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Trichlorfon inhibits proliferation and promotes apoptosis of porcine trophectoderm and uterine luminal epithelial cells☆

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ABSTRACT

Trichlorfon is an organophosphate insecticide widely used in agriculture. Additionally, it is applied to pigs for control of endo- and ectoparasites. Previous studies have shown the effects of trichlorfon in pigs during late stages of gestation; however, little is known about its effects during early pregnancy, including implantation and placentation. We investigated whether trichlorfon affects proliferation and apoptosis of porcine trophectoderm (pTr) and uterine luminal epithelial (pLE) cells. Trichlorfon inhibited the proliferation of pTr and pLE cells, as evidenced by cell cycle arrest, and altered the expression of proliferation-related proteins. In addition, trichlorfon induced cell death and apoptotic features, such as loss of mitochondrial membrane potential and DNA fragmentation, in pTr and pLE cells. Moreover, trichlorfon treatment decreased concentrations of Ca^{2+} in the cytoplasm in both cell lines and increased concentrations of Ca^{2+} in mitochondria of pTr cells. Trichlorfon inhibited the activation of phosphoinositide 3-kinase/AKT and mitogen-activated protein kinase signaling pathways in pTr and pLE cells. Therefore, we suggest that trichlorfon-treated pTr and pLE cells exhibited abnormal cell physiology which might lead to early pregnancy failure.

1. Introduction

Exposure to insecticides during pregnancy in humans and livestock disrupts development of the fetus and myometrial contractions required for parturition (Mayhoub et al., 2014; Perez et al., 2009; Wrobel et al., 2015). Based on epidemiological studies, maternal exposure to the insecticide from one's occupation, environment or diet leads to fetal growth restriction (Mayhoub et al., 2014). In addition, the exposure to insecticides such as aldrin and dieldrin suppresses oxytocin-induced myometrial contraction in cows (Wrobel et al., 2015). Also, exposure of fertilized quail eggs during incubation to dichlorvos, an organophosphate insecticide, induces teratogenicity in the embryos (Lutz-Ostertag and Bruel, 1981). Among the insecticides, trichlorfon has been used widely as an organophosphate insecticide in agriculture and aquaculture since 1952. It has also been used in veterinary medicine as an ecto- and endopesticide in pigs. Trichlorfon residues in animal-derived food have become an important issue owing to its extensive use (Wang et al., 2010). Furthermore, extensive studies of trichlorfon have shown that its toxicity extends beyond insects to non-targeted species (Hjelde et al., 1998; Tian et al., 2011; Xu et al., 2012). Piglets from sows exposed to trichlorfon during mid- and late-stages of gestation showed nervous disorders, including ataxia and locomotor disturbances (Knox et al., 1978; Pope et al., 1986). Moreover, administration of trichlorfon during the gestational period involving active brain development resulted in disturbance of development of the cerebellum in fetal guinea pigs (Mehl et al., 2007). In addition, trichlorfon causes direct damage to the fetus *via* transplacental transfer from mother to fetus in guinea pigs (Berge and Nafstad, 1986). These reports clearly suggest that the mammalian reproductive tract can be exposed to trichlorfon. However, to the best of our knowledge, the effects of trichlorfon during early pregnancy in pigs have not been studied.

In pigs, implantation of conceptus starts on about gestational day 13, and similar to other mammals, conceptus-endometrial interactions are required for successful establishment of pregnancy (Dantzer, 1985; Perry et al., 1976). Bidirectional communication

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between the maternal uterine tissue and conceptus is mediated by various factors, including growth factors, hormones, and cytokines (Bazer and Johnson, 2014; Morawska-Pucinska et al., 2014). Moreover, both maternal uterine endometrial tissue and extra-embryonic conceptus membranes develop into components of the placenta in pigs, whereas blastocysts of rodents and primates penetrate into the uterine endometrium during the initial stages of implantation and placentation (Geisert et al., 2015). Furthermore, most embryonic death loss takes place during the peri-implantation period of pregnancy (Spencer and Bazer, 2004). Therefore, the interaction between and proliferative properties of the trophoblast and uterine endometrial cells should be well-controlled for successful establishment and maintenance of pregnancy.

In this study, we investigated whether trichlorfon could affect the viability of porcine trophectoderm (pTr) and uterine luminal epithelial (pLE) cells. First, we examined the effects of trichlorfon on proliferation and programmed cell death in both cell lines. Next, we investigated its effects on cell cycle distribution and expression of related proteins, including proliferating cell nuclear antigen (PCNA) and cyclin D1, in pTr and pLE cells. We also verified whether DNA fragmentation and mitochondrial membrane potential ($\Delta\psi_m$) disruption, which are features of apoptotic cell death, were induced in pTr and pLE cells by trichlorfon. Finally, we determined the effects of trichlorfon on phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) and mitogen-activated protein kinase (MAPK) cell signaling pathways in pTr and pLE cells.

2. Material and methods

2.1. Chemicals

Trichlorfon was purchased from Sigma-Aldrich, Inc. (98.4% purity; St. Louis, MO, USA). Inhibitors for ERK1/2 (U0126, catalog number: EI282), p38 (SB203580, catalog number: EI286), and JNK (SP600125, catalog number: EI305) were purchased from Enzo Life Sciences Inc. (Farmingdale, NY, USA), and the PI3K/AKT inhibitor (Wortmannin, catalog number: 9951) was purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Antibodies against phosphorylated AKT (Ser^{473}), ERK1/2 (Thr^{202}/Tyr^{204}), P70S6K (Thr^{421}/Ser^{424}), P90RSK (Thr^{573}), S6 (Ser^{235}/Ser^{236}), c-Jun N-terminal kinase (JNK; Thr^{183}/Tyr^{185}), p38 (Thr^{180}/Tyr^{182}), and cyclin D1 (Thr^{286}), as well as total AKT, ERK1/2, P70S6K, P90RSK, S6, JNK, p38, and cyclin D1 were also purchased from Cell Signaling Technology.

2.2. Cell culture

Previously established (Ka et al., 2001; Wang et al., 2000) mononuclear pTr cells from day 12 pig conceptuses and pLE cells from day 12 pregnant gilts were used in the present *in vitro* studies. Briefly, monolayer cultures of pLE cells were grown to 80% confluence in Dulbecco's modified Eagle's medium (DMEM)/F12 1:1 culture medium containing 20% fetal bovine serum (FBS) in 100 mm tissue culture dishes. Monolayer cultures of pTr cells were grown to 80% confluence in DMEM/F12 1:1 medium containing 10% FBS. For assays, *in vitro*–cultured pLE and pTr cells were serum-starved for 24 h and then subjected to various treatments.

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. The pTr and pLE cells were seeded in a 96-well plate, and then incubated for 24 h in serum-free DMEM/F12 1:1 medium. Next, pTr and pLE cells were treated with 0, 5, 10, 20, 50, and 100 μM trichlorfon in a final volume of 100 μL/well, and incubated for 48 h. Also, pTr and pLE cells were simultaneously treated with wortmannin (1 μM), U0126 (5 μM), SP600125 (5 μM) and SB203580 (20 μM) with or without trichlorfon (100 μM) in a final volume of 100 μL/well, and incubated for 48 h. After 48 h of incubation, we analyzed relative amounts of BrdU incorporation into newly synthesized cellular DNA by measuring the absorbance at 370 and 492 nm. These experiments were performed in triplicate.

2.4. Immunofluorescence microscopy

The effects of trichlorfon on the expression of proliferating cell nuclear antigen (PCNA) in pTr and pLE cells were determined using immunofluorescence microscopy. pTr and pLE cells were seeded on confocal dishes (Cat No: 100350, SPL Life Science, Republic of Korea), and incubated for 24 h in serum-free medium. After trichlorfon (100 μM) treatment for 24 h, cells were probed with mouse monoclonal anti-human PCNA antibody (Cat No. sc-56, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted to 1:100 (2 μg/mL). Next, pTr and pLE cells were incubated with goat anti-mouse mouse IgG Alexa 488 (Cat No. A-11001, Invitrogen, Carlsbad, CA, USA), and then stained with 4′,6-diamidino-2-phenylindole (DAPI) (Cat No. D8417, Sigma). Fluorescence of Alexa 488 and DAPI was detected using a LSM710 (Carl Zeiss, Thornwood, NY, USA) confocal microscope. These experiments were performed in triplicate.

2.5. Cell cycle analysis

Cell cycle analyses were performed using serum-starved pTr and pLE cells treated with trichlorfon in a dose-dependent manner (0, 20, 50, and 100 μM) for 48 h. After treatment, the cells were fixed in 70% ethanol. Next, RNase A (Sigma) was used to treat the cells at a final concentration of 100 μg/mL, and then cells were stained with propidium iodide (PI; BD Biosciences, Franklin Lakes, NJ, USA). Fluorescence intensity of PI was detected using a FACS Calibur (BD Biosciences). These experiments were performed in triplicate.

2.6. Apoptosis assay by annexin V and propidium iodide (PI) staining

Induction of apoptosis in pTr and pLE cells by trichlorfon was analyzed using a fluorescein isothiocyanate Annexin V apoptosis detection kit I (BD Biosciences). The cells were seeded in a 6-well plate and incubated in serum-free medium for 24 h before treatment with trichlorfon. Next, pTr and pLE cells were treated with trichlorfon in a dose-dependent manner (0, 20, 50, and 100 μM) and incubated for 48 h. Then, the cells were collected and incubated with FITC Annexin V and PI at room temperature. Fluorescence intensity of FITC Annexin V and PI was analyzed using a flow cytometer. These experiments were performed in triplicate.

2.7. TUNEL assay

The TUNEL (terminal deoxynucleotidyl transferase dNTP nick end labeling) assay was performed on pTr and pLE cells treated with 100 μM trichlorfon for 48 h using the In Situ Cell Death Detection kit, TMR red (Roche). The cells were incubated in serum-free medium for 24 h, and then exposed to 100 μM trichlorfon for 48 h at 37 °C in a CO_2 incubator. Next, the cells were fixed with 4% paraformaldehyde in PBS and then incubated with the TUNEL staining mixture as described previously (Yang et al., 2017, 2018). After DAPI staining, cells were observed under fluorescence microscopy. These experiments were performed in triplicate.