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Högfluorerade ämnens (PFAS) effekt på celldöd under den tidiga embryoutvecklingen in vitro hos gris

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Swedish University of Agricultural Sciences, SLU
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Abstract

The aim of this study was to evaluate the effects of PFHxS (perfluorohexane sulfonic acid) and PFOS (perfluorooctane sulfonic acid) on early embryo development parameters and apoptosis on day 6 post fertilization in porcine embryos. A second purpose was to evaluate, and establish, the TUNEL assay in our IVF laboratory. PFHxS and PFOS belong to the PFAS (per- and poly-fluoroalkyl substances) group, which is a large and diverse group of man-made compounds used in *e.g.* textiles and fire-fighting foam. Embryos were produced *in vitro* using oocytes obtained from abattoir ovaries. Oocytes were matured for 45 hours, with the addition of PFHxS (40 µg/mL) and PFOS (0.1 µg/mL), respectively. Oocytes were then fertilized, and presumed zygotes were cultured. Cleavage rate was recorded 48 hours post fertilization. Developmental stages and grades, as well as number, of resulting blastocysts were recorded for each group on day 5 and 6 post fertilization, respectively.

Blastocysts were then collected, fixated, and stained using a TUNEL assay to detect nuclei of apoptotic cells. Positive and negative controls were included to evaluate the TUNEL assay. Stained blastocysts were evaluated using confocal microscopy followed by computerized image analysis.

Development parameters and proportion of apoptotic cells were analysed statistically. The treatments had no significant effect on cleavage rate, cleavage rate above 2 cells, developmental stage, or grade. PFOS had no effect on blastocyst rate on day 5 or 6 post fertilization. PFHxS tended to result in higher blastocyst rate on day 5 post fertilization ($p=0.07$) and resulted in higher blastocyst rate on day 6 post fertilization ($p=0.05$). Blastocysts with higher total number of nuclei contained a significantly lower proportion of apoptotic nuclei, compared with blastocysts containing lower total numbers of nuclei ($p=0.01$). PFHxS-treatment tended to result in larger total numbers of nuclei ($p=0.062$). Furthermore, blastocysts in the PFHxS group tended to contain higher proportions of apoptotic nuclei than blastocysts in the control group ($p=0.084$). PFOS did not have any effect on any of these parameters.

This study shows that PFHxS likely interferes with some parameters of early embryo development in porcine embryos, although not all results obtained were statistically significant. The concentration of PFOS used in this study (0.1 µg/mL) had no effect on any parameter, although it has been showed to have adverse effects on reproduction in several earlier studies. More studies are needed to investigate the effects of PFAS on apoptosis and early embryo development. Furthermore, the effect of PFAS on continued embryonic and foetal development, as well as on live offspring, also needs to be investigated.

Keywords: Apoptosis, PFAS, PFOS, PFHxS, IVF, IVP, porcine, pig, embryo, TUNEL, reproduction

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Abbreviations

AE	Acrosomal exocytosis
COC	Cumulus oocyte complex
DAPI	2-(4-amidinophenyl)-1H-indole-6-carboxamidine
dbcAMP	Dibutyryladenosine cyclic monophosphate
DNase	Deoxyribonuclease
FSH	Follicle stimulating hormone
IVF	*In vitro* fertilization
IVP	*In vitro* production
LAF hood	Laminar air flow hood
LH	Luteinizing hormone
P10	0,1-10 µL pipette
P100	50-100 µL pipette
P1000	100-1000 µL pipette
PBS	Phosphate buffered saline
PFAS	Per- and polyfluoroalkyl substances
PFHxS	Perfluorohexane sulfonic acid
PFM	Porcine fertilization medium
PFNA	Perfluoro nonanonic acid
PFOS	Perfluorooctane sulfonic acid
PFSA	Long-chain perfluoroalkyl sulfonic acids
POM	Porcine oocyte maturation medium
PPAR	Peroxisome proliferator-activated receptor
PVA	Polyvinyl alcohol
PZM	Porcine zygote medium
SD	Standard deviation
TMR	Tetramethyl rhodamine
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
ZP	Zona pellucida

1. Introduction

Globally, approximately 10% of couples who are attempting to conceive experience infertility (Gnoth *et al.* 2005). Several lifestyle factors including obesity and advanced ages of women trying to become pregnant have been pointed out as contributing to this issue (Silvestris *et al.* 2019). However, in later years, the potential effects on human reproduction of so-called endocrine disrupting chemicals have been discussed more intensively (Sifakis *et al.* 2017).

Per- and polyfluoroalkyl substances (PFAS) constitute a large group of substances, commonly used in everyday household products. PFAS are highly persistent in the environment and have been found to be able to accumulate in organisms (KemI 2015). For instance, PFAS have been detected in human serum and follicular fluid (Heffernan *et al.* 2018). Several studies have also indicated that PFAS can have various negative effects on female reproduction (Ding *et al.* 2020).

In this study, in which the pig served as a model for humans, the aim was to evaluate the effect of two different PFAS; PFOS (perfluorooctane sulfonic acid) and PFHxS (perfluorohexane sulfonic acid) on apoptosis and development in early porcine embryos. Pig ovaries (collected at an abattoir, where these are available in large numbers) were used as a source of oocytes to produce embryos, meaning that no laboratory animals were needed. Oocytes included in the study were subjected to PFHxS and PFOS during their maturation phase. They were then fertilized *in vitro*, and resulting blastocysts were fixed and stained using a TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) staining kit to detect apoptotic cells. Stained blastocysts were imaged using confocal microscopy and analysed to calculate the proportion of apoptotic cells.

2. Literature Review

2.1. Reproduction biology of mammals

2.1.1. Summary of the female reproductive cycle

The estrous cycle can be divided into two phases: the follicular phase and the luteal phase. During the first part of the follicular phase, the secretion of luteinizing hormone (LH) from the hypophysis is increasing, which stimulates follicles to produce and release estrogen. At this stage of the cycle, estrogen has a negative feedback effect on the hypothalamus, and thus, on the production and secretion of LH and follicle stimulating hormone (FSH). As, towards late follicular phase, estrogen levels increase, follicles develop and increase in size, and estrogen switches to exert positive feedback on the hypothalamus. This results in briskly increasing LH concentrations, and, ultimately, the pre-ovalutory LH surge, followed by bursting of the dominant follicle(s) and, thereby, ovulation. In each cycle, only a certain number (depending on the species in question) of the developing follicles are selected to become dominant, *i.e.* to complete the maturation process and finally ovulate (Sjaastad *et al*. 2010).

After ovulation, estrogen-, LH-, and FSH levels decrease. Subsequently, the *corpus luteum* is formed (*i.e.* luteinization), and the luteal phase begins. The *corpus luteum* secretes progesterone, which prepares the uterine tissue to eventually foster an embryo. Progesterone also influences the release of LH and FSH negatively and promotes new follicular growth. At the end of the luteal phase, luteolysis occurs, *i.e.* the corpus luteum regresses, and progesterone levels drop. Here, prostaglandin F2α plays an important role; it is secreted from the endometrium in a pulsatile fashion, diffuses through the vessel walls to the ovarian artery, and induces luteolysis. If pregnancy occurs, however, the corpus luteum persists, and continues to produce progesterone. This is critical in maintaining a pregnancy as progesterone (among other things) hinders myometrial contractions (Sjaastad *et al.* 2010).

Oocyte maturation

At birth, ovaries of mammalian females already contain a large number of so-called "resting" germ cells that have been halted in the phase called prophase I during their first meiotic division; a phase also referred to as meiotic arrest (Sen & Caiazza 2013). All oocytes remain dormant until puberty. Shortly before ovulation, meiotic activity is resurrected, and the first meiosis is completed, with the expulsion of the first polar body (Sjaastad *et al.* 2010). The second meiosis is initiated and progresses to the phase called metaphase II, and, if the oocyte becomes fertilized, is completed. The second polar body is eliminated from the oocyte (Sen & Caiazza 2013). During maturation, developing oocytes also grow significantly in size (Sjaastad *et al.* 2010). See figure 1.

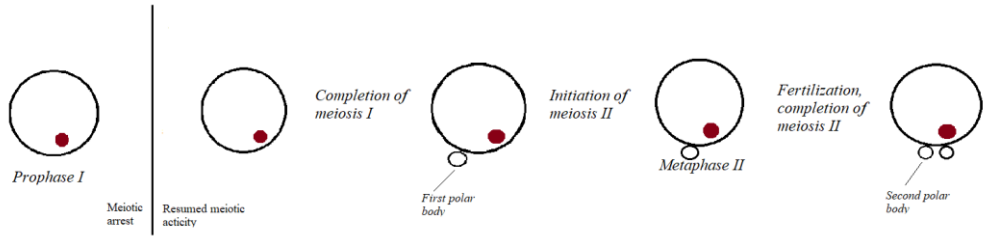

Figure 1. Schematic representation of oocyte maturation.

2.1.2. Fertilization and early embryonic development

In the case of natural fertilization, spermatozoa are transported through the female reproductive tract to the uterine tubes mainly under the influence of myometrial contractions. However, when the fertilization site is approached, spermatozoal movement aid the germ cells to approach the oocytes. Chemotaxis (*i.e.* attraction or repelling of cells in relation to a chemical substance) is a mechanism of importance in leading the spermatozoa in the appropriate direction. In mammalian reproduction, progesterone synthesized by cumulus cells appears to be of significance in attracting spermatozoa (Tosti & Boni 2011).

Once the spermatozoon has reached the cumulus oocyte complex (COC), it travels through the layers of cumulus cells and binds to the zona pellucida (ZP) of the oocyte. Simultaneously, a series of events referred to as acrosomal exocytosis (AE) is initiated. The AE is an irreversible process which includes the exocytosis of a portion of the acrosome of the sperm. The cell membranes of the spermatozoon and the oocyte fuse together, and a so-called zygote is formed (Tosti & Boni 2011).

In some cases, one oocyte can become fertilized by more than one spermatozoon (Evans 2020). This is referred to as polyspermy and can result in ceased zygote development (Coy & Romar 2002). There is evidence of several naturally occurring

mechanisms to avoid polyspermic fertilization, including sperm reservoirs in oviducts (*i.e.* binding of spermatozoa to the epithelium of the uterine tubes) and blockages in the ZP and the cell membrane of the oocyte (Evans 2020). However, high rates of polyspermy is a major challenge in *in vitro* production of pig embryos (Coy & Romar 2002).

Once the zygote is formed, it undergoes several mitotic divisions, forming daughter cells termed as blastomeres. Under *in vivo* conditions, the first division (*i.e.* the establishment of the two-cell stage) is generally completed approximately 24 hours after fertilization. When the two-cell stage is reached, a new division occurs, and the embryo now consists of four blastomeres. Divisions continue to proceed until the cells ultimately constitute a morula, *i.e.* a spherical shape consisted by embryonic cells. Until this stage of embryonic development, all blastomeres are undifferentiated cells that are similar to each other. They are evenly distributed throughout the structure, without any fixed position (McGeady *et al.* 2006).

After a certain number of divisions (which varies depending on the species), the blastomeres are packed more closely together, and tight junctions between cell surfaces form. These events are defined as compaction, and lead to the formation of what is referred to the inner cell mass (ICM). Fluids secreted by blastomeres subsequently pool together to form an extracellular, fluid-filled cavity. The outer embryonic cell layers develop into the trophoblast, *i.e.* the most superficial layer which encloses the embryo. Under *in vivo* conditions, the trophoblast (at later stages of the pregnancy) adheres to the endometrial lining of the uterus. Thus, at this developmental phase, the embryo is consisted by two cell types (*i.e.* the embryonic cells have now, to some extent, undergone differentiation) and is referred to as a blastocyst (see figure 2). Initially, the blastocyst starts to expand within the ZP. However, it cannot continue its development enclosed by the ZP and, as is the case for most mammals, the ZP subsequently commences to decompose while the blastocyst emerges, or "hatches" out of it (McGeady *et al.* 2006). After hatching, the embryo attaches, *i.e.* implants, to the uterine wall (Geisert *et al.* 2015).

As mentioned, in mammals (*i.e.* in pigs as well as humans), the first cleavage of the zygote is generally completed 24 hours post fertilization (McGeady *et al.* 2006). However, in human embryos, the blastocyst hatches from the ZP ca. 5 days post fertilization, and implantation takes place on day 8-9 (Webster & de Wreede, 2016). In the pig, hatching generally takes place around day 8 after fertilization, and implantation occurs on day 13-14 (Geisert *et al.* 2015).

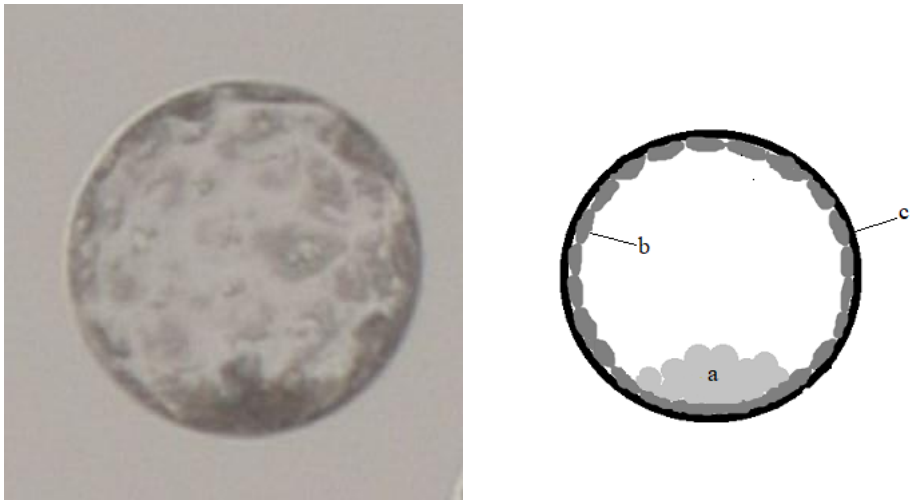

Figure 2. Left: porcine blastocyst on day 6 after fertilization. Right: schematic representation of a blastocyst. a: inner cell mass. b: trophoblast cell. c: Zona Pellucida (ZP).

2.2. *In vitro* fertilization in pigs

Porcine embryos produced using *in vitro* techniques can be used in several research fields. Porcine ovaries can be collected in large quantities at abattoirs and used as a source of oocytes for IVF (Coy & Romar 2002). Advantages of the use of embryos obtained from oocytes of slaughtered pigs for *in vitro* studies include potential reduction of the usage of laboratory animals (Santos *et al.* 2014).

The first steps of pig IVF involve selection and maturation of oocytes. In theory, it is, by flushing uterine tubes of gilts and sows, possible to obtain newly ovulated (and thus, mature) oocytes. However, in practice, the method of choice when obtaining oocytes is (in general) to collect them from follicles, and to allow them to complete their maturation process *in vitro*. This method is more effective and allows the collector to obtain many immature oocytes (Coy & Romar 2002). After selection of oocytes, they are (in most cases) allowed to mature for a total of 42-44 h in 38.5°C, 20% O_2, and 5% CO_2 (Romar *et al.* 2019). This particular amount of time is used to mimic physiologic *in vivo* conditions, where the sow ovulates 38-42 hours after the LH surge (discussed earlier) (Coy & Romar 2002). Maturation is carried out in maturation media, where hormones and dbcAMP generally are used as supplements approximately during the first half of the process (Romar *et al.* 2019).

Subsequently, the oocytes are fertilized. Before fertilization, the spermatozoa need to be separated from *e.g.* cryoprotectants (in the case of using frozen sperm). Motile, functional spermatozoa also need to be separated from defect ones (Romar *et al.* 2019). For this purpose, sperm can be centrifuged in colloid preparations (Morrell

et al. 2019). Prior to fertilization, spermatozoa are capacitated (*i.e.* undergo changes that enable them to fertilize oocytes). *In vitro* fertilization media commonly contain calcium and bicarbonate, which are known to be of importance in the capacitation process. In pig IVF, polyspermy is a major challenge. Thus, the biggest difficulty does not necessarily lay in obtaining the largest possible number of functional spermatozoa (Romar *et al.* 2019) but to avoid polyspermy while still using spermatozoa in such a concentration that will allow successful fertilization of oocytes (Sjunnesson 2020).

After fertilization has been completed, the following step in the process is culturing. Culture media are generally enriched with, among other things, growth factors and nutrients. Studies has shown that under *in vivo* conditions (*i.e.* conditions of the reproductive tract of the female pig), O_2 tension is close to 7%. When culturing embryos under these conditions (5% CO_2, 88% N_2, 7% O_2), development is enhanced compared to when 20% O_2 is used. Regarding temperature, settings between 38.5-39°C are common for porcine IVF (Romar *et al.* 2019). Once the blastocyst has hatched from ZP, continued *in vitro* culturing becomes more difficult as the embryo, at this stage of development, requires an environment that is more similar to that of the uterus (normal development further than hatched blastocysts have never been possible to produce in the VHC IVF-laboratory, SLU, Uppsala, Sjunnesson Y pers. mess., 2020).

Generally, results (in terms of *e.g.* fertilization rate and blastocyst rate) when performing pig IVF are inferior to those of, for instance, systems of bovine IVF. High percentages of polyspermy are, as mentioned, a largely unsolved issue (Sjunnesson 2020). There are also still unanswered questions regarding optimization of methods (*e.g.* composition of media) used in the process. However, when studying matters like human diseases, it is possible that the pig can serve (and already serves) as a suitable model (Romar *et al.* 2019), and research aimed at enhancing pig IVF output is ongoing (Sjunnesson 2020).

2.3. Per- and polyfluoroalkyl substances (PFAS)

PFAS constitute a vast and diverse group of chemical compounds. They can be divided into two smaller groups; polymers and non-polymers, which, in turn, can be furtherly divided into several subgroups based on their molecular structure. Thanks to their water- and grease resistant characteristics, the use of PFAS is widespread in products such as cosmetics, firefighting foam, fabrics, packaging materials, etc. (KemI 2015).

Humans can, for instance, be exposed to PFAS via contaminated drinking water (KemI 2015). Some groups, *e.g.* firefighters and residents of heavily contaminated areas, may be exposed to exceptionally elevated levels of PFAS in relation to the overall population (discussed later) (Kim *et al.* 2020).

PFAS can (as mentioned) be divided into several subgroups, of which long-chain perfluoroalkyl sulfonic acids (PFSAs) and perfluoroalkyl carboxylic acids (PFCAs) belong to the most studied. This study focuses on the effects of PFOS and PFHxS, which are classified as PFSAs. PFSAs are perfluorinated molecules containing a backbone consisted by at least 6 carbons, and an SO_3H group (Buck *et al.* 2011).

Although PFAS have been linked to adverse health effects in humans (effects on female reproduction described below), knowledge about the exact mechanisms of actions is still limited. However, activation of peroxisome proliferator-activated receptors (PPARs) is believed to be involved (Behr *et al.* 2020). Peroxisome proliferator-activated receptors are a group of nuclear receptors involved in (among other things) regulating differentiation of cells and metabolism. There are three known subtypes of PPARs; PPARα, PPARβ/δ, and PPARγ (Cheng *et al.* 2019). Both PFOS and PFHxS have (among other PFAS) been shown to be able to (in relatively high concentrations) activate human PPARα, which is a nuclear receptor involved in *e.g.* cellular differentiation and growth. In the same study, other PFAS including Perfluoro-2-methyl-3-oxahexanoic acid (PMOH) and 3H-perfluoro-3-[(3-methoxypropoxy) propanoic acid (PMPP) (although not PFOS nor PFHxS) were shown to be able to exert weak agonism on the human PPARγ receptor (Behr *et al.* 2020). The PPARγ receptor is, for example, involved in regulating ovarian function and (when activated) increasing insulin sensitivity. PPARγ agonists have, additionally, been showed to have potential as an infertility treatment in women with polycystic ovarian syndrome (PCOS), which is a common disease characterized by *e.g.* insulin resistance and lacking ovulations (Xu *et al.* 2017).

2.3.1. Perfluorooctane sulfonic acid (PFOS)

PFOS is a substance consisted by eight perfluorinated carbons and a SO_3H group (see figure 3). It has documented toxic effects on, among other things, the reproductive system (discussed more below) and in later years, its use has been regulated. For instance, it is no longer permitted to use PFOS in the manufacturing of fire-fighting foam in the European Union. However, as mentioned, PFAS generally persist in the environment to a high extent, and PFOS is still used to produce fire-fighting foam in large parts of the world (KemI 2015).

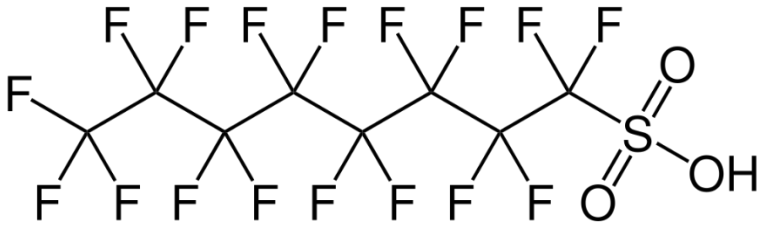

Figure 3. Molecular structure of perfluorooctane sulfonic acid (PFOS) (Leyo, without year). https://commons.wikimedia.org/w/index.php?curid=51707317.

2.3.2. Perfluorohexane sulfonic acid (PFHxS)

PFHxS can be described as PFOS in a shorter form (six perfluorinated carbons whereas PFOS contains eight, see figure 4). Compared to PFOS (discussed above), much less information on usage etc. is available regarding PFHxS (KemI, 2015). However, some evidence has showed that it likely has effects similar to those of PFOS on the reproductive system (discussed in detail below) (Taylor *et al.* 2014; Zhou *et al.* 2017; Zhang *et al.* 2018).

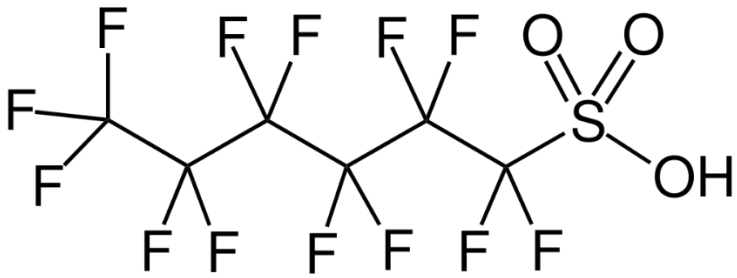

Figure 4. Molecular structure of perfluorohexane sulfonic acid (PFHxS) (Jü, without year). https://commons.wikimedia.org/w/index.php?curid=51246217.

2.3.3. Previous studies on the effects of PFAS on female reproduction

Whilst the use of PFAS has been common and widespread for several decades, it is only in the last 15-20 years that the potential adverse health effects have commenced to be investigated (Domingo & Nadal 2019). For instance, different types of PFAS have been suggested to have negative effects on the reproductive system (Liew *et al.* 2018). In a 2017 study by McCoy *et al.* concentrations of 15 different PFAS were analysed in plasma as well as in follicular fluid of patients receiving IVF treatment in the United States. It was concluded that plasma concentrations positively correlated to follicular fluid concentrations among the participants.

Furthermore, several links between PFAS and disturbed female reproductive function have been found. In a Chinese study aimed at investigating the relations

between ovarian insufficiency and exposure to PFAS, it was concluded that, among the participants (n=240), premature ovarian insufficiency was associated to more elevated plasma concentrations of PFOS, PFHxS and perfluorononanonic acid (PFNA, which also belongs to the PFAS group) (Zhang *et al.* 2018). In a second study, the results showed that women who reported to have irregular or abnormally long menstrual cycles were more likely to have higher serum concentrations of four PFAS, including PFOS and PFHxS, than women with regular menstrual cycles of normal length (Zhou *et al.* 2017). Furthermore, it has been concluded that higher levels of serum PFAS, including PFOS and PFHxS, increases the probability for women to enter menopause at a lower age (Taylor *et al.* 2014).

PFAS has, in addition, previously showed to have effects on embryonic development and oocyte maturation. In a study examining the effect of PFOS treatment on wild-type and mutant zebra fish embryos (respectively), it was found that increasing PFOS concentrations (0, 16, 32 and 64 µM) resulted in elevated apoptosis rates in wild-type embryos (96 h post fertilization). Wild-type embryos exposed to PFOS were also more likely to die than non-exposed embryos (Sant *et al.* 2018). Moreover, in a study on the effects of PFOS on (among other things) pig oocyte maturation and viability, it was concluded that increasing PFOS concentrations (0, 12.5, 25 and 50 µM) resulted in decreasing numbers of matured oocytes. In the same study, PFOS was also linked to significantly diminished percentage of live oocytes after culture (Dominguez *et al.* 2016).

In the bovine, PFNA (mentioned earlier) has, in a study involving IVF of bovine oocytes, showed to affect embryo development. A lower proportion of zygotes that developed to the 2-cell stage, as well as blastocysts, were seen in PFNA-treated groups. Furthermore, before fertilization, oocytes in treated groups were found to exhibit signs of impaired maturation. However, no effects on blastocyst stage nor grade on day 8 post fertilization were seen (Hallberg *et al.* 2019).

Concentrations of PFAS in body fluids

In a study concerning the eventual correlations between PFAS concentrations in follicular fluid and fertility factors conducted by Kim *et al.* (2020), follicular fluid samples of Australian women (n=97) undergoing infertility treatment were individually screened for several PFAS. Of the 32 different PFAS analysed, PFOS was found to be present in the highest mean concentration (4.8 ng/mL) among the participants. The mean concentration of PFHxS in the same study was 1.7 ng/mL. However, large fluctuations throughout the general population appear to exist when it comes to PFAS concentrations in body fluids. In the study by Kim *et al.* (2020), the range of PFOS concentrations measured in follicular fluid was 0.7-22.4 ng/ml, while the range of PFHxS concentration was 0.2-21.3 ng/mL. Zhang *et al.* (2018)

detected PFOS and PFHxS plasma concentrations ranging between 1.03-47.8 ng/mL and 0.09-8.46 ng/mL respectively among their participants.

As an example of high human exposures to PFAS, the "Kallinge PFAS scandal" in Ronneby, Sweden can be mentioned. In this instance, severely elevated PFAS levels were detected in blood samples from inhabitants of this area. This was linked to the fact that municipal drinking water had been contaminated with PFAS-containing fire-fighting foam, used in the Swedish armed forces' activities in a military airport located in the area during several years. In Ronneby residents, mean blood concentrations of 228 ng/mL (PFHxS) and 245 ng/mL (PFOS) have been measured. Concentrations as elevated as 1790 ng/mL (PFHxS) and 1870 ng/mL (PFOS) were detected in some individuals (Li *et al.* 2017).

2.4. Apoptosis

Apoptosis is often referred to as "programmed cell death". It stands in contrast towards necrosis (*i.e.* swelling and bursting of, typically, several cells) in that it is a controlled process, generally involving separate cells, regulated by signalling pathways. Apoptosis can be initiated as a result of several different stimuli, *e.g.* DNA injuries, toxic damage, and nutrient deficiency (Miller & Zachary 2017). Additionally, it is important to remember that apoptosis also occurs in several physiological processes *e.g.* embryogenesis (Kyrylkova *et al.* 2012).

Depending on the triggering stimuli, apoptosis can occur through the extrinsic pathway, or the intrinsic pathway. In the extrinsic pathway, the process is initiated by the binding of an extracellular ligand to a membrane-bound receptor. This triggers an intracellular pathway involving activation of caspases (*i.e.* intracellular proteases that cleave cellular peptides), leading to apoptosis. The intrinsic pathway can, in contrast, be triggered by DNA injuries, for example as a result of toxicity. The process involves induced mitochondrial outer membrane permeability, which leads to leakage of cytochrome c from the mitochondrial intermembrane space. This, in turn, triggers the activation of caspases, and apoptosis occurs (Miller & Zachary 2017). The morphological appearance of the apoptotic cell is characterized by shrinking of the cell and nucleus, and fragmentation of the cell (Kyrylkova *et al.* 2012).

2.4.1. Detection of apoptosis

In apoptotic cells, DNA strands are cleaved in a certain manner, which exposes 3'-hydroxyl ends of DNA. This can be used with the purpose of labelling apoptotic nuclei. The TUNEL (terminal deoxynucleotidyl transferase dUTP nick end

labelling) assay involves binding of dUTP to the exposed ends of the DNA fragments. This reaction is catalyzed by the enzyme terminal deoxynucleotidyl transferase. For the dUTP-labelled apoptotic cells to be visualized (*e.g.* using fluorescence microscopy), different dyeing techniques can be implemented (Loo 2011). Tetramethylrhodamine (TMR) is an example of a fluorescent dye that can be used for this purpose (Roche 2016).

When using TUNEL assays, false positive results can occur, *i.e.* non-apoptotic cells can be labelled and thus, falsely interpreted as apoptotic. As mentioned, the technique relies on labelling of exposed 3'-hydroxyl ends of DNA strands. This particular type of DNA breakage can occur in nuclei in other situations than apoptosis, including necrosis and ongoing DNA transcription. The TUNEL technique will label 3'-hydroxyl DNA ends regardless of the reason they were exposed (Loo 2011). It is advised to include DNase-treated positive controls every time the assay is performed in order to increase reliability (Kyrylkova *et al.* 2012).

2.5. Confocal microscopy

The first version of the confocal microscope, *i.e.* a light microscope which enables visualisation of three-dimensional structures (Paddock 2014), was invented in the 1950s. Since then, significant advancements of the technology have been made (Swaim 2010), and the confocal microscope is now a tool widely used in laboratories where research is done in the field of *e.g.* biomedicine. The confocal microscopy technique involves imaging of individual sections of a (fluorescently stained) subject. Focused laser beams are used to scan sections of the subject, using pinhole to enable scanning of only thin sections of the specimen. This efficiently avoids disturbances from surrounding fluorescent light. Thus, relatively thick specimens can be clearly visualised in a three-dimensional manner (Paddock 2014).

3. Materials and methods

3.1. Project overview

Oocytes were aspirated from porcine ovaries collected at an abattoir. A total of nine batches of oocytes (range: 90-120 oocytes/batch) were then matured, fertilized and cultured (see figure 5). During the maturation phase, two groups of oocytes were exposed to PFOS and PFHxS respectively. One control group was also included in each batch. The percentage of cleavage as well as cleavage above the 2-cell stage was recorded at 48 h post fertilization. The number, stage and grade (discussed more below) of blastocysts were assessed on day 5 and 6 post fertilization, respectively. After culturing, blastocysts were collected, fixed and stained for nuclei (all) and apoptotic nuclei. The stained blastocysts were imaged using confocal laser scanning microscopy.

During selection, maturation, fertilization and culture, all handling of COCs and embryos was performed in a laminar air flow hood (LAF hood) set to 37.5°C. The maturation and incubation of oocytes during fertilization was carried out in 38.5°C in a 5.5% CO_2 incubator. The presumed zygotes (24 hours post fertilization) were incubated in 38.5°C in a 5.5% CO_2 and 6% O_2 incubator. All media (unless indicated otherwise) were manufactured by Research Institute for the Functional Peptides, Osaka, Japan. For more detailed information, see appendix.

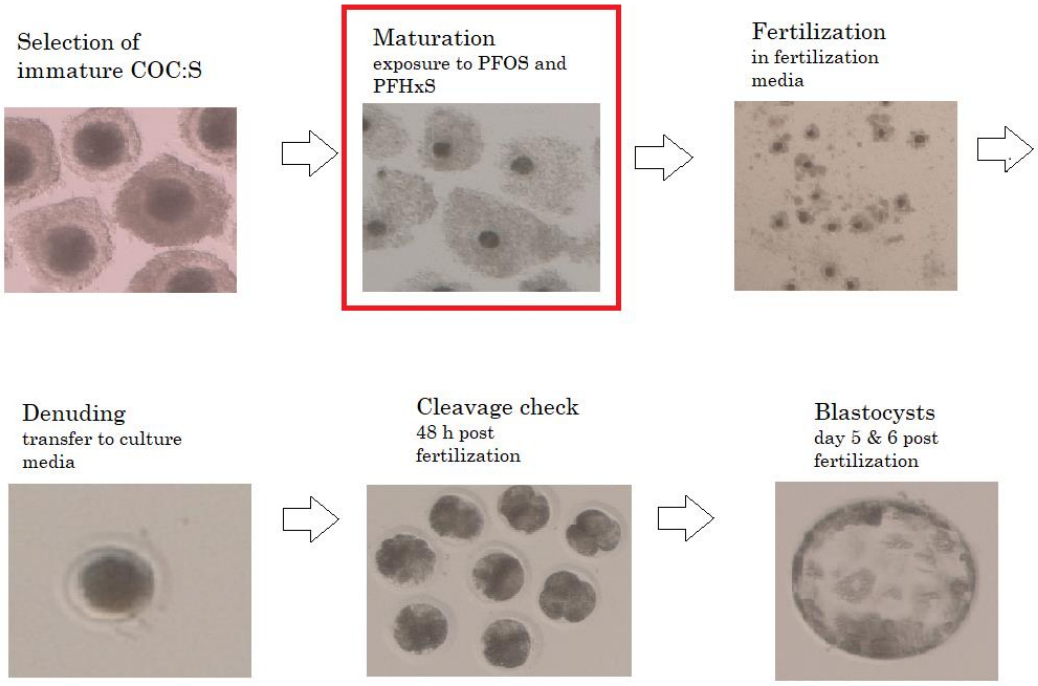

Figure 5. Overview of IVF process.

3.2. Collection of ovaries and maturation of oocytes

3.2.1. Collection and aspiration

Porcine ovaries were collected at an abattoir. The ovaries were collected directly after disposal of the abdominal organs and were immediately placed in a thermos flask containing pre-heated 9 mg/mL NaCl. The temperature in the thermos flask was measured regularly and was kept at approximately 32°C (range: 30-32°C) during collection and transportation of ovaries. The duration between onset of collection at the abattoir and onset of aspiration of the ovaries at the laboratory ranged between 145-390 minutes.

At arrival to the laboratory, the ovaries were washed with NaCl 9 mg/mL and placed in a clean, pre-heated (35°C) thermos flask containing NaCl 9 mg/mL. Subsequently, the ovaries were (in turn) lifted out of the thermos flask and follicular fluid was aspirated using 5 mL syringes and 20 Gauge canulas. Only follicles measuring approximately 3-8 mm were chosen. The aspirated follicular fluid was transferred to 3-4 (depending on number of collected ovaries, range: 42-72) 25 mL universal tubes and diluted in wash medium with heparin (manufactured in the IVF

laboratory, see appendix). The universal tubes and the wash medium with heparin were pre-heated and kept at 37°C during the aspiration process.

Subsequently, oocytes of sufficient quality were selected and matured. All media for use in incubators were pre incubated to allow equilibration at 38°C and 5.5% CO_2 for a minimum of 2 hours.

3.2.2. Selection and maturation of oocytes

Using a P1000 (*i.e.* a 100-1000 µL pipette), the oocytes were aspirated from the bottom of the 25 mL universal tubes and placed into 60 mm petri dishes. Sufficient wash medium (manufactured in the IVF laboratory, see appendix) to cover the bottom of the petri dishes was then added. The petri dishes were then searched for oocytes under stereo microscopes. Oocytes with a distinct, even cytoplasm and a minimum of two layers of cumulus cells (Hirao *et al.* 1994) were selected and washed in three steps using 30 mm petri dishes containing wash medium. Using a 4-well plate containing wash medium and a P10 (*i.e.* a 1-10 µL pipette), the oocytes were randomly assigned into three groups of equal size (range: 28-40 oocytes/group) labelled X (PFHxS group), P (PFOS group), and C (control group). With the use of a P10, the groups of oocytes were separately transferred to, and washed in, a pre-incubated four-well containing 460 µL/well of POM medium (see appendix) with the addition of PFOS (C=0.1 µg/mL) and PFHxS (C=40 µg/mL) respectively. Subsequently, the groups were immediately transferred, in a 20 µL volume of POM medium respectively, to a four-well containing 460 µL/well of POM medium with the addition of (added just prior to oocyte groups): 10 µL of FSH (FSH Porcine, OOPA00171, Insight Biotechnology, Middlesex, United Kingdom) and LH (LH Protein, OOPA00173, Insight Biotechnology) per well as well as 10 µL of dibutyryladenosine cyclic monophosphate (dbcAMP) (dibutyryl-cAMP, sodium salt, 1698950, Biogems, Westlake Village, United States) per well. The final concentration of FSH and LH was 0.05 IU/mL respectively, and the final concentration of dbcAMP was 1 mM. The oocytes were then incubated for 22 hours.

After the initial incubation, the oocyte groups were separately transferred in 20 µL of POM medium to a second, pre-equilibrated four-well plate containing 480 µL/well of POM medium with added PFOS (C=0.1 µg/mL) and PFHxS (C=40 µg/mL) respectively. The oocytes were then incubated for a further 23 hours, without any hormones or dbcAMP.

3.3. *In vitro* fertilization

3.3.1. Preparation of sperm

During fertilization, all manual work was performed in a LAF hood set to 37.3°C. (with the exception of procedures involving Porcicoll (see below), sperm counting and handling of the sperm straw). The porcine fertilization medium (PFM) used was always pre-incubated at 38.5 °C and 5.5% CO_2 for a minimum of two hours.

Semen from the same boar was utilized throughout the experiment to fertilize the oocytes from all batches. The semen was stored in a liquid nitrogen (LN_2) tank. Prior to each fertilization, one straw containing 0.5 mL of semen was collected from the LN_2 tank and transported to the IVF laboratory in a thermos flask containing sufficient LN_2 to cover the entire straw. At arrival to the laboratory, the semen straw was defrosted in regular tap water (35°C) for 30 seconds.

Thereafter, the semen was diluted in a swim up tube containing 4 mL of porcine fertilization medium (PFM, see appendix). Approximately one drop of semen was directly placed on a slide under the stereo microscope. Motility was visually controlled, and the presence/absence of motility was recorded. If the sperm were motile, 2 ml of the semen dilution was carefully added to a 15 ml centrifuge tube containing 4 mL of single layer colloid (SLC) (Porcicoll, prof. Jane Morrell, Uppsala, Sweden) using a P1000. Care was taken not to place any of the semen dilution under the surface of the Porcicoll. The 15 mL tube was then centrifuged for 20 minutes at 300 x G, which caused a “sperm pellet” to form at the bottom of the tube. Using a P1000, excess sperm and Porcicoll were eliminated from the tube. The pellet was then transferred to a swim up tube containing 0.75 mL of PFM.

Using a light microscope and the sperm dilution of which the preparation is described above, sperm was counted in a Bürcher chamber. The concentration was calculated. A dilution of 1.2 x 10e6 spermatozoa/mL was obtained by adding sperm to PFM.

3.3.2. Fertilization

The four-wells containing PFM described below were always pre-incubated for a minimum of two hours in 5.5% CO_2 and 38.5°C.

The oocyte groups, now matured, were transferred from the POM medium to be washed in a four well containing 480 µL/well of PFM (wells labelled X, P, and C, respectively). They were then transferred, in 20 µL of medium/group, to a four well containing 400 µL/well of PFM (wells labelled X, P, and C, respectively). Sperm

solution (described in previous excerpt) was then added to the wells. Volume of sperm solution was adjusted according to a dilution table. The volumes of the wells were, if needed, adjusted to obtain a final volume of 500 µL/well. The spermatozoa and oocytes were then incubated for 24 hours.

3.4. Culturing

After fertilization and incubation, the oocyte groups were transferred to a 4 well with 500 µL wash media/well. The oocytes were then denuded, *i.e.* cumulus cells were removed by pumping with a P100 (*i.e.* a 50-100 µL pipette). One 4 well per oocyte group was used, meaning that each oocyte group was washed through 4 wells.

The oocyte groups were then washed in a 4 well containing 480 µL/well of porcine zygote media (PZM, see appendix 2). Subsequently, the groups were finally transferred in 20 µL/group to a 4 well containing 480 µL/well of PZM and a top layer of 400 µL/well of IVF oil (IVF Bioscience, Falmouth, United Kingdom) and incubated in 38.5°C, 5.5% CO_2 and 6% O_2 for a further 24 hours**.** Before transfer of presumed zygotes, both 4 wells were pre-incubated in 38.5°C and 5.5% CO_2 for a minimum of 2 hours.

3.4.1. Assessment of blastocyst development

On day 2 after fertilization, development was assessed. The percentage of zygotes that had reached 2-cell stage and >2 cell stage, respectively, was recorded for each group. On day 5 and 6, blastocyst development was assessed anew (at 120 and 144 hours post fertilization). Number of blastocysts was recorded, as well as development stage; all blastocysts were labelled as early blastocyst, blastocyst, expanding blastocyst, or hatching blastocyst (see figure 6) (Wright 2010). Blastocyst staged as “early” or “blastocyst” were assigned number 1, and blastocysts staged as “expanding” or “hatching” were assigned number 2 for statistical analyses. Blastocysts were also graded (grade 1: excellent or good quality, grade 2: fair quality, grade 3: poor quality, grade 4: dead or degenerating) (Bo & Mapletoft 2013). When suitable, half grades (*i.e.* 1.5 and 2.5) were assigned. Grade 4 was not used in this study, *i.e.* only grades 1-3 were documented.

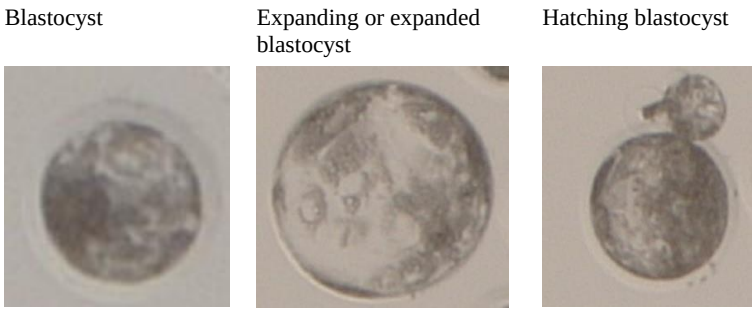

Figure 6. Examples of blastocysts of different stages.

3.5. Fixation and staining

3.5.1. Fixation

On day 6 after fertilization, embryos were fixated overnight at 4°C in 500 µL 2% paraformaldehyde in phosphate buffered saline (PBS). They were then washed twice in 500 µL PBS with 1% polyvinyl alcohol (PVA). Subsequently, they were placed in wells containing approximately 650 µL of PBS with 1% PVA and stored at 4°C in anticipation of staining.

3.5.2. Staining of blastocysts

For staining of apoptotic nuclei, a TUNEL staining kit (*In Situ* Cell Detection kit, TMR red, 12156792910, Roche, Mannheim, Germany) was used. Antifade mounting medium with DAPI (2-(4-amidinophenyl)-1H-indole-6-carboxamidine) (Vectashield with DAPI, H-1200, Vector Laboratories, Burlingame, United States) was used to stain all nuclei.

Staining protocol

All samples and controls were stained according to recommendations from manufacturers. Two-three batches of blastocysts were stained in each staining session. After fixation and washing, blastocysts were permeabilized in room temperature for 1 hour in a solution containing 0.5% surfactant (Triton X-100, 9002-93-1, Sigma Aldrich, St Louis, United States). The surfactant was dissolved in PBS with 0.1% Na Citrate and 1% PVA (prepared fresh for each staining session). Blastocysts were then washed twice in PBS with 1% PVA.

Two blastocysts per staining session were randomly selected to serve as controls. These were added to 90 µL of deoxyribonuclease (DNase) (DNase I, 1073395, Qiagen, Hilden, Germany) solution with the concentration 0.1 UI/µL (in tris

buffer), prepared before each staining. The controls were then left to incubate at 37°C for 1 hour in darkness. Subsequently, they were washed twice in PBS with 1% PVA.

The samples and the positive controls were then incubated at 37°C for 1 hour in darkness in 50 µL of the TUNEL solution (enzyme diluted 1:10 in nucleotide). The negative controls were incubated under the same circumstances but in 50 µL of nucleotide only. The negative controls and samples (including positive controls) were added to the nucleotide and the TUNEL solution (respectively) in 10 µL PBS with 1% PVA. After incubation, they were washed twice in PBS with 1% PVA.

After the TUNEL reaction, blastocysts were placed in black well plates (Diagnostic microscope slides 6.7 mm, ER-208B-CE24, Thermo Scientific, Braunschweig, Germany) in 2 µL PBS with 1% PVA, and 2µL of antifade mounting medium with DAPI was added to each well now containing blastocysts. The wells were covered with cover glasses and sealed, immediately after mounting, using nail polish. Care was taken to expose TUNEL-stained and DNase-treated blastocysts to as little light as possible. Sealed black well plates were stored in darkness and 4°C in anticipation of confocal microscopy.

3.6. Confocal microscopy and image analysis

The blastocysts, now fixated, stained and sealed in black well plates, were scanned using a confocal microscope (LSM 800, Zeiss, Oberkochen, Germany). Each blastocyst was individually imaged using z-stack, *i.e.* portrayal of the three-dimensional structure through multiple sections. A section thickness of 2 µm was used for the imaging of all blastocysts. All images were taken using the 20x objective. The 405 and 561 lasers were used.

The z-stacks were used to obtain a 3D image of each embryo and analysed in Fiji for ImageJ (Schindelin *et al.* 2012). A macro for ImageJ (PerObjectEllipsefit) (Ranefall *et al.* 2016) was adapted to identity nuclei and apoptotic cells automatically. An apoptotic cell was defined as both TUNEL-positive and DAPI-stained (hence, nuclei stain and apoptotic stain overlapping). Manual confirmation of each embryo was performed by the same person, and blinded. The nuclei were found to correspond well to the manual validation, and for statistical analyses, total numbers of nuclei obtained by the macro were used. One embryo was excluded due to issues regarding nuclei validation. Additionally, four embryos were excluded from further analyses due to degeneration. The apoptotic cells interpreted by the macro were validated manually and nuclei that were stained but not validated (for

example, remaining sperm heads) were excluded. The manually corrected proportions of apoptotic cells were used for the statistical calculations.

3.7. Statistical analyses

Prior to statistical analyses, blastocysts staged as “early blastocyst” or “blastocyst” were assigned stage=1, and blastocysts staged as “expanding/expanded blastocyst” or “hatching blastocyst” were assigned stage=2. Logistic regression (programme: R, version 1.1.453) was used to evaluate the effect of treatments on the following parameters: proportion cleaved 48 hours post fertilization, proportion cleaved above the 2-cell stage 48 hours post fertilization, blastocyst rate on day 5, blastocyst rate on day 6, blastocyst stage, and blastocyst grade. On parameters proportion cleaved, proportion cleaved over 2-cell stage, and blastocyst rate (on day 5 and 6, respectively), binominal distribution was used. On the parameters blastocyst grade and blastocyst stage, cumulative link mixed models were used.

P-values <0.05 were considered significant. Results are presented as odds ratio (OR) +/- standard deviation (SD) if not stated otherwise.

For the statistical analysis of proportion of apoptotic cells, the programme Minitab (version 19.2020.1) was used. The data obtained through image analysis of z-stacks were used for total number of nuclei. However, for apoptotic cells, numbers counted in manual validation were used. Random effects for batch and total number of nuclei were used. For analysis of total numbers of nuclei as an effect of treatments, random effect for batch was used. P-values <0.05 were considered significant.

4. Results

Of the total 855 COC:s that were included in this study (COC:s/group, range: 28-40), 341 were found to be cleaved (2-cell stage or higher) 48 hours post fertilization, and 181 had developed beyond the 2-cell stage at the same time. A total of 158 presumed resulting blastocysts were produced. Furthermore, 146 presumed blastocysts were imaged as z-stacks using confocal microscopy and analysed in regards of proportion of apoptotic cells.

4.1. Embryo development

No significant differences between PFAS-treated groups and the control group were observed in cleavage rate or cleavage rate >2 cells. Both PFOS and PFHxS groups had very slightly higher OR (odds ratio) of cleavage than the control group (see table 1), but this was not significant in any of the cases (p=0.86 and p=0.58, respectively) (see figure 7). When it comes to cleavage rate above 2 cells, both treated groups had slightly lower OR of cleavage than control group (see table 1), but this was not significant in any of the cases (p=0.84 (PFOS), p=0.48 (PFHxS)). However, the PFHxS-treated blastocysts tended to have higher blastocyst rate than the control group (OR: 1.688, p=0.07) on day 5 post fertilization, and on day 6 post fertilization, the PFHxS-treated group had significantly higher blastocyst rate than the control group (OR: 1.555, p=0.05). There were no significant differences in blastocyst rate in day 5 nor 6 between the PFOS-treated group and the control group (p=0.31 and p=0.17, respectively) (see figures 8 and 9, as well as table 1).

*Table 1. Mean embryo development parameters (proportions) and odds ratios. Calculated from total number of COC:s (with exception: proportion of cleavage >2 cells calculated from embryos having reached at least 2 cell stage 48 hours post fertilization. *Tendency (higher compared to control group, p=0.07). **Significant value (significantly higher compared to control group, p=0.05).*

Parameter	**Mean (SD)**			**OR (min, max)**		
	Control group	**PFHxS group**	**PFOS group**	**Control group**	**PFHxS group**	**PFOS group**
Cleavage rate	0.379 (0.091)	0.394 (0.150)	0.376 (0.170)	1.000	1.100 (0.784, 1.543)	1.030 (0.733, 1.448)
Cleavage rate >2 cells	0.588 (0.274)	0.498 (0.234)	0.587 (0.285)	1.000	0.862 (0.570, 1.300)	0.958 (0.637, 1.440)
Blastocyst rate day 5	0.074 (0.063)	0.115 (0.070)	0.093 (0.070)	1.000	1.688 (0.957, 3.040)*	1.357 (0.751, 2.487)
Blastocyst rate day 6	0.145 (0.075)	0.205 (0.110)	0.186 (0.090)	1.000	1.555 (1.007, 2.420)**	1.367 (0.878, 2.142)

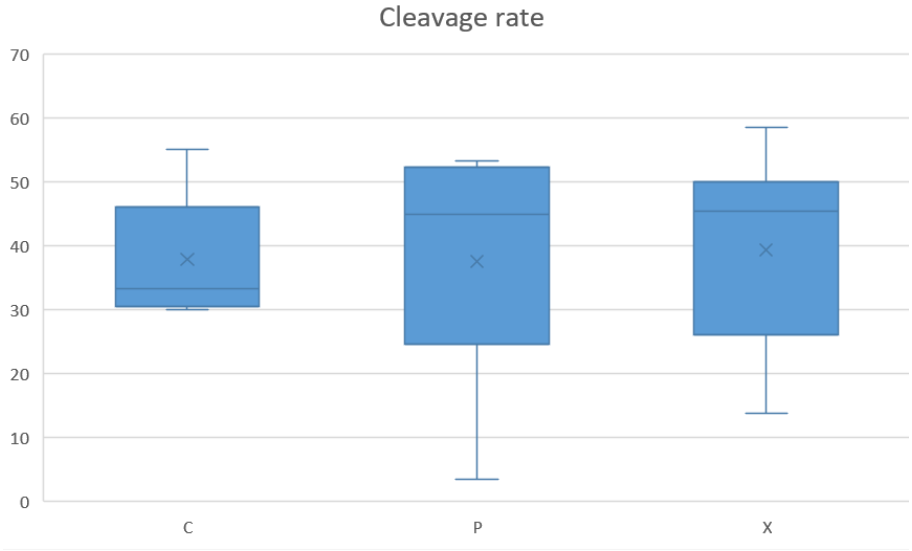

Figure 7. Box plot illustrating distribution of, and mean (x), cleavage rate (in percentage) 48 hours post fertilization for each group. C: control group, P: PFOS group, X: PFHxS group. There were no significant differences in cleavage rate between control group and PFOS group nor PFHxS group.

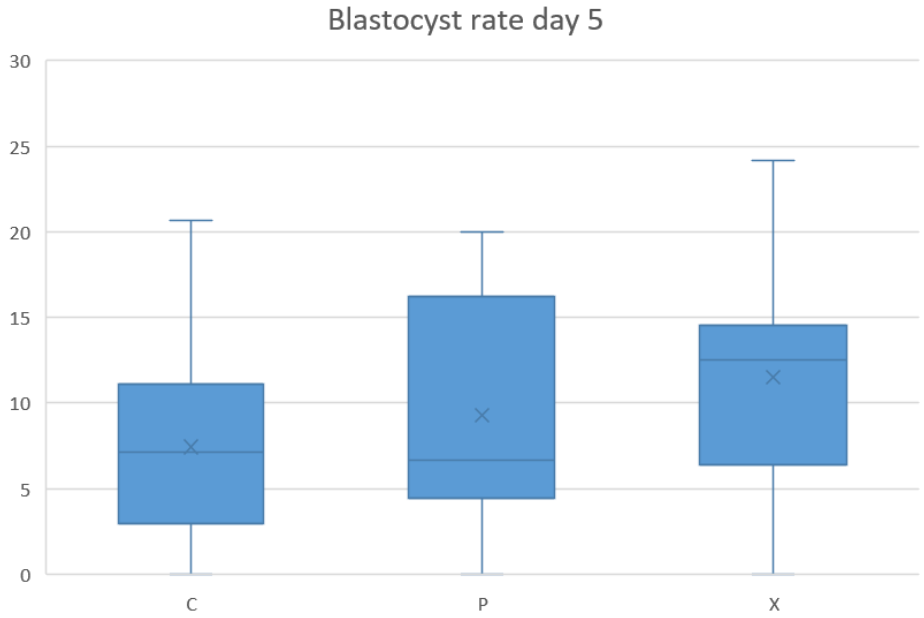

Figure 8. Box plot illustrating distribution of, and mean (x), blastocyst rate (in percentage) on day 5 post fertilization for each group. C: control group. P: PFOS group, X: PFHxS group. The PFHxS group tended to have higher blastocyst rate on day 5 compared to the control group (p=0.07).

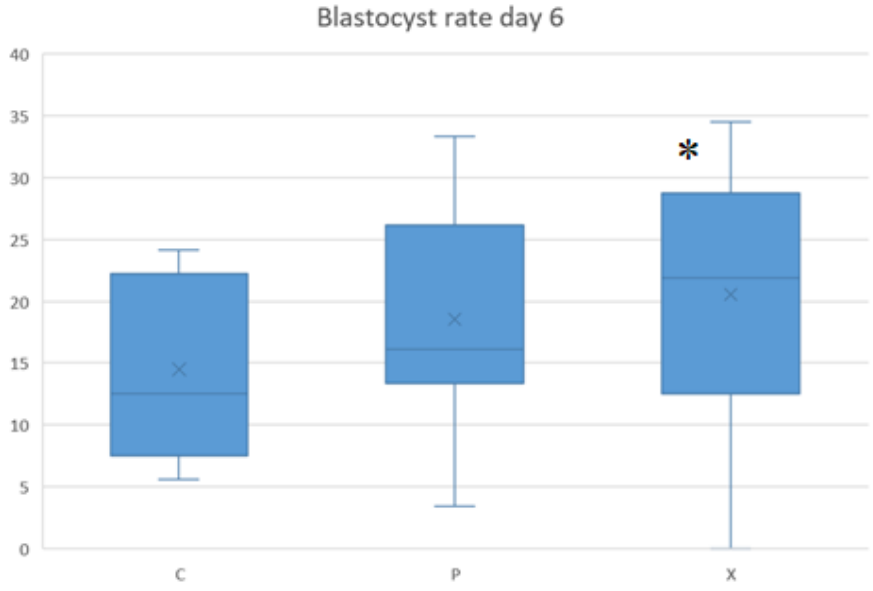

Figure 9. Box plot illustrating distribution of, and mean (x), blastocyst rate (in percentage) on day 6 post fertilization for each group. C: control group, P: PFOS group, X: PFHxS group. The PFHxS group (marked with asterisk) had significantly higher blastocyst rate on day 6 compared to the control group (p=0.05).

Regarding developmental stage of blastocysts, no significant differences between treated groups and control group were seen. There was no risk of changes in stage after exposure to PFOS (OR:0.52, p=0.21) nor PFHxS (OR:1.32, p=0.62). Concerning grade, no risk of changes in grade score were attributable to exposure to PFOS (OR: 0.96, p=0.93) nor PFHxS (OR: 0.78, p=0.56).

4.2. TUNEL assay and apoptotic cells

Of the 4 positive controls that were performed (one for each staining session), all had a TUNEL staining pattern that was (visually) highly similar in shape and distribution to the corresponding DAPI staining pattern of the same blastocyst (see example in figure 10). In the negative controls, no blastocysts were (as expected) visible in the TUNEL channels (see example in figure 11). During manual validation, it was found that the nuclei labelled as apoptotic by the TUNEL assay were morphologically abnormal (*e.g.* fragmented, shrunken, condensated), implying apoptosis (see examples in figures 12 and 13). This indicates that the TUNEL assay of the blastocysts functioned correctly, *i.e.* it labelled exposed 3'-hydroxyl DNA ends accurately.

Concerning image analysis and automated calculations of nuclei and apoptotic cells, the macro used was, through manual validation, deemed to have produced accurate estimations of total numbers of nuclei and apoptotic nuclei in all batches except one. In this batch, some change in staining intensity appeared to be the cause of the incorrect estimations. Because of this, manually obtained numbers of apoptotic nuclei were used to perform statistical analyses for all batches (see examples of interpretations of blastocysts obtained from the macro used in figures 14 and 15). However, for the remaining batches, the macro made satisfactory estimations with manual exclusion mostly of adherent sperm heads to the blastocysts.

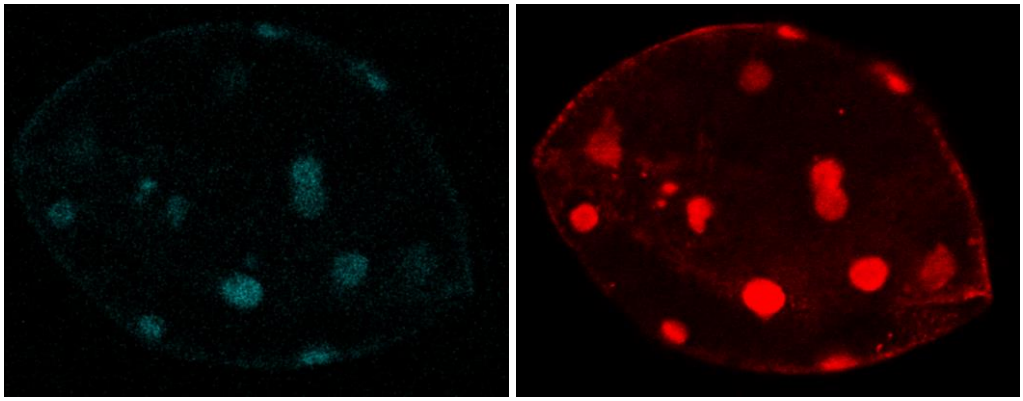

Figure 10. Example of DNase-treated positive control; DAPI channel (left) and TUNEL channel (right). Note that the TUNEL staining pattern corresponds to the DAPI staining pattern, i.e. all nuclei that were stained by DAPI were also stained by the TUNEL assay. The shapes and sizes of nuclei in the DAPI channel are also very similar to those in the TUNEL channel.

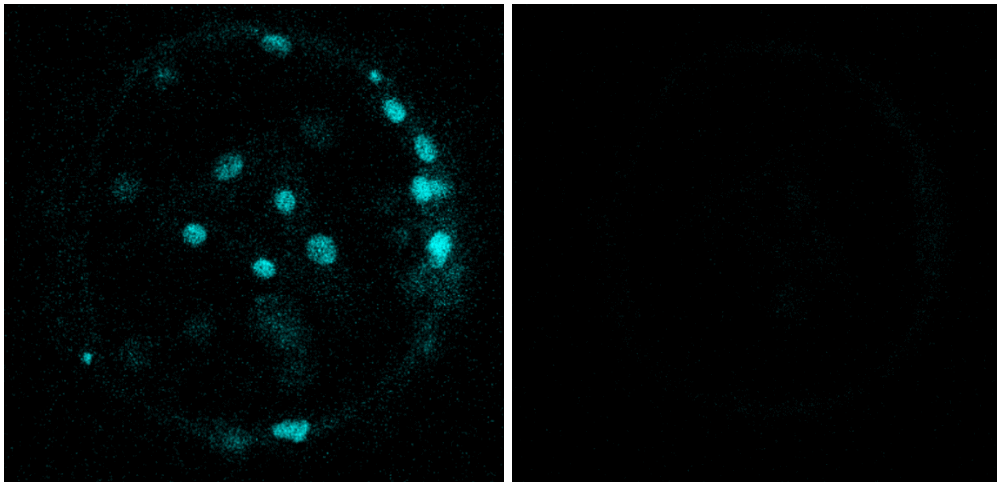

Figure 11. Example of DNase-treated negative control, that was not incubated with the TUNEL reaction mixture, which (as anticipated) resulted in a normally appearing DAPI staining pattern (left) and no visible blastocyst in the TUNEL channel (right).

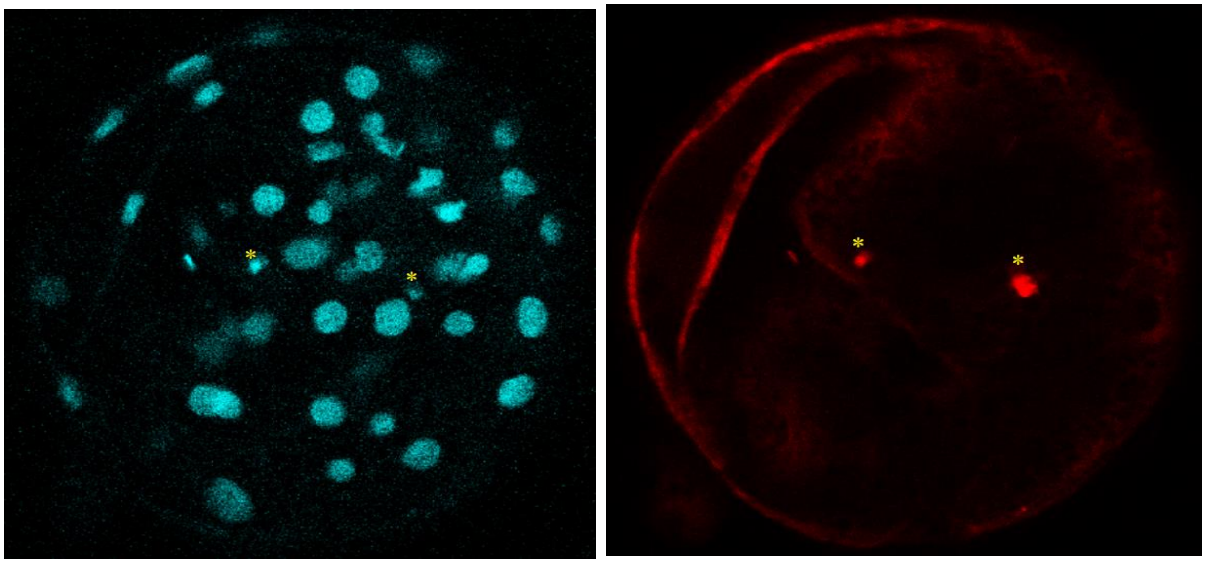

Figure 12. Example of a stained blastocyst, DAPI channel (left) and TUNEL channel (right). In this section of the blastocyst, two apoptotic nuclei are seen (asterisks). Note the abnormally shrunken appearance of the apoptotic nuclei when compared to surrounding, non-apoptotic nuclei in the DAPI channel.

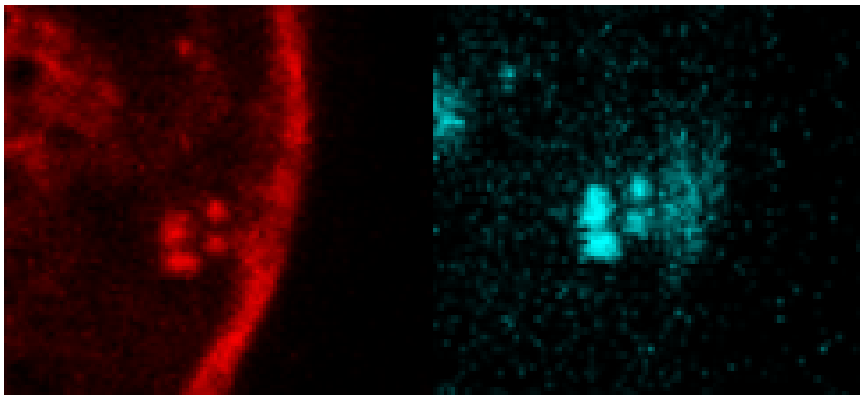

Figure 13. Example of fragmented nucleus in a blastocyst labelled by both TUNEL channel (left) and DAPI channel (right). The fragmented appearance indicates apoptosis, i.e. that the TUNEL assay correctly labelled the nucleus.

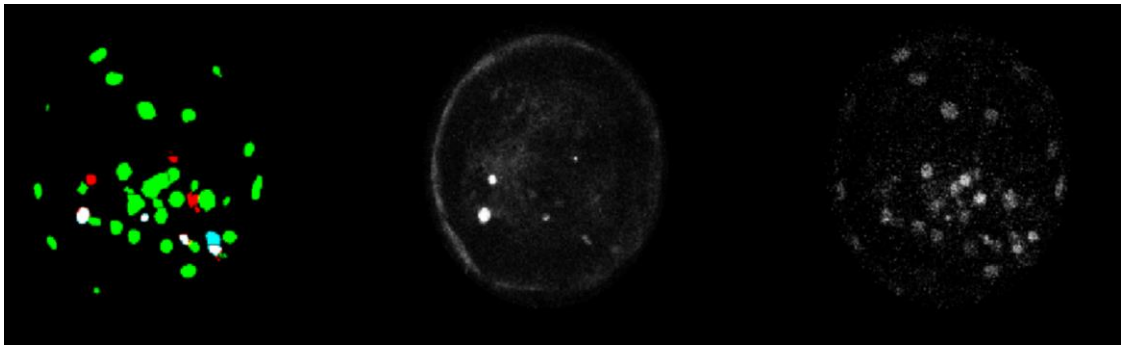

Figure 14. Example of section of z-stack of blastocyst interpreted by the Fiji for ImageJ programme and plugin (left). TUNEL channel (middle) and DAPI channel (right) of the same section are also included for comparison. In this example, the programme has correctly identified 4 nuclei that were labelled as apoptotic (white). The nuclei marked with red by the programme were labelled by the TUNEL assay, however cannot be found in the DAPI channel, and thus, were (correctly so) not identified as apoptotic. Normal nuclei are marked with green by the programme, and this pattern corresponds well with the DAPI channel, indicating that the programme has identified almost all nuclei correctly.

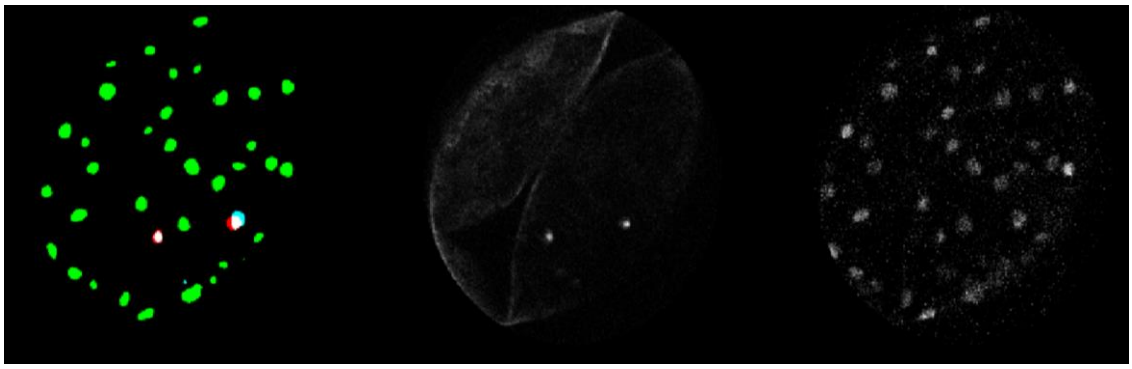

Figure 15. Example of section of z-stack of blastocyst interpreted by the Fiji for ImageJ programme and plugin (left). TUNEL channel (middle) and DAPI channel (right) of the same section also included for comparison. Two nuclei have been correctly identified as apoptotic by the programme (marked in white by programme and clearly visible in both TUNEL and DAPI channels).

During statistical analysis it was found that the number of nuclei in all blastocysts ranged between 11-93. The mean number of nuclei in all embryos was approximately 39. It was also found that larger embryos (*i.e.* embryos that were consisted by larger total amounts of cells) generally contained lower proportions of apoptotic cells ($p=0.01$).

Furthermore, PFHxS tended to result in larger embryos, *i.e.* embryos that contained larger numbers of nuclei than the control group ($p=0.062$, see figure 16). As mentioned, the total number of nuclei was related to the proportion of apoptotic nuclei in blastocysts ($p=0.01$, see figure 17). When this was taken into account, embryos in the PFHxS group also tended to have higher proportions of apoptotic

cells (p=0.084, see figure 18). For the PFOS group, embryos did not differ from the control group in terms of total number of nuclei (p=0.29) nor proportions of apoptotic cells (p=0.28).

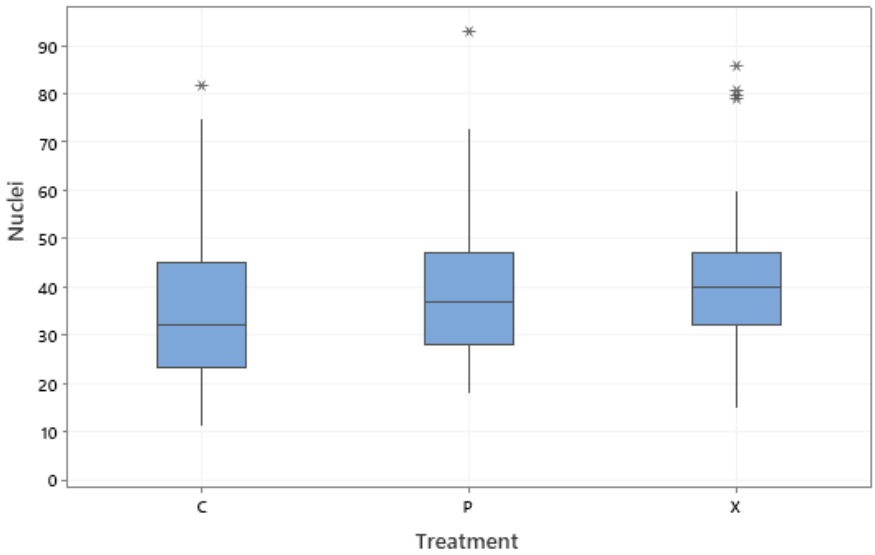

Figure 16. Box plot illustrating the relationship between treatment and number of nuclei in blastocysts (C: control group, P: PFOS group, X: PFHxS group). When batch was used as random effect, blastocysts in the PFHxS group tended to contain larger total numbers of nuclei (p=0.062) than blastocysts in the control group. PFOS had no effect on this parameter (p=0.29).

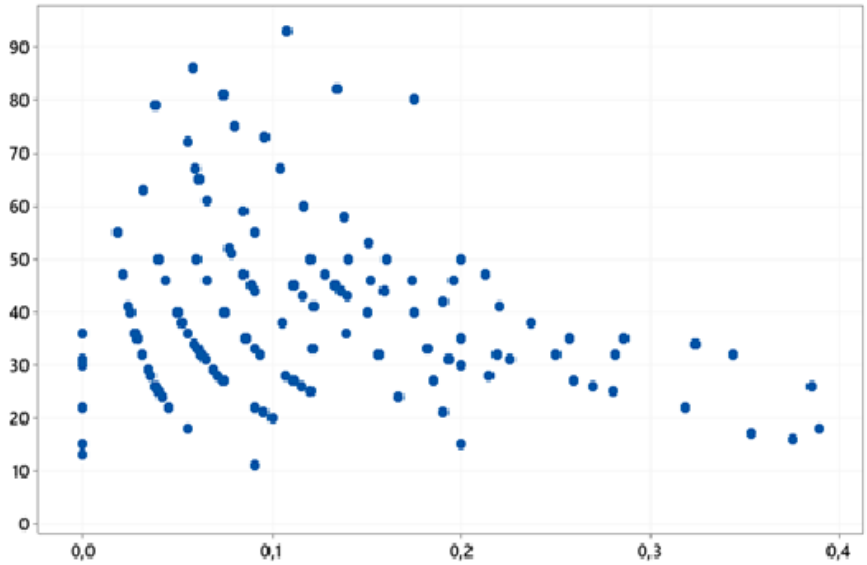

Figure 17. Scatter plot illustrating the relation between total number of nuclei (Y axis) and proportion of apoptotic cells (X axis) in blastocysts. Blastocysts with larger total numbers of nuclei had significantly lower proportions of apoptotic nuclei (p=0.01).

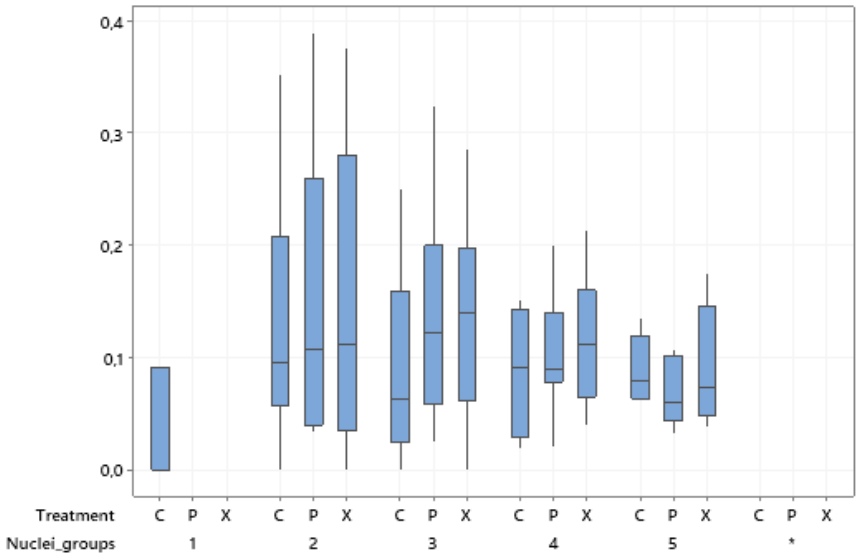

Figure 18. Box plot illustrating the proportion of apoptotic cells (Y axis) in blastocysts in relation to treatment (X axis, C: control group, P: PFOS group, X: PFHxS group). To illustrate the difference in proportion of apoptotic cells (that is related to the size of the embryo), blastocysts are divided into groups based on total numbers of nuclei (X axis, group 1: <15 nuclei, group 2: 15-30 nuclei, group 3: 30-45 nuclei, group 4: 45-60 nuclei, group 6: >60 nuclei). Blastocysts in the PFHxS group tended to have larger proportions of apoptotic cells than blastocysts in the control group (p=0.084). PFOS had no effect on the proportion of apoptotic cells (p=0.28).

5. Discussion

In this study, the effects of PFHxS and PFOS on early embryo development and apoptosis in *in vitro* produced porcine embryos were investigated. Oocytes were exposed to these compounds during maturation. Following IVF, zygotes were cultured, and resulting blastocysts were stained using a TUNEL assay in order to detect apoptotic cells.

Differences between treated groups and control group regarding cleavage rate, cleavage rate above 2 cells, blastocyst stage, and blastocyst grade were not significant, *i.e.* the treatments had no effects on these parameters. Furthermore, PFOS had no effect on blastocyst rate on day 5 nor 6 post fertilization. However, the PFHxS group had a significantly higher blastocyst rate than the control group on day 6 post fertilization and tended to have a higher blastocyst rate than the control group on day 5 post fertilization. Additionally, PFHxS resulted in significantly larger blastocysts, and larger blastocysts were found to have significantly lower proportions of apoptotic nuclei. When taking this into consideration, addition of PFHxS during oocyte maturation tended to result in a larger proportion of apoptotic cells in blastocysts when compared to the control group.

5.1. Fertilization and cleavage

The results of this study can be regarded in relation to results of previous experiments. Firstly, in this study, there were (as mentioned) no significant differences in cleavage rate when comparing treated and non-treated groups. The mean cleavage rates of each group were also very similar, further indicating no effect from treatments on this parameter, although there was a larger distribution of values in the treated groups compared to the control group (see figure 7). In one previous study involving IVP of embryos, where maturing bovine oocytes were exposed to PFNA, a significantly lower cleavage rate was seen in the treated group (Hallberg *et al.* 2019). Thus, it has previously been showed that PFAS (although not the molecules investigated in this study) can in fact have effects on cleavage rates in *in vitro* produced embryos. It is not possible to surely determine whether PFOS and/or PFHxS can alter these processes in early embryo development. Although not highly

probable, it cannot be entirely precluded that *e.g.* a larger study sample, or less variation (in terms of oocyte quality) within groups and/or between groups (see discussion concerning study design below), would have resulted in significant differences between groups regarding this parameter. It is also, naturally (which is indicated by the results in this study), possible that exposure to PFOS and PFHxS during the maturation phase of oocytes do not have any effects on cleavage in porcine embryos.

When it comes to cleavage rate above the 2-cell stage, no significant differences between treated and non-treated groups were seen, although the PFHxS group exhibited a lower mean proportion of embryos above the 2-cell stage 48 hours post fertilization than both the PFOS group and the control group, which had very similar mean cleavage above 2-cell stage rates (see table 1). It must also be noted that standard deviations in this parameter were relatively high for all groups (see table 1). It cannot be precluded that a larger study sample, and/or less heterogenous material, would have given rise to different results.

5.2. Blastocyst development

Furthermore, as mentioned, PFHxS treatment tended to result in a higher blastocyst rate on day 5 post fertilization ($p=0.07$) and resulted in a higher blastocyst rate on day 6 post fertilization ($p=0.05$) when compared to the untreated group. In the 2019 study by Hallberg *et al.*, PFNA treatment resulted in lower blastocyst rates, indicating that PFNA had a suppressing effect on embryo development. Furthermore, in a study involving porcine oocytes by Dominguez *et al.* (2016), PFOS treatment resulted in impaired oocyte maturation as well as significantly decreased survival rates of oocytes (which, if these oocytes would have been fertilized, could possibly be assumed to have resulted in decreased blastocyst rates). In this study, however, PFHxS appears to have had a stimulant effect on blastocyst development, which, accordingly, could be said to stand in conflict to these previous results. This could possibly be attributed to the fact that PFNA (and, although to a lesser extent, PFOS) has a different molecular structure than PFHxS (and thus, possibly, different effects on living organisms), although exact mechanisms of PFAS toxicity are still incompletely understood (Behr *et al* 2020), *i.e.* this is difficult to investigate further. It is also important to note that the study by Hallberg *et al.* (2019) uses the bovine as model animal (as opposed to this study, which uses the pig); species differences could possibly have given rise to different results. It cannot be precluded that, for instance, the porcine species has lower sensitivity to PFAS than bovines. This matter also needs to be further researched before definitive conclusions can be drawn. Furthermore, in the studies mentioned, different concentrations of PFAS are used, which, likely, have impact on results (discussed later).

Moreover, the potential effect of PFHxS as a PPAR agonist can be discussed. As mentioned, PPAR agonists have been attracting attention as potential treatments of some conditions involving female infertility and anovulation (Xu *et al.* 2017). Additionally, PFHxS has showed evidence of being able to activate certain types of PPAR receptors (Behr *et al.* 2020). These aspects could be discussed as a possible reason to the fact that PFHxS appears to have had a stimulatory effect on early embryonic development in this study. As mentioned, the concentrations of PFAS that organisms are subjected to could possibly be of importance for the outcomes. It cannot, for instance, be precluded that lower doses of PFAS could have a stimulatory effect on *e.g.* embryo development, while higher doses would be detrimental. Further research on exact mechanisms of actions of PFAS, and if/how the above-mentioned previous findings could be of relevance in early embryo development, are needed.

Concerning developmental stage and grade of blastocysts, no effects of treatments were seen in this study. This is consistent with results from the previously mentioned 2019 study by Hallberg *et al.*, where no effects on blastocyst stage or grade following exposure to PFNA were seen.

5.3. Apoptosis and nuclei

Regarding assessment of z-stacks of blastocysts with stained nuclei, PFHxS tended to result in larger embryos (in terms of total number of nuclei). Furthermore, blastocysts consisted by larger total numbers of nuclei contained significantly lower proportions of apoptotic nuclei. This can be viewed as highly logical; all blastocysts were cultured for the same amount of time. However, the total number of nuclei in blastocysts ranged between approximately 10-90. Thus, it is clear that there were fluctuations among embryos regarding what developmental point was reached. It can be regarded as reasonable to assume that larger embryos that reached further developmental stages were more viable, and thus, contained lower proportions of apoptotic cells.

Furthermore, PFHxS-treated blastocysts tended to contain higher proportions of apoptotic cells than blastocysts in the control group. This trend was enhanced when the fact that larger embryos generally contained lower proportions of apoptotic cells was taken into account (see above). It should be noted that large variations within each group were present (see figure 18), and it cannot be precluded that less heterogenous study material and/or a larger study sample would have resulted in statistically significant results in this parameter. More studies on the effect of

PFHxS on apoptosis in early embryo development are needed before definitive conclusions can be drawn.

Moreover, PFOS did not tend to have any effect on this parameter. In an earlier study, PFOS treatment resulted in significantly higher proportions of apoptotic cells in wild-type zebra fish embryos (Sant *et al.* 2018). This could be said to correspond to the results of this study, as the molecular structures for PFHxS and PFOS are similar, and thus, they could be expected to have similar effects (*i.e.* elevated rates of apoptosis, see above). However, the fact that PFOS had no effect on apoptosis in this study could be said to be standing in conflict with the results obtained by Sant *et al.* (2018). As mentioned, species differences, as well as the fact that different concentrations are used in different studies, should always be considered as a factor that could give rise to conflicting results when comparing studies where different animal models and/or concentrations of PFAS were used.

When taking all the above-mentioned results into consideration, it appears as PFHxS has had a stimulant effect on embryo development (as it resulted in a significantly larger number of embryos that tended to be larger in size). However, PFHxS also tended to result in elevated apoptosis rates. This indicates that PFHxS alters, rather than impairs, early embryo development in the pig. Here, it is of great importance to emphasize that this is an *in vitro* study that only evaluates a relatively brief period of embryo development. From the results that were obtained, it is not possible to determine how/if *e.g.* later stages of embryonic and foetal development and/or the health of live offspring would have been affected by the treatments used. However, as embryo development was altered, it is reasonable to suspect that some adverse effects could be present later in development, and further studies are needed to investigate these matters.

In this study, the PFOS treatment had no significant effect on any of the parameters investigated, although it is a chemical with well documented adverse effects on living organisms. It has (as recently mentioned) been showed to result in increasing apoptosis rates in zebra fish (Sant *et al.* 2018), and in impaired oocyte maturation and survival in porcine oocytes (Dominguez *et al.* 2016). In addition, its use has been restricted in some parts of the world because of known toxic effects in humans (KemI 2015). With this in consideration, it could be intuitive to hypothesize that significant impairment should be seen in several developmental parameters in a study that exposes living cells to relatively high concentrations of this chemical. However, in this study, this was not the case. This could, possibly, partly be explained by species differences (discussed earlier). Although the pig and the human are often seen as relatively similar, the possibility of porcine cells being less sensitive to PFOS compared to human cells cannot be precluded. In the case of this

being true, the pig could be viewed as unsuitable to serve as a model for humans when studying toxicological effects of PFOS, as similar responses are desirable in these studies. When it comes to zebra fish, there are many large and obvious differences when compared to mammalian species, which could possibly explain differences in response to PFOS. However, in the 2016 study by Dominguez *et al.*, PFOS had significant, impairing effects in porcine oocyte maturation and survival, which could be assumed to have effects on resulting embryos in the case of fertilization. This could possibly be explained by the fact that the material used for this study is relatively heterogenous, which could have affected results (see discussion regarding study design below), and also by differences in PFOS concentrations used.

5.4. Staining and image analysis

In this study, 146 embryos were stained using the TUNEL assay (for apoptotic nuclei) and DAPI (for all nuclei). DNase-treated positive controls were used to confirm that the assay was functioning appropriately (*i.e.* DNase treatment created DNA breaks, causing the TUNEL assay to stain all nuclei in positive controls). In all positive controls that were performed, the TUNEL staining pattern appeared identic to the DAPI staining pattern (assessed manually), indicating that the TUNEL assay functioned appropriately throughout the experiment, *i.e.* it labelled exposed 3'-hydroxyl DNA ends in blastocyst nuclei correctly.

The TUNEL assay is based on labelling of exposed 3'-hydroxyl DNA ends in nuclei of apoptotic cells. However, as mentioned, these DNA ends can be exposed for reasons unrelated to apoptosis, which can potentially give rise to false positive results (Loo 2011). For this reason, it has been argued that it could be of benefit to use other staining techniques along with TUNEL with the purpose of confirming apoptosis (Kyrylkova *et al.* 2012). As discussed, however, nuclei of apoptotic cells generally have certain morphological characteristics (*i.e.* a shrunken appearance) (Kyrylkova *et al.* 2012), which was exploited to visually confirm apoptosis. However, it can be assumed that some degree of uncertainty regarding this matter is still present.

When it comes to analysis of z-stacks of blastocysts, computerized and automated methods were used, which adds some degree of uncertainty. However, as mentioned, all blastocysts were controlled manually after analysis, and embryos where numbers of nuclei could not be assessed with certitude were excluded prior to statistical analyses. It should also be mentioned that during the usage of computerized methods, the same settings were used for all images, which is an advantage in relation to manual counting.

5.5. In vitro model, PFAS concentrations and implications for human health

5.5.1. *In vitro* production of embryos

In vitro production (IVP) of embryos includes many steps, and different approaches and study designs can be used. It is reasonable to assume that these matters can influence final results in studies involving IVP of embryos.

Firstly, the choice of animal species for this study can be discussed. When performing toxicological studies, it is generally regarded as beneficial to use animal models that physiologically resemble humans. The pig is often regarded as an animal that, in many ways, is relatively similar to the human species (Romar *et al.* 2019). However, there are species differences regarding points at which some important steps of embryo development take place. For instance, hatching from the ZP takes place approximately 3 days later in the pig than in the human (Geisert *et al.* 2015). However, establishment of the 2-cell stage takes ca. 24 hours in both species (McGeady *et al.* 2006). Thus, there are both differences and similarities between porcine and human embryos during the developmental phases that are studied here. However, it is unclear whether these differences influence the effects of PFOS and PFHxS on the development of embryos in the two species.

Furthermore, the influence of PFAS concentrations used can be discussed. In this study, groups of maturing oocytes were subjected to 40 µg/mL of PFHxS and 0.1 µg/mL of PFOS, respectively. Viewed in relation to mean PFOS and PFHxS levels detected in human body fluids in previous studies (e.g. PFOS: 4.8 ng/mL in follicular fluid and PFHxS: 1.7 ng/mL in follicular fluid (Kim *et al.* 2020)), the concentrations used in this study can be regarded as very elevated. Although, it is important to note that there are large variations in measured PFAS concentrations in human body fluids. For instance, Zhang *et al.* (2018) detected PFOS concentrations in human plasma ranging from 1.03 to 47.8 ng/mL. Furthermore, following events of heavy environmental contamination, people can be subjected to extremely elevated PFAS concentrations (Li *et al.* 2017). With this in consideration, it can be viewed as appropriate to evaluate the effects on physiological processes of relatively elevated PFAS concentrations. It is also generally regarded as desirable to, to some degree, employ "safety margins" when evaluating toxicological effects on humans (particularly when using animal models), which, in practice, translates to using elevated concentrations of (potentially) toxic compounds in experiments of this nature. Furthermore, in this study, COC:s were only exposed to PFAS during their maturation phase, *i.e.* for 45 hours. In *in vivo* conditions, it is safe to assume that oocytes can be exposed to PFAS for

significantly longer periods of time. For these reasons, it can be argued that relatively elevated concentrations of PFAS should be used in *in vitro* studies evaluating toxicological effects, as an attempt to compensate for this difference in exposure time. However, oocyte maturation could possibly be a particular "window" where oocytes (and resulting embryos) have increased sensitivity to PFAS. In this case, it should be sufficient to only expose oocytes to relevant doses of PFAS during this period and still obtain physiologically relevant results. More studies on this subject are needed.

Moreover, when executing studies of this genre, some external factors that can potentially influence the results have to be taken into consideration. *In vitro* production of embryos involves many steps, of which some can have impact (which cannot be controlled) on results. Firstly, it is reasonable to believe that the quality of ovaries (and thus, oocytes that can be aspirated) obtained from the abattoir for each batch differs significantly; both within and between batches. It could be argued that eventual variations within batches should not impact the results greatly, as oocytes were randomised into three groups (control group, PFOS group and PFHxS group) for each batch, which should aid in distributing oocytes of varying quality evenly between groups. However, it should be noted that this randomisation was based solely on manual observation of COC:s, *i.e.* they were divided based on visual appearance with the aim of obtaining equal groups. In spite of this effort, it can most likely not be precluded that there were variations in terms of *e.g.* COC quality between groups within the same batch, which, naturally, could have had impact on the final results, although it is not possible to know if/to what extent the results were affected. It should also be pointed out that in this study, embryos and batches were not excluded without extraordinary reasons. This choice of inclusion criteria can result in isolated data points that differ substantially from the remaining values describing the same parameter. Thus, it is possible that more strict inclusion criteria would have resulted in somewhat different results.

With all of the above in consideration, it also needs to be highlighted that the PFAS investigated in this study have previously documented negative effects on female reproduction, including increased risk of premature ovarian insufficiency (Zhang *et al.* 2018), irregular menstrual cycles (Zhou *et al.* 2017) and early menopause (Taylor *et al.* 2014). Thus, it is reasonable to assume that PFOS and PFHxS can (notwithstanding the results of this study) have negative effects on human reproduction, and thus, to at least some extent, consequences on human health and wellbeing. It is also important to point out that PFOS and PFHxS are far from the only compounds that humans are subjected to. This also applies to PFAS in general; other groups of substances with suspected, and documented, adverse health effects can be found in the environment. Accordingly, there may be so-called "cocktail

effects" that are unknown, which also needs to be taken into consideration when interpreting results from this study, and other studies evaluating the effects of single substances. More studies are needed to investigate effects from simultaneous exposure to multiple compounds.

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Populärvetenskaplig sammanfattning

Ofrivillig barnlöshet är mycket vanligt bland par över hela världen. Många faktorer kan påverka fertiliteten, men på senare år har s.k. hormonstörande ämnen och deras betydelse för fruktsamheten börjat diskuteras och studeras. Högfluorerade ämnen (PFAS) är stor grupp av kemikalier med fett- och vattenavstötande egenskaper som har använts i produkter som t.ex. brandskum och textilier i flera decennier. Flera PFAS har i studier visat sig påverka levande organismer på flera olika sätt.

Målet med denna studie var att undersöka hur tidig embryoutveckling samt celldöd påverkas av två olika typer av PFAS, med grisen som modelldjur för människan. Grisen är ett djur som generellt anses vara relativt likt människan, varför den av många anses vara lämplig som modelldjur i denna typ av studier. Eftersom embryon framställdes genom provrörsbefruktning, eller IVF, behövdes inga försöksdjur till studien.

Äggstockar från grisar av honkön samlades in från ett slakteri. Från dessa sögs äggceller ut med hjälp av sprutor och kanyler. Utvalda äggceller fick sedan mogna under sammanlagt 45 timmar, med tillsats av perfluorooktansulfonsyra (PFOS) respektive perfluorohexansulfonsyra (PFHxS). En kontrollgrupp, som inte utsattes för några sådana tillsatser, fanns också med i studien. Efter mognadsprocessen befruktades äggcellerna med spermier från en galt. Spermierna förvarades frysta i s.k. plaststrån, i flytande kväve, och tinades upp inför varje befruktning. De befruktade äggcellerna fick sedan utvecklas till embryon under 6 dagar. Under utvecklingsprocessen dokumenterades hur stor andel av embryona som delats till 2 eller fler celler 48 timmar efter befruktning, samt hur många som utvecklats till blastocyster (ett utvecklingsstadie hos embryon) på dag 5 respektive 6 efter befruktning. Vid dessa tidpunkter bedömdes även blastocysterna med avseende på kvalitet och mognadsgrad.

På dag 6 efter befruktning samlades de embryon som utvecklats till blastocyster ihop, fixerades och färgades med två typer av färgningstekniker. Den ena tekniken (s.k. TUNEL-teknik) användes för att färga cellkärnor i döende, eller s.k. apoptotiska, celler. Den andra färgningstekniken färgade in samtliga cellkärnor. De färgade blastocysterna monterades på objektsglas, och avbildades sedan med hjälp

av ett s.k. konfokalmikroskop, d.v.s. en speciell typ av mikroskop som tar 3-dimensionella bilder genom att fotografera tunna ”skivor”, eller sektioner, av objekt. På så sätt är det möjligt att avbilda relativt tjocka, 3-dimensionella objekt, som t.ex. blastocyster. Andelen apoptotiska celler hos blastocysterna togs fram med hjälp av ett datorprogram för bildanalys. Manuell kontroll av de 3-dimensionella bilderna av blastocysterna samt resultatet från bildanalysen visade att bildanalysen fungerat tillfredsställande, och att TUNEL-tekniken färgat in apoptotiska celler på ett korrekt sätt. Manuell kontroll är viktigt för att säkerställa tillförlitlighet vid användande av automatiska analysmetoder.

Därefter utfördes statistiska analyser, för att se om tillsatsen av PFAS-ämnen haft någon effekt på embryoutvecklingen. Vi fann då att fler blastocyster utvecklats i den grupp som utsatts för PFHxS. Det verkade även som att PFHxS resulterade i större blastocyster, samt blastocyster innehållande fler celler som befann sig i celldöd, men detta kunde inte med säkerhet statistiskt säkerställas**.** Bland övriga uppmätta parametrar fanns inga statistiskt säkerställda effekter av PFAS-behandlingen. Det går inte att helt säkert avgöra om detta beror på att behandlingen inte har någon effekt alls på dessa parametrar, eller om en skillnad hade kunnat uppmätas om studien t.ex. innefattat ett större, och/eller mer homogent (med avseende på t.ex. kvalitet hos äggcellerna), studiematerial.

Denna studie visar att PFHxS förefaller kunna påverka vissa parametrar i den tidiga embryoutvecklingen hos gris. Mer forskning behövs för att säkerställa detta samt för att avgöra vad detta kan ha för betydelse för den fortsatta utvecklingen av embryot, och för den levande avkomman. Det behövs även fler studier för att ta reda på mer exakta mekanismer för hur PFAS påverkar levande organismer på cell- och molekylnivå.

Appendix 1

Wash media pig

Reagents	**Cat**
Hepes TCM 199	M 7528
Gentamycine	G 1264
Heparin 180 USP Units/mg	H 3149
L-glutamine	G 8540
PVA	P 8136

Basic wash media

Reagents	**1000 ml**	**2000 ml**	**Final concentration**
Hepes TCM 199	1000 ml	2000 ml	
Gentamycine sulphate (50mg/ml stock)	200 µl	400 µl	10 µg/ml
L-glutamine MW 146.14	0.1461 g	0.2923 g	1mM
PVA	3 g	6 g	3 mg/ml

Wash media with heparin

Reagents	**200 ml**	**400 ml**	**Final concentration**
Basic wash	200 ml	400 ml	
Heparin	0.0222 g	0.0444 g	20 U/ml

Information regarding commercial media used in this study are available on http://www.fujihira.co.jp/english/index.html (accessed 2020-12-08).