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Pro-apoptotic Effect of Pifithrin-α on Preimplantation Porcine *In vitro* Fertilized Embryo Development

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ABSTRACT: The aim of this study was to investigate the impact of a reported p53 inhibitor, pifithrin-α (PFT-α), on preimplantation porcine *in vitro* fertilized (IVF) embryo development in culture. Treatment of PFT-α was administered at both early (0 to 48 hpi), and later stages (48 to 168 hpi) of preimplantation development, and its impact upon the expression of five genes related to apoptosis (*p53*, *bak*, *bcl-xL*, *p66Shc* and *caspase3*), was assessed in resulting d 7 blastocysts, using real-time quantitative PCR. Total cell numbers, along with the number of apoptotic nuclei, as detected by the *in situ* cell death detection assay, were also calculated on d 7 in treated and non-treated control embryos. The results indicate that PFT-α, when administered at both early and later stages of porcine IVF embryo development, increases the incidence of apoptosis in resulting blastocysts. When administered at early cleavage stages, PFT-α treatment was shown to reduce the developmental competence of porcine IVF embryos, as well as reducing the quality of resulting blastocysts in terms of overall cell numbers. In contrast, at later stages, PFT-α administration resulted in marginally increased blastocyst development rates amongst treated embryos, but did not affect cell numbers. However, PFT-α treatment induced apoptosis and apoptotic related gene expression, in all treated embryos, irrespective of the timing of treatment. Our results indicate that PFT-α may severely compromise the developmental potential of porcine IVF embryos, and is a potent apoptotic agent when placed into porcine embryo culture media. Thus, caution should be exercised when using PFT-α as a specific inhibitor of p53 mediated apoptosis, in the context of porcine IVF embryo culture systems. (**Key Words:** Apoptosis, *In vitro* Fertilization, Pifithrin-α, Blastocyst, Preimplantation Embryo, Pig)

INTRODUCTION

The *in vitro* fertilization (IVF) of *in vitro* matured (IVM) porcine oocytes is a popular and inexpensive method for generating viable embryos in the porcine species, and is routinely employed as a research tool in many laboratories (Lee et al., 2010). However, porcine IVF embryos exhibit delayed development, reduced total cell numbers, fewer cells in the inner cell mass (ICM), and a greater degree of fragmentation in comparison to *in vivo* counterparts (Hao et al., 2008; Mateusen et al., 2005). Various studies have been conducted in order to underline the stressors experienced by *in vitro* produced (IVP) embryos in culture (Hardy et al., 1989; Favetta et al., 2007), often with the intention of modifying existing culture conditions in order to alleviate the impacts of stress and improve the quality of resulting embryos (Choi et al., 2008; Abdelrazik et al., 2009; Lee et al., 2010). These studies are essential if we are to improve IVP embryo quality but it is also of fundamental importance to investigate the underlying mechanisms by which embryos respond to stress (Brison, 2000). One measurable outcome of embryo stress in culture is an increase in apoptosis (Jurisicova et al., 1998).

Extensive apoptosis is generally assumed to be incompatible with successful embryo development (Hao et al., 2003), and controlling apoptosis has been identified as a key factor in determining the successful outcome of IVF (Jurisicova 1998; Brison, 2000). Despite this, there remains a lack of knowledge regarding the specific cellular signalling mechanisms that govern apoptosis in the developing embryo, and how embryonic programmed cell death (PCD) is carried out and controlled (Isom et al., 2007). Studies in humans and in the mouse have revealed a distinct relationship between *in vitro* culture and the expression of the tumor suppressor protein 53 (p53), particularly in

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embryos susceptible to the stressors of culture (Chandrakanthan et al., 2006). The removal of the negative regulator of p53, *mdm2*, results in early embryonic lethality in a mouse knockout model (Jones et al., 1995). This embryonic loss is reversible upon simultaneous deletion of *p53* (Luna et al., 1995), demonstrating that the *p53* response pathway plays a direct role in embryonic loss in the mouse.

Furthermore, hyperglycemia induced cell death in mouse blastocysts is dependent upon *p53* expression (Keim et al., 2001). Higher levels of *p53* mRNA transcripts have also been associated with greater incidences of embryo fragmentation in human IVF embryos, and p53 protein levels, as revealed by immunostaining procedures, are elevated in human embryos that have undergone cryopreservation (Chandrakanthan et al., 2007). All of these lines of evidence outline a prominent role for p53 during the stress response of embryos in culture, and point to p53 and its associated signalling pathways as good candidates for further study into the molecular mechanisms by which embryos deal with adversity.

Pifithrin-α (PFT-α), is a stable water soluble chemical with a molecular weight of 367, it was named PFT-α (an abbreviation of p-fifty three inhibitor-α), for its ability to reduce the transactivation of p53 responsive genes (Komarov et al., 1999). This compound has been shown to abrogate the p53 related apoptosis pathway by altering p53 related gene expression (Kelly et al., 2003), however the exact mechanism of action of PFT-α remains unclear (Walton et al., 2005). It has even been shown that PFT-α treatment, may in fact activate p53 mediated apoptosis in various murine cancer cell lines (Kaji et all, 2003), demonstrating the need for caution when using this compound as a specific inhibitor of p53 *in vitro*.

Therefore, the primary aim of this study was to investigate the effects of PFT-α, a reported p53 specific inhibitor (Komarov et al., 1999; 2003), on the developmental ability and quality of IVF porcine blastocysts during culture. In this study, we assessed the developmental competence of porcine IVF embryos that were cultured in the presence of PFT-α for 48 h post insemination (hpi), as well as normal embryos that were selected 48 hpi and then cultured with PFT-α up to the blastocyst stage (48 to 168 hpi). We also assessed the impact of PFT-α treatment upon the incidence of apoptosis and apoptotic related gene expression in resulting blastocysts. Our results indicate that PFT-α severely compromises the developmental competence of porcine IVF embryos when administered at both early (0 to 48 hpi), and at later stages (48 to 168 hpi) of development. In the porcine IVF system, PFT-α acts as a pro-apoptotic stimulant, inducing increased apoptosis and apoptotic related gene expression in PFT-α treated blastocysts, irrespective of the timing of treatment. These findings demonstrate that caution should be exercised when using this compound as an inhibitor of apoptosis, p53-related or otherwise, in the porcine model.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents were purchased from the Sigma Aldrich Corp (St. Louis, MO, USA) unless otherwise stated. This study was conducted in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching, published by the Consortium for Developing a Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching, 3rd ed., 2010.

Recovery of oocytes and *in vitro* maturation

Ovaries were retrieved from pre-pubertal gilts at a local slaughterhouse and transported to the laboratory in physiological saline at 30 to 38°C, within 2 h of extraction. The follicular fluid and cumulus-oocyte complexes (COCs) within follicles 3 to 6 mm in diameter were aspirated using an 18-gauge needle. Compact COCs were selected and cultured in tissue culture medium (TCM-199; Life Technologies, Rockville, MD, USA), supplemented with 10 ng/ml epidermal growth factor (EGF), 4 IU/ml equine chorionic gonadotropin (eCG) (Intervet, Boxmeer, The Netherlands), human chorionic gonadotropin (hCG) (Intervet) and 10% (v/v) porcine follicular fluid (pFF).

The pFF was aspirated from prepubertal gilt ovaries, with only ovarian follicles 3 to 7 mm in diameter utilized for this purpose. After centrifugation at 300×*g* for 30 min, supernatants were collected and filtered using 0.20 μm syringe filters (Gelman Sciences, Ann Arbor, MI, USA). Prepared pFF was stored at -20°C until use. Each group of 50 COCs was cultured in TCM-199 and incubated at 38°C in a humidified atmosphere of 5% CO_2 and 95% air. After culturing for 22 h, COCs were cultured in eCG- and hCG-free TCM-199 medium for a further 22 h.

In vitro fertilization

Fresh boar semen was collected for all experiments and was stored at a temperature below 8°C. Prior to use, sperm samples were diluted with Dulbecco's PBS (Life Technologies) supplemented with 0.1% bovine serum albumen (BSA) and centrifuged twice. The sperm pellet was re-suspended in modified Tris-buffered medium (mTBM) containing 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2 \cdot 2H_2O$, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate, and 0.1% (w/v) BSA to give a concentration of 2 $\times 10^7$ sperm/ml. Following 42 h of *in vitro* maturation (IVM), oocytes in the maturation culture were freed from

cumulus cells. Twenty to twenty-five oocytes were randomly placed in mTBM droplets covered with pre-warmed paraffin oil, and the sperm suspension was added to each fertilization drop to give a final sperm concentration of 2×10^6 sperm/ml. After co-incubation of gametes for 6 h, the oocytes were washed, and transferred into an *in vitro* culture (IVC) medium.

In vitro culture and chemical treatment

Embryos were cultured at 39°C, in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 for 7 d. The basic IVC medium was North Carolina State University (NCSU)-23 medium supplemented with 4 mg/ml fatty acid-free BSA (Petters and Wells, 1993). For all experiments, a single 30 μM dose of PFT-α was added to the culture drop at the beginning of either treatment period, at 0 to 48 h post insemination (hpi) or 48 to 168 (hpi), respectively 30 μM represents the highest usable PFT-α concentration possible under typical culture conditions (Walton et al., 2005).

Measurement of apoptosis

Terminal deoxynucleotidyltransferase (TdT)-mediated deoxy-Uridine Nick-End Labelling (TUNEL) is the enzymatic addition of fluorescently labelled nucleotides to the free 3'-ends of DNA strands made available by the DNA fragmentation that typically accompanies programmed cell death or apoptosis.

In order to visualize apoptotic nuclei, embryos were washed four times at room temperature in phosphate buffered saline (PBS) supplemented with 1 mg poly-vinyl pyrrolidone (PVP) per milliliter PBS (PBS-PVP). Embryos were then fixed for 1 h at room temperature in 4% (w/v) paraformaldehyde in PBS (pH 7.4). After fixation, embryos were washed a further two times in PBS-PVP. Embryos were then permeabilized for 10 min at room temperature in a 0.5% (v/v) Triton X-100 solution in PBS. After permeabilization, embryos were washed one time in PBS-PVP in preparation for the TUNEL procedure. Fixed and permeabilized embryos were then subject the TUNEL assay procedure using an *in situ* cell death detection kit (Roche; Mannheim, Germany) according to the manufacturers instruction. Briefly, embryos were incubated with terminal transferase enzyme and labelled nucleotide solution (mixed in a 1:10 ratio) in a humidified, sealed chamber in the dark at 37°C for 1 h.

After completion of the TUNEL reaction procedure, embryos were washed four times for 2 min in PBS-PVP and then transferred to a solution containing 10 mg/ml Hoechst 33342. Nuclear staining by Hoechst 33342 was allowed to proceed for approximately 15 min at room temperature in the dark. Embryos were then mounted onto slides using 3 μl of glycerol in-between a vaseline bridge, and compressed gently under a microscope slide. Slides were then visualized and photographed under UV illumination, using an inverted microscope equipped with differential interference contrast optics (Nikon; Tokyo, Japan). Nuclei displaying distinct labelling and condensed or pyknotic morphology were considered to be TUNEL-positive. Average embryo cell numbers were determined by counting the number of nuclei stained with the fluorescent nuclear dye Hoechst 33342.

Quantitative real-time PCR

To analyze the relative abundance of the mRNA transcripts of *p53*, *bcl-xL*, *bak*, *caspase3* and *p66Shc* both treated and control blastocysts were harvested at 168 hrs post insemination. Messenger RNA was extracted using the Dynabeads mRNADirect Kit (Dynal Asa, Oslo, Norway) according to the manufacturer's instructions. For reverse transcription, the total mRNA of 10 blastocysts was used in a final volume of 20 μl using a high capacity RNA-to-cDNA Kit (Applied Biosystems, Forster City, CA, USA) according to the manufacturer's instructions. Reverse transcription was carried out at 37°C for 60 min, the reaction was stopped by incubation of the reaction mixture for 5 min at 95°C. The products were stored at -20°C until required for real-time polymerase chain reaction (PCR) analysis.

Real-time PCR amplification was conducted using the ABI 7300 real time PCR System (Applied Biosystems). A QuantiTect SYBER Green PCR kit (Finnzymes, Espoo, Finland) was used to provide real-time quantification of the desired PCR product. The real-time PCR reaction mixture was comprised of 2 μl of cDNA, and 300 nM each of forward and reverse primers (Table 1) in a total volume of 20 μl. Three replications were conducted and the mRNA level of each sample was normalized to that of β-actin. The relative levels of mRNA were analyzed by the Δ-Ct method (Livak and Schmittgen, 2001).

Statistical analysis

Statistical analysis was performed using the "R" program (http://www.R-project.org). The development of Treated and non-treated embryos to the blastocyst stage of development was analyzed using the *x*-squared test. The cell numbers and mRNA expression levels were analyzed by the one-way repeated ANOVA test and data are presented here as mean±SEM. Treatment differences were considered to be significant when the p value was less than 0.05.

RESULTS

The developmental competence of treated and non-treated embryos

In a preliminary study, investigating the appropriate treatment dosage, it was found that concentrations lower

Table 1. Primer sequences and annealing temperature conditions used in real-time PCR

Genes	Primer sequences	Annealing temperature (°C)	Products size (bp)	Accession number
β-Actin	5'-GTGGACATCAGGAAGGACCTCTA-3'	58	135	U07786
	5'-ATGATCTTGATCTTCATGGTGCT-3'			
p53	5'-GCAATGGATGAGGCGCAGTC-3'	58	195	NM_213824.2
	5'-TGGCACTCTGGAGGCGTCAT-3'			
Bak	5'-CAGCACCATGGGGCAGGTAG-3'	58	150	AJ001204.1
	5'-AGGCTGGAGGCGATCTTGGT-3'			
Bcl-xL	5'-TGAAGCAAGCGCTGAGGGAG-3'	58	150	AF216205.1
	5'-TCACCCCATCCCGGAAGAGT-3'			
Caspase3	5'-GCCGAGGCACAGAATTGGAC-3'	58	180	AB029345.1
	5'-GCGCTGCACAAAGTGACTGG-3'			
p66Shc	5'-AAACAGATCATCGCCAACCA-3'	58	161	U73377.1
	5'-CTGATGACATCCTGGGCAAG-3'			

than 20 μM, failed to cause a differential outcome in either cleavage, or blastocyst formation rates, amongst treated and non-treated groups. During this trial period, a concentration of 30 μM elicited the greatest differential response amongst treated and non-treated groups. A concentration of 30 μM was therefore chosen for subsequent experiments in this study.

The developmental rates of both treated and non-treated porcine IVF embryos are displayed in Table 2 and 3. Cleavage and blastocyst development rates were calculated at 48 hpi and 168 hpi respectively. As can be seen in Table 2, cleavage rates amongst 0 to 48 hpi treated IVF embryos were significantly ($p<0.05$) reduced, when compared with the non-treated controls (57.1±7.6 vs 65.1±7.9). Further to this, the percentage of embryos reaching the blastocyst stage was also significantly reduced amongst treated embryos in comparison with the control group (14.2±1.4 vs 21.3±1.7).

In Table 3, it is shown that the percentage of embryos reaching the blastocyst stage is significantly increased ($p<0.05$) amongst cleavage stage embryos treated from 48 to 168 hpi (52.5±6.9), as compared to the non-treated control group (44.1±6.7).

Table 3. The *in vitro* developmental potential of pifithrin-α treated (48 to 168 hpi) porcine embryos*

PFT- α concentration (μM)	No. cleaved embryos [1]	No. blastocysts (% mean±SEM)[2]
0	268	116 (44.1±6.7) [a]
30	223	117 (52.5±6.9) [b]

* The number of replicates was five.
[1] Normal cleaved embryos at the 2 to 4 cell stage of development were selected 48 hpi and then cultured up to the blastocyst stage with or without PFT-α supplementation in NCSU-23 culture media.
[2] The blastocyst development rate was calculated on d 7.
[a-c] Values with different letters within each column are significantly different, $p<0.05$.

Total cell numbers and frequency of apoptotic cell detection in treated and non-treated controls

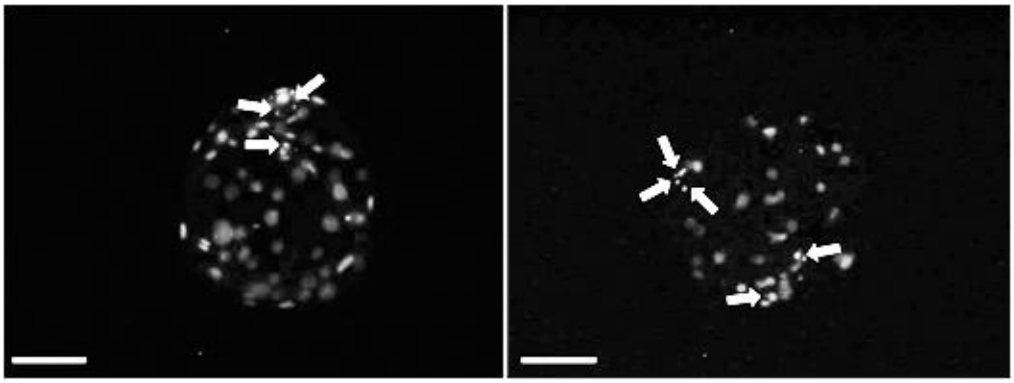
The TUNEL assay was used to determine the frequency of apoptotic cells, and the average numbers of cells were counted using Hoechst 33342 staining in treated and non-treated porcine IVF blastocysts at 168 hpi (Figures 1 and 2). As can be seen in Table 4, the total cell numbers recorded in blastocysts resulting from 0 to 48 hpi treatment are significantly lower ($p<0.05$), in comparison with the non-treated controls (51.2±6.93 vs 72±4.37). Furthermore, the percentage of apoptotic nuclei detected in these treated blastocysts was greater ($p<0.05$) than that of the untreated controls (16±1.68 vs 6.5±1.76).

Table 5, shows that the overall cell numbers recorded in blastocysts resulting from the 48 to 168 hpi treatment group did not significantly differ from those recorded in the untreated controls (72±4.88 vs 73±3.21). However, the incidence of apoptosis in these blastocysts was significantly higher ($p<0.05$), when compared with

Table 2. The *in vitro* developmental potential of pifithrin-α treated (0 to 48 hpi) porcine embryos*

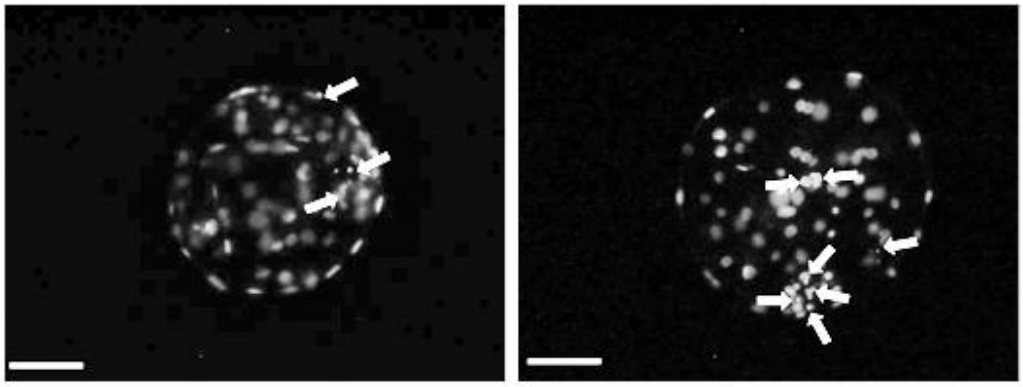
PFT-α concentration (μM)	No. zygotes[1]	No. cleaved (% mean±SEM)	No. blastocysts (% mean±SEM)[2]
0	258	168 (65.1±7.9) [a]	55 (21.3±1.7) [a]
30	268	153 (57.1±7.6) [b]	38 (14.2±1.4) [b]

* The number of replicates was five.
[1] Presumed zygotes showing good morphology were selected following 6 h of co-incubation with sperm.
[2] The blastocyst development rate was calculated on d 7.
[a-c] Values with different letters within each column are significantly different, $p<0.05$.



A **B**

Figure 1. Apoptosis in pifithrin-α treated (0 to 48 hpi) d 7 *in vitro* fertilized blastocysts. Following IVF presumed zygotes were cultured for 48 h in NCSU-23 media in the absence (A) or presence (B) of pifithrin-α (30 μM). Cleaved embryos were washed and transferred to fresh media where they were cultured until d 7. Harvested d 7 blastocysts were subject to nuclear staining (Hoechst 33342) and the *in situ* cell death TUNEL assay. TUNEL positive nuclei (red) are marked by arrows. Scale bars = 100 μm.



A **B**

Figure 2. Apoptosis in pifithrin-α treated (48 to 168 hpi) d 7 *in vitro* fertilized blastocysts. Porcine cleavage stage IVF embryos (48 hpi) were cultured in NCSU-23 media for 5 d in the absence (A) or presence (B) of pifithrin-α (30 μM). Nuclei were stained with Hoechst 33342 and TUNEL positive nuclei (red) are marked by arrows. Scale bars = 100 μm.

Table 4. The effects of pifithrin-α treatment (0 to 48 hpi) on total cell numbers and apoptosis in d 7 porcine *in vitro* fertilized embryos*

PFT-α concentration (μM)	Blastocysts (n)	No. total cells in blastocysts (mean±SEM)	Apoptotic nuclei (mean±SEM)	Apoptotic cell rate (% mean±SEM)
0	15	72±4.37[a]	4.8±1.25	6.5±1.76[a]
30	15	51.2±6.93[b]	6.6±0.88	16±1.68[b]

* The number of replicates was three. [a-b] Values with different letters within each column are significantly different, $p<0.05$.

Table 5. The effects of pifithrin-α treatment (48 to 168 hpi) on total cell numbers and apoptosis in d 7 porcine *in vitro* fertilized porcine embryos*

PFT-α concentration (μM)	No. Blastocysts (n)	No. total cells in blastocysts (mean±SEM)	No. apoptotic nuclei (mean±SEM)	Apoptotic cell rate (% mean±SEM)
0	23	73±3.21[a]	4.8±0.72	6.6±0.99[a]
30	16	72±4.88[a]	7.3±0.96	10.6±1.48[b]

* The number of replicates was three. [a-b] Values with different letters within each column are significantly different, $p<0.05$.

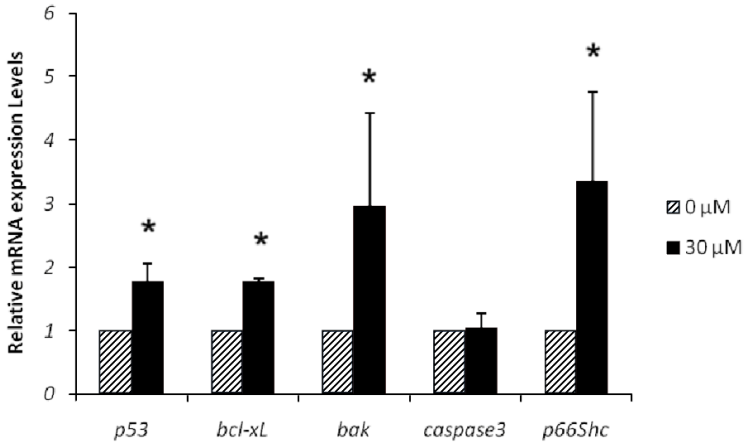


Figure 3. Relative gene expression levels of treated (0 to 48 hpi) and non-treated porcine *in vitro* fertilized embryos. The mRNA levels of *p53*, *bcl-xL*, *bak*, *caspase3* and *p66shc* were analyzed in d 7 *in vitro* fertilized blastocysts cultured with or without 30 µM pifithrin-α from 0 to 48 hpi. Results are displayed as fold changes in treated blastocysts relative to non-treated controls. Significant difference is marked with an asterisk (*) ($p<0.05$).

non-treated controls (10.6±1.48 vs 6.6±0.99).

Expression pattern of apoptosis related genes in treated and non-treated *in vitro* fertilized blastocysts

In order to analyze the relative and quantitative differential expression of five selected genes related to apoptosis, in both treated and non-treated porcine embryos, gene specific primers were designed and used for real-time PCR (Table 1). For each analysis, pools of 10 embryos were selected and used for mRNA isolation, cDNA synthesis and quantitative real-time PCR. A total of three replicates were conducted for each experiment.

As illustrated in Figure 3, it can be seen that in 0 to 48 hpi treated blastocysts, *p53*, *bak* and *p66shc* mRNA abundance is significantly increased ($p<0.05$), whilst caspase3 mRNA abundance is reduced ($p<0.05$), relative to the non-treated controls. Surprisingly, the abundance of *bcl-xL* mRNA is greater in treated embryos ($p<0.05$).

For 48 to 168 hpi treated embryos, a similar pattern of apoptotic gene expression was observed (Figure 4), with *p53*, *bak*, and *p66shc* transcripts being up-regulated in treated embryos ($p<0.05$). However, caspase3 mRNA expression was in this case similar to that of the non-treated control embryos, whilst *bcl-xL* mRNA abundance was also greater in treated embryos ($p<0.05$).

DISCUSSION

The present study was designed in order to investigate the effects of a commonly used p53 inhibitor, PFT-α, on

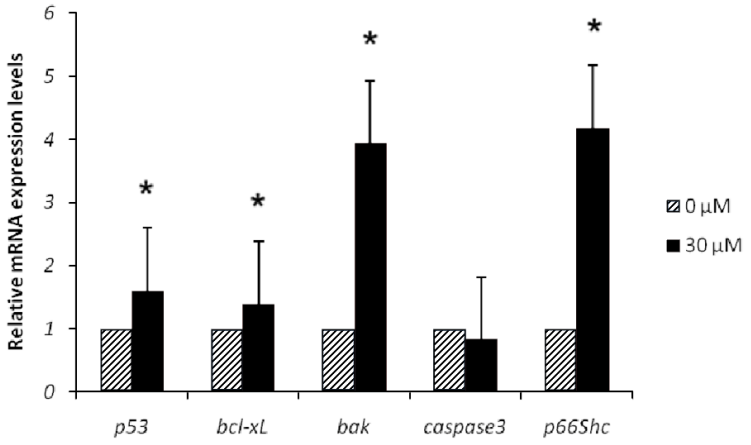


Figure 4. Relative gene expression levels of treated (48 to 168 hpi) and non-treated porcine *in vitro* fertilized embryos. The mRNA levels of *p53*, *bcl-xL*, *bak*, *caspase3* and *p66shc* were analyzed in d 7 *in vitro* fertilized blastocysts cultured with or without 30 µM pifithrin-α from 48 to 168 hpi. Results are displayed as fold changes in treated blastocysts relative to non-treated controls. Significant difference is marked with an asterisk (*) ($p<0.05$).

preimplantation porcine IVF embryo development in culture. PFT-α has been widely used as a ubiquitous and reversible inhibitor of p53 and p53 mediated apoptosis (Kelly et al., 2003; Eipel et al., 2005; Gupta et al., 2007; Luo et al., 2009; Waters et al., 2010). This compound has also been used in order to ameliorate the severity of embryonic loss experienced as a result of activation of the p53 stress response pathway (Jin et al., 2009). In the mouse, PFT-α treatment from the zygote stage has been proven capable of ameliorating p53 dependent embryonic loss in the C57BL/6 (B6) strain, which is susceptible to the stressors of culture (Jin et al., 2009). PFT-α treatment improve the development of mouse embryos as well as improving the number of outgrowths formed from mouse somatic cell nuclear transfer (SCNT) embryos, indicating a positive effect on trophectoderm function. However, PFT-α treatment has not been demonstrated to have any discernible effect upon blastocyst formation rates in nuclear transfer embryos (Esteves et al., 2011). PFT-α treatment has also been shown to rescue porcine SCNT embryos from p53 mediated embryonic arrest, occurring during early cleavage stages. Porcine SCNT embryos injected with an anti-DJ-1 antibody (a-DJ1-NT) are compromised developmentally and typically undergo arrest, which is characterized by the up regulation of p53 and that the embryonic arrest of a-DJ1-NT blastocysts can be reversed with PFT-α treatment (Miyamoto et al., 2011). Therefore, we hypothesized that this compound was potentially capable of reducing culture stress induced, apoptosis in porcine IVF embryos. However, on the contrary, our results indicate that PFT-α may in fact severely compromise the developmental potential of porcine IVF embryos, and is in fact a potent apoptotic agent when introduced into porcine IVC media.

In this study, we investigated the effects of PFT-α treatment during both early embryo cleavage stages (0 to 48 hpi), as well as during later development (48 to 168 hpi). For these experiments, a single 30 μM dose of PFT-α was administered at the beginning of the treatment period, as this was determined to be the optimum concentration in a preliminary study. Lower concentrations (0 to 5 μM), have been used in order to protect cultured embryos from various apoptosis inducing stimuli, such as UV (Davidson et al., 2008), however, at these concentrations, PFT-α did not affect porcine IVF embryo viability in preliminary experiments. PFT-α becomes unstable at concentrations greater than 30 μM under typical culture conditions (Walton et al., 2005), and so 30 μM also represented the maximum usable PFT-α concentration in this study. At this concentration, PFT-α has proven able to protect human cancer cell lines from DNA damage induced apoptosis *in vitro* (Sohn et al., 2009), and has also been used to ameliorate the severity of embryonic loss experienced as a result of activation of the p53 stress response pathway in cultured mouse embryos (Jin et al., 2009).

Our results demonstrate, that when PFT-α is administered for 48 h immediately following IVF (0 to 48 hpi), it severely compromises porcine embryo development *in vitro*, reducing both the cleavage and blastocyst formation rates amongst treated embryos (Table 2). Conversely, PFT-α, when administered to normal 2 to 4 cell cleavage stage embryos from 48 to 168 hpi, results in marginally increased blastocyst formation rates amongst treated embryos (Table 3). These results may reflect observations that have been made during studies investigating the radioprotective effects of PFT-α upon zebrafish embryos. It has been found that PFT-α treatment of zebrafish embryos at 24 hpi protects this species from radiation induced embryo lethality (Duffy and Wickstrom, 2007).

In contrast, PFT-α treatment of zebrafish embryos at 3 hpi results in disorders affecting the head, brain, eyes and kidney function and does not confer radioprotective effects (Davidson et al., 2008). Therefore, it may be inferred that the susceptibility of porcine embryos to the negative impacts of PFT-α may also depend upon the stage at which the drug is administered, with porcine IVF embryos proving susceptible to the negative impacts of treatment at early, but not later stages of development, in a similar manner to zebrafish embryos (Davidson et al., 2008; Duffy and Wickstrom, 2007).

It has been previously reported that PFT-α is capable of improving the developmental potential of *in vivo* derived, B6 mouse zygotes, that display enhanced p53 expression, and which otherwise develop poorly in culture (Jin et al., 2009). However, these authors merely noted that PFT-α appears to increase development to the blastocyst stage in these culture susceptible embryos. Considering that various modes of action have been proposed for PFT-α (Komarova et al., 2003; Murphy et al., 2004; Davidson et al., 2008; Sohn et al., 2009), and that the p53 specific nature of this compound has been questioned (Gary and Jensen, 2005; Walton et al., 2005), further analysis may be necessary in order to draw firm conclusions about whether these findings indicate a p53 inhibitory, or anti-apoptotic role for PFT-α in this context. To our knowledge, no study has yet assessed the impact of PFT-α on the quality of mammalian embryos, in terms of gene expression or the frequency of apoptotic cells within resulting blastocysts. We, therefore, aimed to assess the impact of PFT-α upon the incidence of apoptosis and apoptotic related gene expression in blastocysts that were cultured in the presence of PFT-α.

The incidence of apoptosis has been found to correlate with overall cell numbers and embryo quality (Jurisicova et al., 1998; Levy, 2005). The TUNEL assay, along with

complimentary nuclear staining may therefore be a useful tool in determining the relative quality of different blastocysts (Van Soom et al., 2003). The TUNEL assay procedure was used in this study in order to determine the incidence of apoptotic cell nuclei, along with total cell numbers in blastocysts resulting from both 0 to 48 hpi and 48 to 168 hpi PFT-α treatment. Treated blastocysts resulting from 0 to 48 hpi treatment, showed fewer cell numbers, along with a corresponding increase in apoptotic cell rates (Figure 1 and Table 4), suggesting that these embryos are inferior in quality in comparison to normal non-treated IVF porcine embryos.

Interestingly, those blastocysts resulting from embryos treated from 48 to 168 hpi, which showed elevated developmental potential in culture (Table 3), also displayed greater levels of apoptosis (Figure 2), whilst cell numbers within these embryos were similar to non-treated controls (Table 5). This is a striking result considering that greater levels of apoptosis are generally considered to negatively affect embryo development (Jurisicova et al., 1998; Mateusen et al., 2005). It must therefore be concluded that some other mechanism of action by PFT-α, uninvolved with apoptosis, is responsible for the enhanced development recorded in these embryos. It has been shown that PFT-α may act to reduce the transactivation of the p53 responsive cell cycle inhibitor gene, *p21* (Komarov et al., 1999; Proietti De Santis et al., 2003).

In view of the fact that the cyclin-dependent kinase inhibitor *p21* induces G1 arrest (Vasey et al., 2011), *p21* inhibition presents one possible mechanism by which PFT-α may enhance porcine embryo development *in vitro*, although this possibility was not investigated in the present study. Our results indicate that PFT-α behaves as a potent apoptotic stimulant when administered during the culture of porcine preimplantation IVF embryos. In a study conducted by (Kaji et al., 2003), PFT-α treatment was found to induce p53-dependent apoptosis in murine JB6 cells, whilst PFT-α treatment in conjunction with DOX or UV, enhanced the induction of apoptosis by these agents in this cell line. To date no reports have investigated how PFT-α may affect apoptotic gene expression in preimplantation embryos in culture. In this study we investigated the expression of five genes (*p53*, *bak*, *bcl-xL*, *caspase3* and *p66shc*), related to the intrinsic mitochondrial apoptosis pathway, in blastocysts resulting from PFT-α treatment during culture.

The regulation of apoptosis may involve stress induced gene expression and/or cell cycle modulators (Betts and King, 2001). Members of the *bcl-2* gene family fall into two categories including pro-apoptotic and anti-apoptotic members (Cory and Adams, 1998) reported that members of the *bcl-2* gene family play key roles in regulating apoptosis, and at least 15 mammalian *bcl-2* gene family members have been identified. These have been categorized into two subgroups, anti-apoptotic (*bcl-2*, *bcl-w*, *bcl-xL*, *a1*, *mcl-1*) and pro-apoptotic (*bax*, *bak*, *box*, *bik*, *blk*, *hrk*, *bnip3*, *bim*, *bad*, *bid*, *bcl-xs*). Among the *bcl-2* gene family, *bcl-xL* and *bak* are known as anti-apoptotic and pro-apoptotic genes, respectively, in the pig (Boise et al., 1993; Jurisicova et al., 1998).

In this study it was shown that PFT-α consistently induced the up-regulation of the pro-apoptotic regulator *bak*, in treated embryos. PFT-α treatment for 48 h following *in vitro* fertilization (0 to 48 hpi), resulted in a 2.8 fold increase in *bak* expression (Figure 3), whilst treatment for 48 to 168 hpi resulted in a 3.9 fold increase in resulting blastocysts (Figure 4). Interestingly, the expression of *bcl-xL* was also increased in both groups (1.4 fold and 1.7 fold respectively). The abundance of *p53* mRNA was also found to be greater in both 0 to 48 hpi and 48 to 168 hpi treated blastocysts (1.5 and 1.6 fold respectively), whilst *caspase3* expression was not significantly affected in either group. However, expression of *p66shc* mRNA was highly induced by PFT-α treatment.

The oxidative stress adaptor *p66shc* induces apoptosis by increasing the level of intercellular ROS, which results in mitochondrial Cytochrome c release and caspase activation (Zhang et al., 2010). As a known downstream target of p53, *p66shc* is a propagator of p53 induced proapoptotic oxidative signalling (Orsini et al., 2004). It is noteworthy, that even transient treatment with PFT-α (0 to 48 hpi) resulted in a 4.7 fold increase in the expression of this gene (Figure 3), in resulting d 7 blastocysts, 5 d after exposure to this compound. This data suggests that PFT-α treatment when administered to developing embryos during early cleavage stages, may irreparably damage any resulting blastocysts. In the present study blastocysts resulting from the 0 to 48 hpi treatment period, displayed aberrant apoptotic gene expression, reduced cell numbers, and increased rates of apoptosis in comparison with non-treated embryos. It is highly likely that these factors contributed to the reduced viability that was recorded in these embryos.

Surprisingly, even when PFT-α treatment appeared to marginally increase the blastocyst formation rate in 48 to 168 hpi treated embryos (Table 3), further assessments revealed that apoptotic related gene expression was up regulated in resulting blastocysts (Figure 4), along with a corresponding increase in the incidence of apoptotic cells within these embryos (Figure 2 and Table 5). This appears to indicate that whilst PFT-α may act to improve the rate of blastocyst formation in cleavage stage pre-implantation porcine IVF embryos, it does not do so by inhibiting the process of apoptosis. These results support earlier findings in zebra fish embryos which demonstrate that PFT-α treatment, particularly at the earliest cleavage stages, may

result in developmental abnormalities (Davidson et al., 2008). Our observation that PFT-α may actually improve blastocyst formation rates when administered at later stages, may be therefore be consistent with these reports, given that the protective effects of this compound may be stage, timing and dose dependent.

Overall, we conclude that PFT-α treatment has a detrimental effect upon porcine IVF embryos in culture. PFT-α treatment resulted in blastocysts of an inferior quality in terms of apoptotic cell rates and apoptotic gene expression profiles in both 0 to 48 hpi and 48 to 168 hpi treatment groups. This study provides evidence that should serve as a caution when using this inhibitor as a specific inhibitor of p53 mediated apoptosis, in the context of mammalian embryo culture systems.

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