addition, chemicals contained in personal care products and food additives such as propyl gallate, butyl paraben and decanoic acid can lead tomitochondrial defects and death of human trophoblast cells [11–13]. It is also known that pathways involving AKT or ERK1/2 signaling proteins are important for the survival and growth of human trophoblast cells, but it is unclear whether external environmental factors alter cell characteristics by modulating signal transduction pathways [14,15].

Therefore, the objectives of this study were to determine whether avobenzone affects the proliferation and death of human trophoblast HTR8/SVneo cells and whether AKT and ERK1/2 activities are regulated by avobenzone. We also measured mitochondrial membrane

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potential and mitochondrial Ca^{2+} concentration to analyze the effect of avobenzone on mitochondrial functions in HTR8/SVneo cells. Finally, we verified the expression of genes that may be targeted by avobenzone in HTR8/SVneo cells.

2. Materials and methods

2.1. Chemicals

Avobenzone was purchased from Selleckchem (Houston, TX, USA). Antibodies against phosphorylated AKT (Ser^{473}), P70S6 kinase (P70S6K, Thr^{421}/Ser^{424}), ribosomal protein 6 (S6, Ser^{235}/Ser^{236}), glycogen synthase kinase 3 beta (GSK3β, Ser^{9}), and extracellular signal-regulated protein kinase 1 and 2 (ERK1/2, Thr^{202}/Tyr^{204}), and total AKT, P70S6K, S6, GSK3β, and ERK1/2 were purchased from Cell Signaling Technology (Beverly, MA, USA). LY294002 was purchased from Cell Signaling Technology and U0126 was purchased from Enzo Life Science (Farmingdale, NY, USA).

2.2. Cell culture

HTR8/SVneo cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in RPMI-1640 with 2.05 mM L-glutamine (Cat No: SH30027.01, HyClone, Logan, UT, USA) with 5% fetal bovine serum 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 avobenzone. In each assay, dimethyl sulfoxide was used as a vehicle.

2.3. Proliferation assay

Proliferation assays were conducted using a Cell Proliferation ELISA BrdU kit (Cat No: 11647229001, Roche, Basel, Switzerland) according to the manufacturer's recommendations as described previously [16]. HTR8/SVneo cells were added in triplicate to a 96-well culture dish and treated with the following concentrations of avobenzone for 48 h: 0, 1, 2, 5, 10, 20, and 50 μM. Reaction products were quantified by measuring absorbance values at 370 and 492 nm using an enzyme-linked immunosorbent assay (ELISA) reader.

2.4. Immunofluorescence microscopy

The effects of avobenzone on the expression of proliferating cell nuclear antigen (PCNA) in HTR8/SVneo cells were determined by immunofluorescence microscopy as described previously [16]. Cells were treated with avobenzone (20 μM) for 24 h. Experiments were performed in triplicate. Images were captured using an LSM710 (Carl Zeiss, Oberkochen, Germany) confocal microscope fitted with a digital microscope Axio-Cam camera with Zen2009 software. Relative fluorescence intensity was measured by obtaining the green/blue ratio using MetaMorph software (Molecular Devices, Sunnyvale, CA, USA).

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

Induction of apoptosis in HTR8/SVneo cells by avobenzone was analyzed using a fluorescein isothiocyanate Annexin V apoptosis detection kit I (BD Biosciences, Franklin Lakes, NJ, USA) as described previously [16]. Cells were treated with different avobenzone concentrations (0, 5, 10, 20 and 50 μM) for 48 h. Fluorescence intensity was analyzed using a flow cytometer (BD Biosciences). Data are representative 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 avobenzone as described previously [16]. Cells were treated with avobenzone (20 μM) for 48 h, and 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 conducted in triplicate.

2.7. Western blot analysis

Western blot analysis of HTR8/SVneo cells treated with avobenzone was performed as described previously [16]. Multiple exposures of each western blot were performed to ensure linearity of chemiluminescent signals. Data are representative of three independent experiments.

2.8. JC-1 mitochondrial membrane potential assay

The JC-1 mitochondrial membrane potential was determined using a mitochondria staining kit (Cat No: CS0390, Sigma-Aldrich, St. Louis, MO, USA) as described previously [16]. Cells were treated with a range of avobenzone concentrations (0, 5, 10, 20, and 50 μM) for 48 h at 37 °C in a CO_2 incubator. Fluorescence intensity was analyzed using FACS-Calibur (BD Biosciences). Data are representative of three independent experiments.

2.9. Measurement of intracellular Ca^{2+} concentration

HTR8/SVneo cells (4×10^5 cells) were seeded onto 6-well plates, grown to 70–80% confluence, and incubated for 24 h in serum-free medium. Cells were then treated with avobenzone (0, 5, 10, 20, and 50 μM) for 48 h at 37 °C in a CO_2 incubator. Vehicle was added up to the highest treated dose. Supernatant was removed from culture dishes and adherent cells were detached with trypsin-EDTA. Cells were collected by centrifugation. For intracellular Ca^{2+} analysis, collected cells were resuspended using 3 μM Fluo-4 AM (Cat No: F14201, Invitrogen, Carlsbad, CA, USA) and incubated at 37 °C in a CO_2 incubator for 20 min. The stained cells were washed with phosphate-buffered saline. To determine mitochondrial Ca^{2+} levels, collected cells were resuspended using 3 μM Rhod-2 AM (Cat No: R1244, Invitrogen) and incubated at 4 °C for 30 min. The stained cells were washed with Hank's balanced salt solution. Fluorescent intensity was analyzed using a flow cytometer (BD Bioscience). Data are representative of three independent experiments.

2.10. siRNA knockdown experiment

For mRNA interference against *IFI27*, HTR8/SVneo cells (5×10^5 cells) were seeded in 6-well plates and transfected with nontargeting control siRNA (siCTR) (Cat No: SR30004, OriGene, Rockville, MD, USA), siRNA directed against IFI27 (siIFI27) (Cat No: 1072552, Bioneer, Daejeon, Korea) using transfection reagent Lipofectamine 2000 according to the manufacturer's instructions. Briefly, cells were cultured with siRNA and Lipofectamine 2000 diluted in Opti-MEM reduced serum medium (Cat No: 32985070, Gibco, Grand Island, NY, USA). After 6 h incubation at 37 °C in a CO_2 incubator, the media were removed and media were added containing 20 μM avobenzone or vehicle for 18 h at 37 °C.

2.11. Quantitative 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 [17]. Specific primers for *IFI27* (forward: 5'-TGT GAT TGG AGG AGT TGT GG-3'; reverse: 5'-CAT CAT CTT GGC TGC TAT GG-3'), *BTBD7* (forward: 5'-TTG GAA TGG AGG ACT CAA GG-3'; reverse: 5'-TCA AGG GAA TTT GGT GTT CC-3'), *IFI27L2* (forward: 5'-CTC TGT TGG GTC AGT GTT GG-3'; reverse: 5'-GTT TTG GAG GTT CAC CTT GG-3') and *SERPINA1* (forward: 5'-AAA GGC AAA TGG GAG AGA CC-3'; reverse: 5'-TCA TCA TAG GCA CCT TCA CG-3') were designed from sequences in the GenBank data base using Primer 3 (ver.4.0.0). All primers were synthesized by Bioneer Inc. (Daejeon, Korea). Using the standard curve method, we determined the expression levels of genes using the standard curves and CT values and normalized expression levels based on *GAPDH* expression. ROX dye (Invitrogen) was used to normalize variations in the well-to-well reaction volumes. Sequence-specific products were identified by generating a melting curve in which the CT value represented the cycle number at which a fluorescent signal was statistically greater than background, and relative gene expression was quantified using the $2^{-\Delta\Delta CT}$ method.

2.12. Statistical analysis

Data were subjected to analysis of variance according to the general linear model (PROC-GLM) of the SAS program (SAS Institute, Cary, NC, USA) to determine whether differences in response to avobenzone were significant. Differences with a probability value of $P < 0.05$ were considered statistically significant. Data are presented as the mean ± standard error of the mean unless otherwise stated.

3. Results

3.1. Avobenzone inhibits proliferative activities of human trophoblast cells

First, we performed a proliferation assay based on BrdU ELISA to determine whether avobenzone inhibits human trophoblast proliferation. HTR8/SVneo cells were treated with various concentrations (0, 1, 2, 5, 10, 20, and 50 μM) of avobenzone with 50 μM being the maximum concentration. A significant decrease in cell proliferation was observed at 5 μM (Fig. 1A). Avobenzone at 10 μM produced more than 50% inhibition of cell proliferation and 74.6% ($P < 0.001$) and 77.2% ($P < 0.001$) cell proliferation were observed at 20 and 50 μM, respectively. Next, we investigated the expression of PCNA, which plays a role in DNA clamping during cell proliferation, by immunofluorescence analysis (Fig. 1B). PCNA in HTR8/SVneo cells was not detected in response to avobenzone (20 μM). Fluorescence intensity decreased by approximately 61.7% ($P < 0.01$). These results indicate that avobenzone blocks the proliferation of human trophoblast cells.

3.2. Avobenzone induces the death of human trophoblast cells

Moreover, we investigated whether avobenzone can cause the death of human trophoblast cells. Flow cytometric analysis of apoptotic status was performed by Annexin V and PI staining (Fig. 2A). In the quadrant, the lower left (LL) shows normal cells, upper left (UL) shows necrotic cells, lower right (LR) shows early apoptotic cells, and upper right (UR) shows late apoptotic cells. Compared with the number of Annexin V-positive cells, indicating apoptotic cells, the apoptosis of HTR8/SVneo cells was dose-dependently increased by treatment with avobenzone. Apoptotic cells were increased 2.6-fold ($P < 0.001$), 4.7-fold ($P < 0.001$), and 5.3-fold ($P < 0.001$) by 10, 20, and 50 μM of avobenzone, respectively. Next, we analyzed DNA fragmentation, which is a typical feature of apoptosis, by conducting a TUNEL reaction (Fig. 2B). The TUNEL reaction was dynamically increased by approximately 2.4-fold by treatment of HTR8/SVneo cells with 20 μM avobenzone ($P < 0.05$). These results suggest that exposure of human trophoblast cells to avobenzone leads to apoptosis.

3.3. Avobenzone regulates AKT and ERK1/2 pathways in human trophoblast cells

To estimate the effect of avobenzone on the AKT and ERK1/2 signaling pathways, alterations in the phosphorylation in HTR8/SVneo cells were time-dependently examined. AKT phosphorylation peaked at 5 min after avobenzone treatment, and then gradually decreased and then increased again at 120 min (Fig. 3A). Phosphorylation of GSK3β and P70S6K, which are regulated by AKT, also peaked at 5 min and gradually decreased by 120 min, although GSK3β phosphorylation slightly increased at 60 min (Fig. 3B and C). Phosphorylation of S6, a downstream protein regulated by P70S6K, peaked at 15 min, which is slightly slower than that of other kinases, and decreased to 120 min compared to controls, as did P70S6K (Fig. 3D). The activity of ERK1/2 also peaked at 5 min following treatment with avobenzone and then gradually decreased to 120 min, which was much lower than the control (Fig. 3E). To investigate whether the activity of avobenzone-regulated signaling transduction proteins regulates the proliferation of HTR8/SVneo cells, LY294002 (20 μM) and U0126 (20 μM), inhibitors of AKT and ERK1/2 were treated with avobenzone to determine cell proliferation levels (Fig. 3F). As a result, cell proliferation decreased by avobenzone (50 μM) was restored by LY294002 and U0126. These results suggest that the AKT and ERK1/2 pathways regulated by avobenzone in human trophoblast cells are involved in regulating cell proliferation.

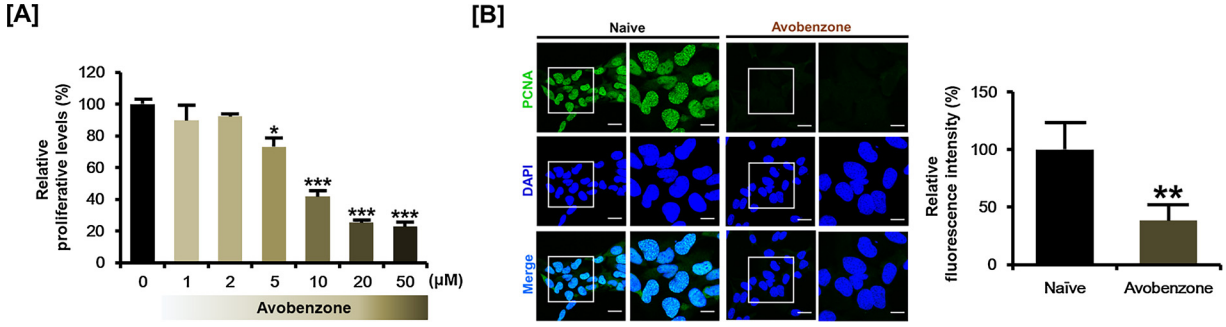

Fig. 1. Effects of avobenzone on proliferation of HTR8/SVneo cells. [A] Dose-dependent effects of avobenzone on the proliferation of HTR8/SVneo cells were determined and the data are presented as percentages relative to vehicle (100%). Cells were treated with avobenzone (0, 1, 2, 5, 10, 20, and 50 μM) for 48 h in triplicate. [B] PCNA protein was detected (green) and nuclei were counterstained with DAPI (blue) in HTR8/SVneo cells treated with 20 μM avobenzone for 24 h. Data are representatives of three independent experiments. Asterisks indicate a significant effect of treatment (***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$). *Scale bar* represents 40 μm (first and third vertical panels) and 20 μm (second and fourth vertical panels) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

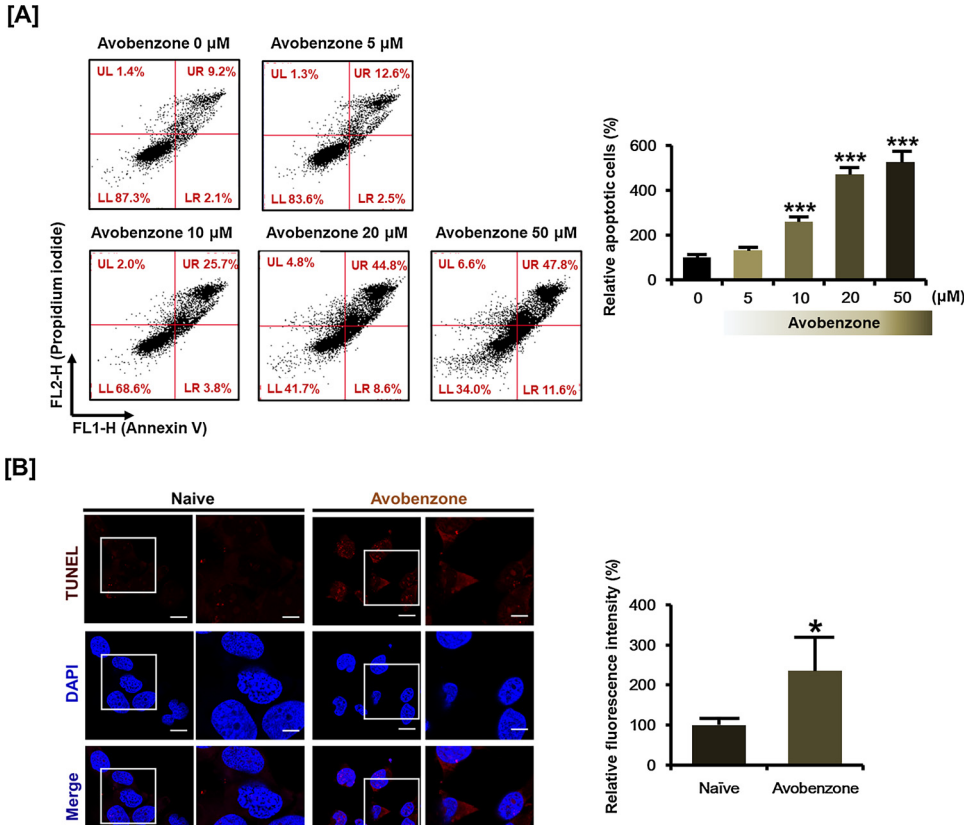

Fig. 2. Avobenzone induces apoptosis in HTR8/SVneo cells. [A] Flow cytometric detection of apoptosis in HTR8/SVneo cells in response to avobenzone. Annexin V and propidium iodide (PI) fluorescence values were estimated at various concentrations of avobenzone (0, 5, 10, 20, and 50 μM) *via* flow cytometry. The percentages of apoptotic cells (upper right and lower right quadrants) were compared to those for control cells. Data are representative of three independent experiments. [B] TUNEL fluorescence identified apoptotic cells (red), and nuclei were counterstained with DAPI (blue). Cells were treated with 20 μM avobenzone. Data are representative of three independent experiments. The asterisks indicate significance (***$P < 0.001$ and *$P < 0.05$). The *scale bar* represents 40 μm (first and third vertical panels) and 20 μm (second and fourth vertical panels) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

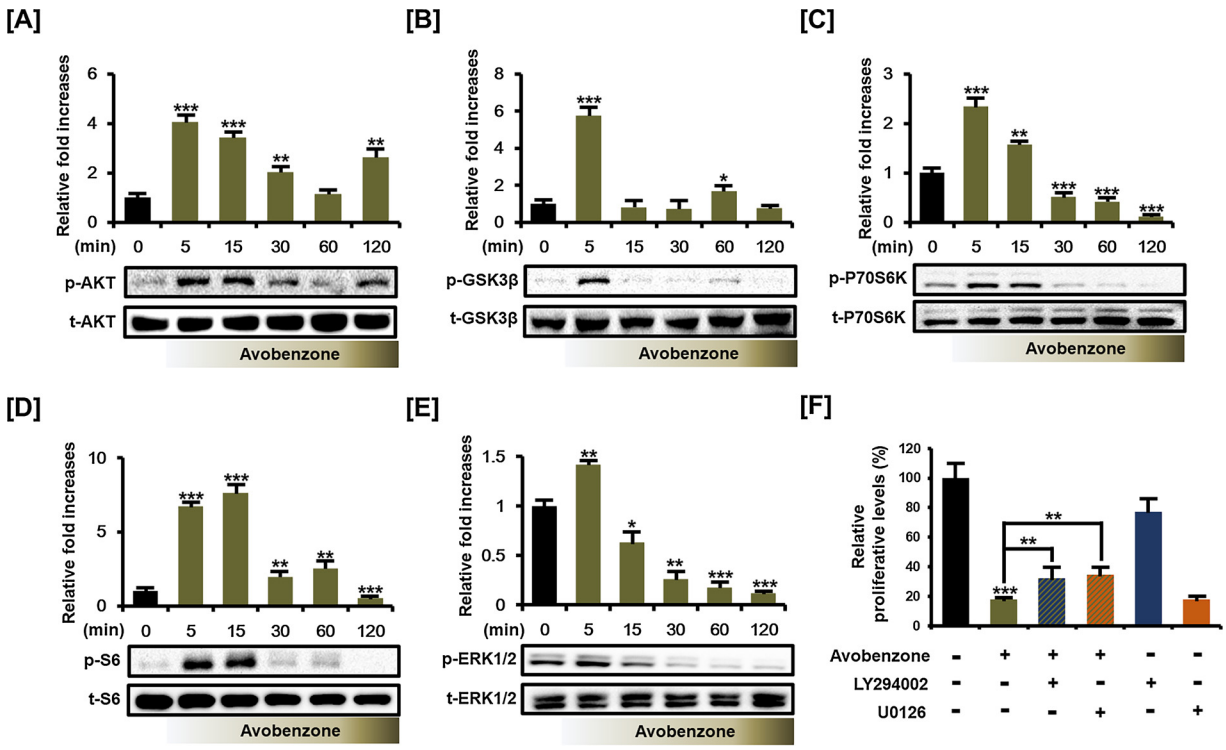

Fig. 3. Avobenzone time-dependently affects phosphorylation of protein kinases in HTR8/SVneo cells. [A–E] Time-dependent phosphorylation of AKT [A], P70S6K [B], S6 [C], GSK3β [D], and ERK1/2 [E] in response to 20 μM avobenzone. Immunoblots were captured to calculate normalized values by estimating the abundances of phosphorylated proteins relative to total proteins. Data are representative of three independent experiments. [F] The effects of avobenzone (20 μM) alone or with selective inhibitors against AKT (LY294002, 20 μM) and ERK1/2 (U0126, 20 μM) on proliferation of HTR8/SVneo cells. Asterisks indicate significant differences compared to control cells (***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$).

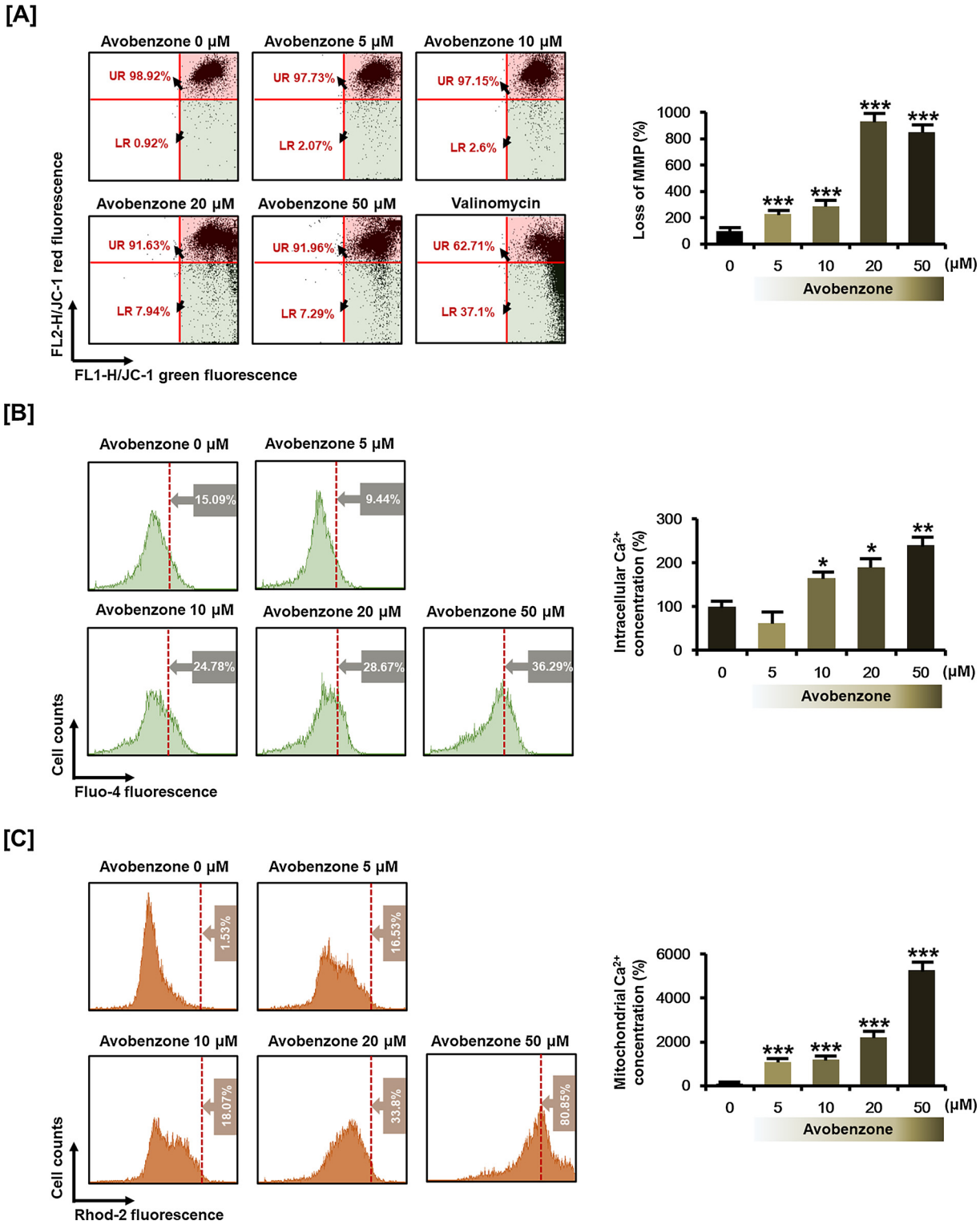

Fig. 4. Avobenzone induces mitochondrial disruption and mitochondrial Ca^{2+} overload in HTR8/SVneo cells. [A] Alterations in mitochondrial membrane potential (MMP) in response to avobenzone (0, 5, 10, 20, and 50 μM) for 48 h in HTR8/SVneo cells were detected by flow cytometry, and JC-1 staining levels were quantified as the relative ratio of lower right/upper right quadrants. Valinomycin (1 μM) was used as a positive control. Data are representative of three independent experiments. [B] Flow cytometric detection of intracellular Ca^{2+} in response to avobenzone in a dose-dependent manner (0, 5, 10, 20, and 50 μM) for 48 h using Fluo-4 staining analysis. Data are representative of three independent experiments. [C] Rhod-2 fluorescence in response to avobenzone (0, 5, 10, 20, and 50 μM) for 48 h was identified *via* flow cytometry in HTR8/SVneo cells to detect mitochondrial Ca^{2+} concentrations. Data are representative of three independent experiments. The asterisks indicate significance (***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$).

3.4. Avobenzone induces mitochondrial membrane disruption and promotes mitochondrial Ca^{2+} overload

A typical pathway for apoptosis involves mitochondria disruption, preventing its function. We investigated changes in matrix metalloprotease (MMP) loss in HTR8/SVneo cells in response to avobenzone (Fig. 4A). The ratio of low right (LR), which are cells with MMP loss, was dose-dependently increased in avobenzone. Additionally, 20 and 50 μM avobenzone increased MMP loss by 9.3-fold ($P < 0.001$) and 8.5-fold ($P < 0.001$), respectively, compared to the control. Excessive Ca^{2+} influx into the mitochondria may have negative effects. We performed flowcytometric analysis of intracellular- and mitochondrial-specific Ca^{2+} concentrations. Intracellular Ca^{2+} concentrations were increased by 1.9-fold ($P < 0.05$) and 2.4-fold ($P < 0.01$) by 20 and 50 μM avobenzone, respectively (Fig. 4B). The concentration of Ca^{2+} in the mitochondria was more sensitive and increased by 22.1-fold ($P < 0.001$) and 52.8-fold ($P < 0.001$), respectively, by 20 and 50 μM of avobenzone (Fig. 4C). These results suggest that mitochondrial collapse because of high Ca^{2+} concentration in the cells may be a major mechanism of the cell death of human trophoblasts.

3.5. Avobenzone regulates IFI27 mRNA expression and target genes for enhancer targeting IFI27

We attempted to identify genes targeted by avobenzone in human trophoblast cells. Interferon alpha inducible protein 27 (*IFI27*) is a gene induced by interferon family proteins, which is mainly expressed in the mitochondria at the protein level. Transient expression of IFI27 decreases cell viability and MMP, leading to apoptotic cell death [18]. Furthermore, *IFI27* is the most highly expressed gene in extravillous trophoblast cells, represented by HTR8/SVneo cells, compared to villous trophoblast cells, suggesting that it may be important for physiological functions of trophoblast cells [19]. In this study, avobenzone increased the mRNA expression of *IFI27* (Fig. 5A). We investigated the expression of genes associated with IFI27 after interference with *IFI27* mRNA. Based on the GeneHancer database in GeneCards, we selected genes targeted by the enhancer of the *IFI27* gene [20]. Next, we transfected siIFI27 and confirmed the mRNA expression of selected genes. As a result, the expression of BTB domain containing 7 (*BTBD7*) was reduced by avobenzone and reduced a greater amount following transfection with siIFI27 (Fig. 5B). Expression of interferon alpha inducible protein 27 like 2 (*IFI27L2*), which is also a paralog of *IFI27*, was significantly increased by avobenzone (Fig. 5C). However, siIFI27 did not significantly differentiate the expression of *IFI27L2* from siCTR transfection. Serpin family A member 1 (*SERPINA1*) expression was not significantly different following avobenzone treatment or siIFI27 transfection (Fig. 5D). These results suggest that the expression of *IFI27* is increased by avobenzone in human trophoblast cells and the expression of genes that are targets of the same enhancer as *IFI27* are also regulated by avobenzone treatment or *IFI27* regulation.

4. Discussion

In present study, we found that avobenzone suppresses proliferation and induces apoptosis in human trophoblast cells. Moreover, avobenzone modulated the AKT and ERK1/2 signaling pathways. Furthermore, avobenzone induced depolarization of the mitochondrial membrane and elevated intracellular and mitochondrial Ca^{2+} concentrations in HTR8/SVneo cells as illustrated in Fig. 6. Finally, we found that avobenzone increased the expression of *IFI27* and that expression of genes linked to enhancer to IFI27 is regulated by avobenzone.

Because human trophoblast cells form the placenta during early pregnancy, proper molecular and genetic control should be maintained [21]. Therefore, if the characteristics of human trophoblast cells are altered extensively by exogenous factors, the trophoblast cells do not function normally. Next, disproportionate placenta formation is detrimental to fetal health. In addition, trophoblast cells are also affected by molecules that mimic the function of various hormones and disturb the endocrine system of the human body. Recent studies showed that avobenzone acts as an androgen antagonist, a glucocorticoid agonist, and a thyroid hormone antagonist [5]. A previous study demonstrated that endocrine disruptors such as bisphenol A and TCDD induce human trophoblast cell death [10,22]. Avobenzone is widely used in personal

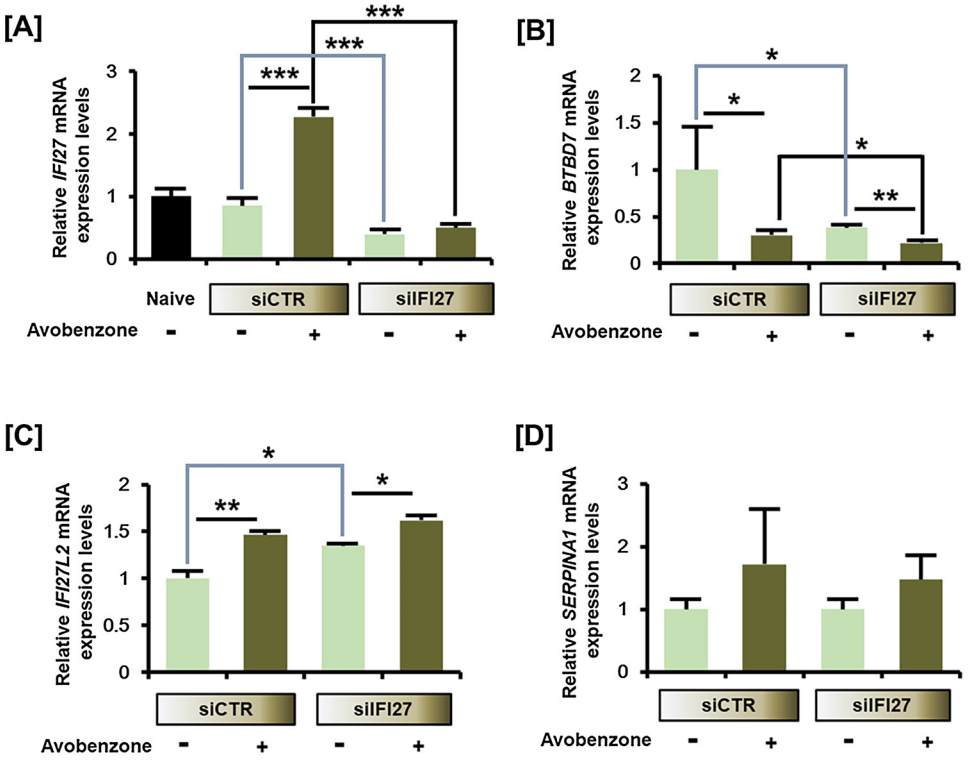

Fig. 5. Avobenzone regulates expression of *IFI27* mRNA and its 'enhancer-gene' network-related genes. [A] Expression of mRNAs for *IFI27* was estimated for siRNA transfected HTR8/SVneo cells by quantitative RT-PCR analyses. [B–D] Expression of mRNAs for IFI27-related genes was estimated for non-targeting control siRNA (siCTR)- or siIFI27-transfected HTR8/SVneo cells in the presence or absence of avobenzone (20 μM). Quantitative RT-PCR analyses were performed for *BTBD7* [B], *IFI27L2* [C], and *SERPINA1* [D]. All genes were analyzed in triplicate. Asterisks indicate an effect of treatment (***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$).

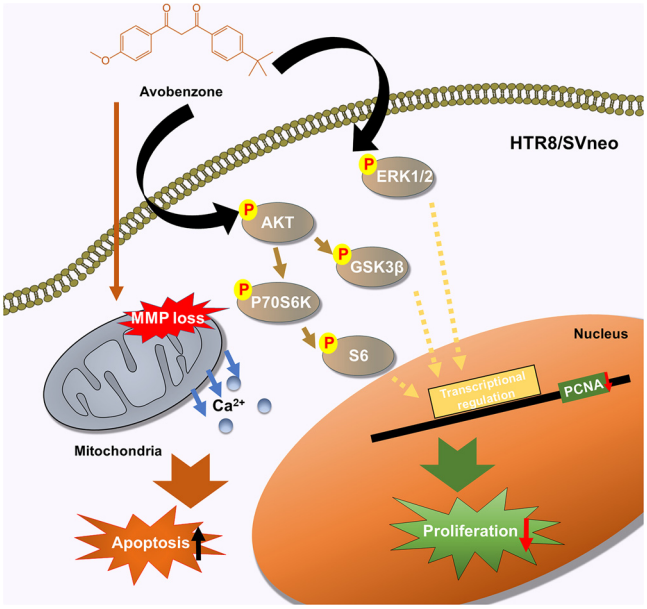

Fig. 6. Schematic illustrating the current working hypothesis regarding the effects of avobenzone on human trophoblast cells. Avobenzone stimulates AKT and ERK1/2 signal transduction proteins and likely undergoes transcriptional regulation. Avobenzone also inhibits PCNA expression and cell proliferation. Moreover, avobenzone damages the mitochondria and is involved in the excessive uptake of Ca^{2+} by cells. Avobenzone eventually induces mitochondrial dysfunction that causes apoptosis of human trophoblast cells.

care products, but its cytotoxicity has been reported only in immune cells [9]. Avobenzone has been detected in fish such as crucian carp, which can be consumed as foods, or in seawater swimming pools, but few studies have examined the intracellular physiological activity of avobenzone [6,23]. We demonstrated that avobenzone increases DNA fragmentation and induces apoptosis in human trophoblast cells, as well as controls the activation of AKT signaling protein. This is the first report on the mechanism underlying the regulation of the intracellular signaling pathway by avobenzone. We then measured cell proliferation using selective inhibitors of AKT and ERK1/2. Interestingly, inhibition of AKT and ERK1/2 activity alleviated the anti-proliferative effect of avobenzone. This suggests that avobenzone-activated AKT and ERK1/2 proteins may inhibit cell proliferation, although previous studies suggested that both signaling pathways are required for the growth of trophoblast cells. In our previous study, we found that chrysophanol, which activates AKT and ERK1/2, reduces viability of trophoblast cells [16]. This is probably due to increases in oxidative stress induced by activation of ERK1/2. The effects of avobenzone-regulated signaling pathways on cell properties require further analysis.

Mitochondria play an important role not only in ATP production but also in ROS generation, Ca^{2+} signaling, and differentiation of various cell types. Because the proliferation and invasiveness of trophoblast cells change with the differentiation process of human trophoblast cells, fine regulation of trophoblast differentiation is essential for normal invasion into the maternal endometrium [24]. Human trophoblast cells differentiate into extravillous trophoblasts with invasive properties and villous trophoblast with proliferative properties. Morphological and functional changes in the mitochondria occur during the differentiation of trophoblast cells [25]. Dysfunction of human trophoblast cells because of abnormal differentiation leads to pregnancy-related diseases such as preeclampsia and intrauterine growth retardation [26]. Excessive oxidative stress and a lack of ATP occur in these diseases. Thus, mitochondria dysfunction in human trophoblast cells may adversely affect placenta formation. In our previous study, one mechanism causing human trophoblast cell death is disruption of the mitochondria caused by excessive Ca^{2+} influx [27]. External stimuli and excessive release of Ca^{2+} from the endoplasmic reticulum cause Ca^{2+} to enter the mitochondria to a damaging level. We verified that avobenzone not only disrupts mitochondrial membranes, but also promotes mitochondrial Ca^{2+} overload. Thus, the apoptotic mechanism induced by avobenzone may be mediated by mitochondrial dysfunction. Further studies using chemicals such as Ca^{2+} chelators are necessary to determine whether mitochondrial Ca^{2+} overload mitigates the apoptosis of human trophoblast cells.

IFI27 is a type 1 interferon-inducible gene that is a member of the interferon-stimulated genes family, which includes proteins mainly expressed in the mitochondria. IFI27 is known to have a pro-apoptotic function [28]. Transient expression of *IFI27* increases the susceptibility to DNA damage-induced apoptosis by altering mitochondrial function [18]. Moreover, inhibition of *IFI27* expression using siRNAs has been reported to mitigate etoposide-induced apoptosis. However, no studies have examined the regulation or function of IFI27 in human trophoblast cells. As the expression of IFI27 increases with avobenzone treatment, we hypothesized that avobenzone-induced apoptosis mediates IFI27 located in the mitochondria in human trophoblast cells. However, when we suppressed *IFI27* expression in HTR8/SVneo cells, *IFI27* expression did not affect cell death, regardless of the presence of avobenzone (data not shown). We examined the large database-based enhancer-gene relationship and identified changes in the expression of genes targeted by enhancers that regulate *IFI27* expression after *IFI27* knockdown and avobenzone treatment [20]. The function of IFI27 in the mitochondria of trophoblast cells requires further study. If the target gene is discovered such that avobenzone can induce changes in cell properties such as proliferation and apoptosis, further studies of the genetic mechanism of avobenzone-mediated cytotoxic effects can be conducted.

In this study, we found that avobenzone inhibits the proliferation of human trophoblast cells and induces apoptosis. Because few studies of the toxicity of avobenzone in human cells have been conducted, our results provide a foundation for studies examining the cytotoxicity of avobenzone in other human cell types. Additionally, it remains unclear whether avobenzone is associated with changes in cell characteristics, but studies of the signaling pathways and genes regulated by avobenzone will lead to further analysis of the genetic regulation mechanisms of avobenzone. Because avobenzone is widely used in personal care products, further studies of its effects are necessary to ensure the health of the mother and fetus during early pregnancy.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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