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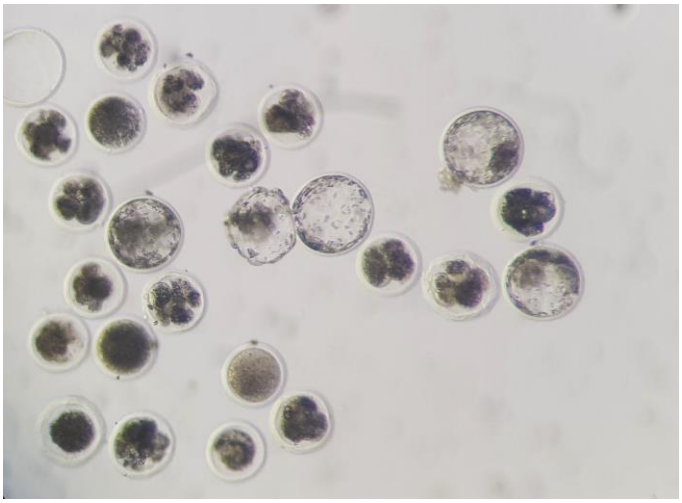

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SUMMARY

Perfluorooctance sulfonic acid (PFOS) is a chemical that has been widely used in products like food packaging, textile, grease proofing treatments and fire fighting foams. Although its usage is now banned in many countries, including the EU, it is still present in nature, animals and humans due to its persistent and bioaccumulating properties. Several studies on laboratory animals have shown PFOS to be endocrine disruptive and have toxic effects on reproduction, while human cohorts show contradictory results. The aim of this study was to examine the effect of PFOS on bovine early embryo development *in vitro* as a possible model for human early embryo development.

Oocytes were matured, fertilized and cultured in a bovine *in vitro* embryo production (IVP) model. The oocytes (n=847) were collected from fresh ovaries, transported to the laboratory from a slaughterhouse. Three groups of oocytes were followed throughout the trial, two treatment groups exposed to either 20 nM PFOS (P20) or 200 nM PFOS (P200) during *in vitro* maturation and one control group (C) without the addition of PFOS during maturation. The used concentrations in this experiment were based on the PFOS levels found in human follicular fluid in a previous study.

Several parameters were used to assess the development of the oocytes and embryos including cleavage rate 44 hours post fertilization and day 7 and 8 blastocyst development and morphology. Nuclear and lipid stains were used to stain the day 8 blastocysts after fixation in paraformaldehyde. A confocal laser scanning microscopy was used for analysis of the stained blastocysts. Evaluations of the number of nuclei, total lipid volume, lipid volume of each blastocyst and lipid droplet size were made.

From 13 batches with a total of 847 oocytes, 162 blastocysts were developed. Cleavage rate and cleavage rate above the 2-cell stage were found to be significantly lower in the P200 group compared to the control group, although no significant difference could be seen on blastocyst development on day 7 and 8 between the same groups or between the control group and the P20 group. The blastocyst developmental stage was significantly lower in the P200 group compared to the C group. In addition, in the P200 group PFOS had an effect on lipid droplet size in the lower blastocyst stage.

Altogether the results from this experiment indicates that clinically relevant concentrations of PFOS shows toxic effects on bovine early embryo development by impairing the developmental process. PFOS also seems to impact the size of lipid droplets. More studies are needed to elucidate the mechanisms and effects of PFOS on the early embryo development.

SAMMANFATTNING

Perfluoroktansulfonsyra (PFOS) är en kemikalie som har använts flitigt i produkter såsom matförpackningsmaterial, textilier, läder och brandskum. Trots att användningen av PFOS nu är förbjuden i många länder, EU inkluderat, finns det fortfarande kvar i miljön, djur och människor på grund av dess persistenta och bioackumulerande egenskaper. Flertalet studier på försöksdjur har visat att PFOS verkar hormonstörande och har toxiska effekter på reproduktion, samtidigt som kohortstudier på människor visar motsägande resultat. Syftet med den här studien var att undersöka PFOS effekt på tidig embryonal utveckling hos nöt *in vitro* som en möjlig modell för embryoutveckling hos människa.

Oocyter fick mogna, bli befruktade och utvecklas vidare i en *in vitro* modell för produktion av nötembryon. Oocyterna (n=847) togs från färska äggstockar som transporterats till labbet från ett slakteri. Tre oocytgrupper följdes under totalt 13 försöksomgångar, varav två var behandlingsgrupper som exponerades för antingen 20 nM PFOS (P20) eller 200 nM PFOS (P200) under mognadsfasen *in vitro* och en var kontrollgrupp (C) utan tillsats av PFOS under mognadsfasen. Koncentrationerna i detta experiment baserades på de PFOS-nivåer som uppmätts i follikelvätska hos kvinnor i en tidigare studie.

Andelen oocyter som hade delat sig 44 timmar efter fertilisering, blastocystutveckling och morfologi på dag 7 och 8 efter fertilisering var parametrar som användes för bedömning av oocyt- och embryoutveckling. Kärn- och lipidfärger användes för att färga blastocysterna på dag 8 efter fixering i paraformaldehyd. Ett konfokalmikroskop användes för analys av de färgade blastocysterna. Blastocysterna bedömdes därefter avseende antal kärnor, total lipidvolym, lipidvolym hos varje enskild blastocyst samt lipiddroppsstorlek.

Av totalt 847 oocyter utvecklades 162 stycken till blastocyster. Andelen oocyter som delat sig en gång och fler än en gång var signifikant lägre i P200-gruppen jämfört med kontrollgruppen, trots att ingen signifikant skillnad kunde ses på andelen utvecklade blastocyster dag 7 eller 8 mellan kontrollgruppen eller någon av behandlingsgrupperna. Blastocysternas utvecklingstadie var också signifikant lägre i P200-gruppen jämfört med C-gruppen. PFOS visade sig också ha en effekt på lipiddroppstorleken i de lägre utvecklingsstadierna av blastocyster i P200-gruppen.

Sammantaget visar resultaten från detta experiment indikationer på att kliniskt relevanta koncentrationer av PFOS har en toxisk effekt på tidig embryoutveckling hos nöt genom att försämra utvecklingsprocessen. PFOS verkar också i viss mån påverka storleken på lipiddroppar i blastocysterna. Ytterligare studier behövs dock för att kunna klargöra PFOS effekter på tidig embryoutveckling samt dess bakomliggande mekanismer.

CONTENT

ABBREVIATIONS

BSA	Bovine serum albumine
C	Control group, no addition of PFOS during *in vitro* maturation
CO_2	Carbon dioxide
COCs	Cumulus oocyte complexes
FSH	Follicle stimulating hormone
ICM	Inner cell mass
IVF	*In vitro* embryo fertilization
IVM	*In vitro* embryo maturation
IVP	*In vitro* embryo production
LH	Luteinizing hormone
LipidTOX	HCS LipidTOX™ Green Neutral Lipid Stain
Zeiss LSM 800	Confocal laser scanning microscope from Zeiss, Oberkochen, Germany
mM	Millimolar
mOsmo	Milliosmolality
mSOF	Modified synthetic oviductal fluid
N_2	Nitrogen
nM	Nanomolar
O_2	Oxygen
P20	Treatment group with the addition of 20 nM PFOS (0.01 µg/mL) during *in vitro* maturation
P200	Treatment group with the addition of 200 nM PFOS (0.1 µg/mL) during *in vitro* maturation
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PFAAs	Perfluoroalkyl acids
PFASs	Perfluoroalkyl and polyfluoroalkyl substances
PFOS	Perfluorooctane sulfonic acid

PPAR	Peroxisome proliferator-activated receptor
PVA	Polyvinyl alcohol
TCM	Tissue culture medium

INTRODUCTION

Over the past decades, human exposure to chemicals through the environment, food, water and various products has increased (Governini *et al.*, 2011). One commonly used chemical has been perfluorooctane sulfonic acid (PFOS) which is a man-made surfactant with proven stability and persistancy in nature. Due to its long half-life it bioaccumulates and is now omnipresent in human body fluids (Domínguez *et al.*, 2016) and can be found in the environment and in other living creatures all over the world (Jensen & Leffers, 2008). There is evidence from studies on laboratory animals that PFOS and other perfluoroalkylated and polyfluoroalkylated substances (PFASs) might affect reproduction and impair fertility, but the mechanisms behind are not well established or investigated and epidemiological studies on humans are very few (Bach *et al.*, 2016).

The use of experimental animals for reproductive toxicity tests is continuously increasing. One estimation is that 70% of all animals used in toxicological studies will be used for reproductive toxicity purposes (Spielmann, 2009). Rodents are commonly used as laboratory animals, but when it comes to using a model for human oocyte maturation and early embryo development, the use of a bovine *in vitro* embryo production (IVP) model is more suitable than the use of a murine model (Ménézo & Hérubel, 2002). The process of maturation and fertilization in bovine oocytes show more similarites to the human process than the murine does (Santos *et al.*, 2014). By collecting oocytes from cows and heifers after slaughter the use of a bovine IVP model without the use of experimental animals is possible.

The aim of this study was to investigate the effect of PFOS on early embryo development *in vitro* using a bovine IVP model. Embryo development evaluation was performed by morphology assesment and staining with nuclear and lipid stain. No experimental animals were used in the study.

LITERATURE REVIEW

Perfluorooctane sulfonic acid (PFOS)

Perfluorooctane sulfonic acid or perfluorooctane sulfonate (PFOS) is a substance belonging to the perfluoroalkylated and polyfluoroalkylated substances (PFASs) which are a big family of fully (per) or partly (poly) flourinated carbon chains with different functional groups at the end. The carbon- fluorine bond makes the substances extremely strong, stable and persistant in the environment (Borg *et al.*, 2012). They are both water and oil repellent and therefore popular chemicals to use in many different products such as surfactants, grease proofing treatments, textile, leather, paper (Bull *et al.*, 2014), food packaging, cosmetics and dental restorative materials. The industrial use of PFASs started in the 1950s and since then they can be detected in nature, humans and other live organisms (Buck *et al.*, 2011). One of the big contaminators has been the use of fire-fighting foams where PFASs (including PFOS) have been a common component, which has lead to a direct contamination of the environment, ground- and drinking water (KemI, 2015). Humans exposure to PFAS has been suggested to be associated with both endocrine disruption, metabolic, cancerogenic and developmental health effects (Blake *et al.*, 2018).

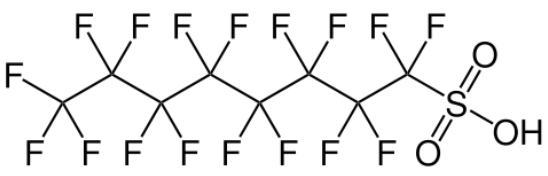

Figure 1. *Structural formula of perfluorooctane sulfonic acid, $C_8F_{17}SO_3H$.* (image source: Wikipedia).

PFOS is a man-made surfactant with an eight-carbon backbone and a charged sulfonate group at one end *(Figure 1)* (Lau *et al.*, 2007). It is together with perfluorooctanoate (PFOA) the most well-known and widely studied PFAS (Borg *et al.*, 2012) and fulfills the criteria for a vPvB substance which means that it is very persistant and very bioaccumalative (KemI, 2004). Due to its persistency and its former globally high production volume it is commonly found in both humans and animals around the world, even in remote arctic areas (Jensen & Leffers, 2008). PFOS shows toxicity in laboratory animals and have a long half-life in humans which is two of the reasons why human exposure is of concern (Lau *et al.*, 2007). In a review by Bull *et al.* (2014) of toxicological

studies done on PFOS, signs of both endocrine disruption, immune, reproductive and carcinogenic toxicity are described. Human accumulation of PFOS mainly occurs by oral intake of food and water (KemI, 2015). Because of the lipophobic characteristics of PFOS, it does not accumulate in fatty tissue, like PCB, dioxins and many other well-known persistent organic pullutants do. Instead it binds to proteins and tends to accumulate in the blood, liver and kidneys (Jensen & Leffers, 2008). PFOS has also been found in very high levels in fish-eating top predators, suggesting biomagnification along the food chain (Bossi *et al.*, 2005; Persson *et al.*, 2013). The production of PFOS stopped in 2000 and since 2008 the use of PFOS is banned in most products in the European Union, but because its persistant nature, we are still being exposed. As substitutes for PFOS, related and sometimes unregulated chemicals that are less known and studied are being used (Jensen & Leffers, 2008).

PFOS and reproduction

A toxicology study on rabbits found that exposure to a daily high dose of PFOS was associated with increased abortion rates and reduced fetal weight (Case *et al.*, 2001). In male mice PFOS exposure has been associated with decreased serum testosterone concentrations (Wan *et al.*, 2011) and in male monkeys with decreased estradiol levels (Seacat *et al.*, 2002). An *in vitro* studie on human adrenocortical cells showed that exposure to PFOS instead resulted in a dose-responsive increased estradiol secretion (Kraugerud *et al.*, 2011). A studie on both cell lines and zebrafish embryos by Du *et al.* (2013) also indicates that PFOS has endocrine disruptive effects both *in vitro* and *in vivo* due to its ability to act as an estrogen receptor agonist, thyroid hormone receptor antagonist and alter the expression of genes involved in steroidogenesis and estrogen receptor production.

Domínguez *et al.* (2016) investigated the effects of PFOS on maturation of porcine oocytes *in vitro,* showing inhibiton on viability in a dose-dependent manner (50 % of the oocytes had died at 32 μM (32 000 nM)). PFOS also inhibited maturation of 50 % of the oocytes at 22 μM (22 000 nM) PFOS, probably as a result of inhibiting the gap junctional intercellular communication between the oocytes and the granulosa cells during the first 8 hours of maturation.

A review by Bach *et al.* (2016) summarized recent findings in epidemiologic studies investigating the association between PFOS exposure and human fertility. Although no consistent results were found, associations between high PFOS levels in men and abnormal semen morphology or lower testosterone levels could not be ruled out. In women, half of the included studies found associations between high PFOS concentrations and prolonged time to pregnancy. In those studies the associations were only found between PFOS levels and women who already had given birth once or more in their lives. Only one study found the same association between high PFOS concentrations and prolonged time to pregnancy of women who had not given birth before. In another study on human fertility (Governini *et al.*, 2011) found that a higher concentration of perfluorinated compunds in follicular fluid of women undergoing *in vitro* fertilization and embryo transfer was correlated to lower fertilization rates. Only the total concentration of perfluorinated compunds was measured in the study, hence the concentration and role of PFOS remains unknown.

PFOS concentrations in humans

Petro *et al.* (2014) investigated the presence of perfluoroalkyl acids (PFAAs), which is an undergroup to PFASs to which PFOS belongs, in follicular fluid and serum from women undergoing assisted reproductive technologies. They found PFOS to be the PFAA with the highest concentration in follicular fluid, resulting in oocytes being directly exposed to a median PFOS level of 7.5 (0.1-30.4) ng/mL. PFOS was also the highest measured PFAA in serum (7.6 (2.8-12.5) ng/mL). In another studie on women in Sweden, PFOS was also found to be the PFAS with the highest level in maternal serum with a median concentration of 20.7 (8.2-48.0) ng/mL (Kärrman *et al.*, 2007). These serum levels are in the range of what they have found in several other studies in different parts of the world, where the median concentrations have been between 3 and 36 ng/mL (Inoue *et al.*, 2004; Vestergaard *et al.*, 2012; Okada

et al., 2013; Toms *et al.*, 2014). Ronneby in Sweden is an example of where considerably higher serum levels have been detected since one third of the households have been exposed to municipal drinking water contamined with PFOS (and other PFAAs) since the mid- 1980s. The contamination was not discovered until 2013 and was caused by fire-fighting foams that had been used in an airfield close to the water source. The median serum concentration from 106 participants was 345 ng/mL, with a range of 24-1500 ng/mL (Li *et al.*, 2018).

Bovine early embryo development

In the female gonad or ovary, there are primitive, primordial follicles that function as a reserve of oocytes. The primordial follicles are oocytes surrounded of one layer of flattened epithelial cells. The epithelial cells proliferate and form several cell layers as the primordial follicles develop into primary and secondary follicles during the estrous cycle. The inner layers consist of granulosa cells and the outer layers of theca cells, separated by a basal lamina. Both cell types participate in the production of estrogen but the granulosa cells also nourish the oocyte and create a layer of non- cellular material, a zona pellucida, to protect it. As the secondary follicle grows the granulosa cells secrete fluid into a forming cavity, creating a tertiary, antral follicle. The fluid contains nutrients and enzymes needed for the follicle to develop and finally ovulate. Multiple follicles in the ovary develop at the same time due to an increase of follicle stimulating hormone (FSH) in the blood, but usually only one becomes dominant and continues to grow under ideal hormonal conditions, becoming a mature, preovulatory follicle (Sjaastad *et al.*, 2012). During maturation of the oocyte the granulosa cells differentiate into cumulus cells that form a cloud around the oocyte and mural granulosa cells that remain in contact with the basement membrane surrounding the follicle. The dominant follicle undergoes its final maturation and ovulates when luteinizing hormone (LH) in plasma is increased (Gordon, 2003). When released into the peritoneal cavity the cumulus cells make the transportation of the oocyte to the oviduct possible by sticking to the oviduct fimbriae. The fertilization takes place further down the oviduct as the sperm passes the cumulus cell layer of the oocyte, penetrates the zona pellucida and finally fuses with the oocyte membrane (Sjaastad *et al.*, 2012).

When an oocyte is fertilized it becomes a zygote which immediately starts to divide, initially once a day. Before it reaches about 40-50 cell stage it is called a morula and its cells are undifferentiated. Reaching day 7-8 it turns into a blastocyst with an inner cell mass (ICM) which will become the embryo and an outer cell layer called trophoblasts which will become the fetal part of the placenta. As the blastocyst grows larger, the zona pellucida gets thinner and thinner until it ruptures, resulting in a hatched blastocyst, on day 7-10 (Sjaastad *et al.*, 2012).

In vitro embryo production (IVP)

The first week of embryo development can be mimicked *in vitro* in a laboratory using oocytes collected through aspiration of follicles from abattoir-derived ovaries or through ovum-pick-up techniques from live animals using ultrasound. The oocytes are then incubated in a specialized medium in an optimal environment for a 22 hours long *in vitro* maturation process. *In vitro* fertilization of the oocytes takes place by adding sperm and letting the oocytes incubate in a medium that is supposed to simulate the oviduct environment for another 22 hours. After fertilization the oocytes are transferred to a new medium for *in vitro* culturing until day 8 post fertilization (Gordon, 2003). Despite the use of specialized media and incubators it is hard to simulate the different *in vivo* environments completely, usually resulting in a 30-40% reduction of oocytes developing into blastocysts using *in vitro* techniques compared to *in vivo* (Rizos *et al.*, 2002).

Evaluation of *in vitro* produced oocytes and embryos

The best way to evaluate oocyte or embryo quality is to transfer the oocyte to a recipient to see if it is capable of resulting in a living healthy offspring. From a practical point of view, alternative methods are needed to be able to assess oocyte quality without having to create a possible pregnancy in live

animals. Commonly used and reliable parameters that are thought to be well associated with oocyte quality are for example the timing of the first cleavage of the zygote, the morphological appearance of the cumulus cells and cytoplasm, lipid content, cell number and ultrastructural evaluation of the nuclear stage and cytoplasm (Leroy *et al.*, 2008).

Using a bovine model

When studying female reproductive toxicology and screening toxic agents, bovine oocytes as a model for human oocytes during *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* embryo production (IVP) have been used in several studies and laboratories. It has been suggested to be a valuable alternative method to testing live laboratory animals which at the present is the most reliable tool for assessing the hazardness of reproductive toxicants (Luciano *et al.*, 2010; Beker van Woudenberg *et al.*, 2012; Tessaro *et al.*, 2015). If oocytes can be collected from abattoir-derived ovaries that are leftover material after slaughter of animals entering the food production chain, the use of a bovine IVP model is a way of following the principles of the 3R (replacement, reduction and refinement of the use of laboratory animals) (Santos *et al.*, 2014). *In vivo* reproductive toxicity tests, usually performed on rodents, does not only require a large number of mice and rats (Santos *et al.*, 2014), but rodents are also less suitable as model animals for humans because of their differences during oocyte maturation and fertilization (Ménézo & Hérubel, 2002). Bovine and porcine models show more similarities to a human model than a murine does when comparing the size of the oocyte, the time to reach 2-cell stage, blastocyst stage and hatching. Further, when considering oocyte maturation and initial embryo development, the time frame for the bovine model is closer to the human one. The use of a bovine model for IVM, IVF and IVP can not eliminate *in vivo* studies completely, but it can be used as a reliable and complex risk assessment option (Santos *et al.*, 2014).

MATERIAL AND METHODS

Experimental Design

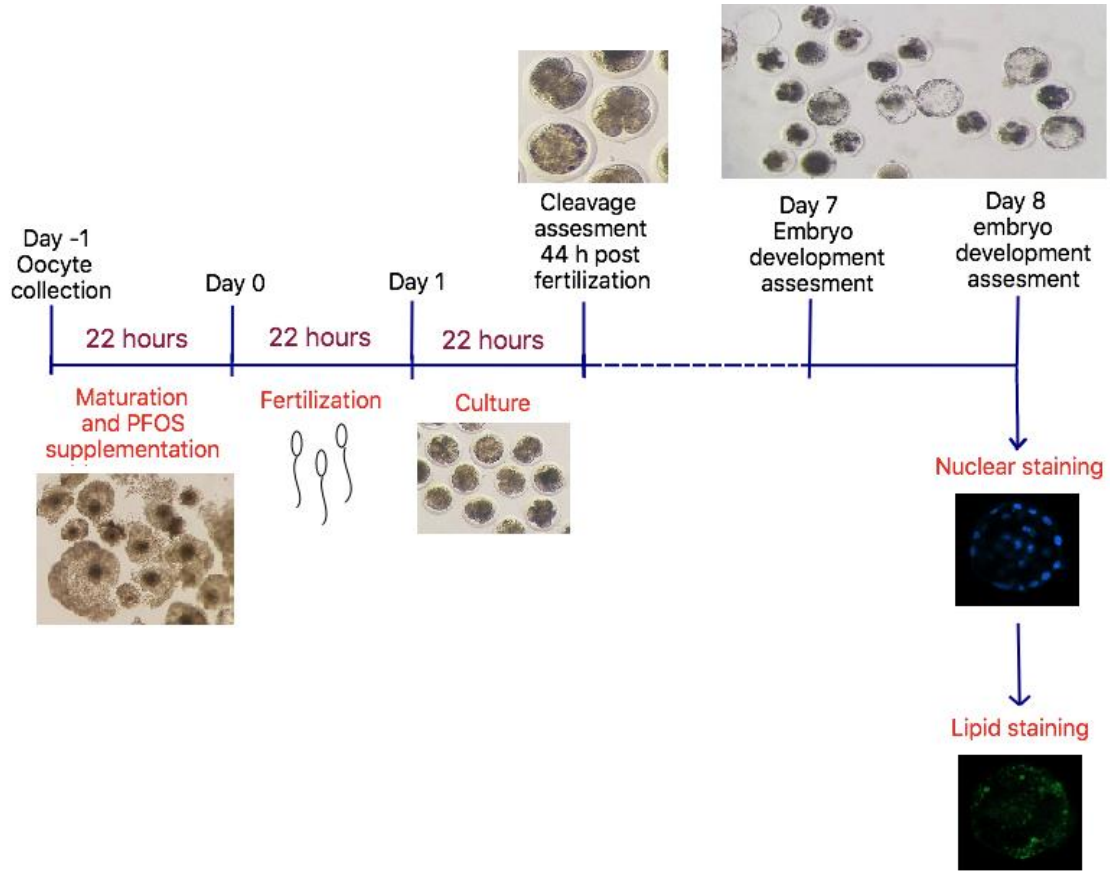

Figure 2. *Experimental timeline.*

Ovaries were collected from cows and heifers independent of breed or age after slaughter at an abattoir. The ovaries were transported to the laboratory where COCs were aspirated from the follicles. The COCs were then randomly divided into three groups prior to *in vitro* maturation; one treatment group with supplement of 20 nM PFOS (P20) during maturation, one treatment group with supplement of 200 nM PFOS (P200) and one control group without supplement of PFOS (C). After maturation the oocytes were fertilized with semen from a bull with proven *in vivo* and *in vitro* fertility. 13 batches were run during 8 weeks (September – October 2018). Each batch contained three groups with 19-30 COCs in each group. A total of 847 COCs were included in the trial. Oocyte maturation and embryo development were assessed according to set parameters; cleavage rate 44 hours post fertilization and blastocyst development and morphology on day 7 and 8 post fertilization. On day 8 blastocysts were fixed in paraformaldehyde (PFA) and then stored in phosphate buffered saline (PBS) containing polyvinylalcohol (PVA) before they were stained with nuclear and lipid stains *(Figure 2).* A confocal laser scanning microscope was used to analyze the stained embryos.

Media

All of the chemicals used were received from Sigma Aldrich, Stockholm, Sweden, if not stated otherwise. Completed media were filtered through a 0.2 µm filter unit and stored at 4 °C (except for maturation media that was only filtered). At least 1 hour before usage the media was equilibrated in 5 % CO2 incubator at 38.5 °C, or pre-warmed in a heat-box (Search and Wash2 Media).

Search

Search medium *(Table 1)* was used for preservation and searching of the COCs after aspiration. pH was measured and corrected if needed to 7.3–7.4 and osmolality to 290–300 mOsmo. The medium was made up weekly.

Table 1. *Search medium*

Reagents	Concentration	Sigma number
TCM 199 with Hepes (modified buffer system)		M7528
Albumin, bovine, 96-99%, BSA (fraction V)	0.2% w/v	A3311
Gentamicin stock (Gentamicin Sulfate salt)	50 µg/ml	G1264

Maturation

Maturation medium *(Table 2)* was used for *in vitro* maturation. pH was measured and corrected if needed to 7.3–7.4 and osmolality to 280–300 mOsmo. The medium was made up fresh every day of ovary collection.

Table 2. *Maturation medium*

Reagents	Concentration	Sigma number
TCM 199 without hepes		M2154
L- Glutamine, non animal source	0.68 mM	G8540
Albumin, bovine, 96-99%, BSA (fraction V)	0.4% w/v	A3311
Gentamicin stock (Gentamicin Sulfate salt)	50 µg/ml	G1264
FSH (stimufol stock)	50 µg/ml	Partnar Animal Health
LH (stimufol stock)	10 µg/ml	Partnar Animal Health

Two different PFOS stock solutions were made by dissolving PFOS in SQ water *(Table 3).* In the first treatment group (P20), 1 µg/ml PFOS stock was added, creating a final concentration of 0.01 µg/mL PFOS (20 nM). In the second treatment group (P200), 10 µg/ml PFOS stock was added to the maturation medium, creating a final concentration of 0.1 µg/mL PFOS (200 nM). In the control group only SQ water was added so that all three groups had the same concentration of 10 µL/mL SQ water *(Table 3).*

Table 3. *PFOS concentrations in maturation media (Sigma number: 77282)*

	C	P20	P200
PFOS stock solution	-	1 µg/ml	10 µg/ml
PFOS final concentration	-	0.01 µg/ml (20 nM)	0.1 µg/mL (200 nM)
SQ water	10 µL/mL	10 µL/mL	10 µL/mL

Fertilization

Three media were used for *in vitro* fertilization (*Table 4*). They were all made up weekly. Oocytes were washed and handled in Wash2 Medium. pH was measured and corrected if needed to 7.3–7.4 and osmolality to 270–290 mOsmo.

Capacitation Medium was used for the selection of motile sperm. pH was measured and corrected if needed to 7.3–7.4 and osmolality to 290–310 mOsmo.

Fertilization Medium was used for *in vitro* fertilization. pH was measured and corrected if needed to 7.8 before the addition of heparin and PHE stock. Osmolality was measured and corrected if needed to 290 – 310 mOsmo.

Table 4. *Wash2 (W2), Capacitation (Cap) and Fertilization (Fert) media*

Reagents	W2	Cap	Fert	Sigma number
Albumin, bovine, 96-99%, BSA (Fraction V)	0.3% w/v	0.6% w/v	-	A3311
Albumin, bovine serum, (FAF: Fatty Acid Free)	-	-	0.6% w/v	A7030
Gentamicin stock (Gentamicin Sulfate salt)	50 µg/ml	50 µg/ml	50 µg/ml	G1264
Sodium DL-lactate	16 mM	16 mM	16 mM	L7900
Sodium Pyruvate, >= 99%	0.5 mM	1 mM	0.5 mM	P4562
Sodium chloride (NaCl)	114 mM	110 mM	114 mM	S5886
Potassium chloride (KCl)	3.19 mM	2.68 mM	3.19 mM	P5405
Sodium bicarbonate (NaHCO3)	2 mM	25 mM	24.88 mM	S5761
Sodium phosphate monobasic, >= 99.0% (NaH2PO4)	0.40 mM	0.40 mM	0.40 mM	S5011
Magnesium chloride hexahydrate (MgCl2 6H2O)	0.49 mM	0.49 mM	0.49 mM	M2393
Calcium chloride dihydrate, minimum 99% (CaCl2 2H2O)	2 nM	2 mM	2 mM	C7902
Hepes, minimum 99.5% tritation	10 mM	5 mM	10 mM	H3375
Phenol red solution	1 µl/ml	1 µl/ml	1 µl/ml	P0290
D-(+)-Glucose	-	6.9 mM	-	G6152
Heparin	-	-	3 µg/ml	H3149
Epinephrine (PHE stock)	-	-	0.06 µM	E1635
Hypotaurine (PHE stock)	-	-	0.4 µM	H1384
Pencillamine (PHE stock)	-	-	0.8 µM	P4875
Sodium Chloride (NaCl) (PHE stock)	-	-	0.072 mM	S5886
Sodium DL-lactate (PHE stock)	-	-	0.42 mM	L7900
Sodium Metabisulphite (PHE stock)	-	-	1.68 µM	S9000

Culture

Modified synthetic oviductal fluid (mSOF medium) (Gordon, 2003) *(Table 5)* was used for *in vitro* culture of presumptive zygotes and embryos. pH was measured and corrected if needed to 7.4 and osmolality to 270 – 280 mOsmo. The medium was made up weekly.

Table 5. *mSOF medium*

Reagents	Concentration	Sigma number
Albumin, bovine serum (FAF: Fatty Acid Free)	0.4% w/v	A7030
Gentamicin stock (Gentamicin Sulfate salt)	50 µg/ml	G1264
BME Amino Acids Solution (50x)	20 µl/ml	B6766
MEM Non-essential Amino Acids Solution (100x)	10 µl/ml	M7145
Sodium chloride (NaCl)	0.11 M	S5886
Potassium chloride (KCl)	7.2 mM	P5405
Potassium phosphate monobasic, >= 99.0% (KH2 PO4)	1.19mM	P5655
Calcium chloride dihydrate, minimum 99% (CaCl2 2H2O)	1.71 mM	C7902
Magnesium chloride hexahydrate (MgCl2 6H2O)	0.49 mM	M2393
D-(+)-Glucose	1.5 mM	G6152
Sodium DL-lactate	9.9 mM	L7900
Sodium pyruvate, >= 99%	0.33 mM	P4562
Sodium bicarbonate (NaHCO3)	25 mM	S5761
Phenol red solution	0.03 µl/ml	P0290
L-Glutamine, non animal source	1 mM	G8540

In vitro embryo production

In vitro *maturation and PFOS supplementation*

The ovaries used in this study were collected for about an hour, immediately after slaughter, at an abattoir. The breed, age and reproduction status of the slaughtered cows and heifers were unknown. After 3-4 hours of transportation to the laboratory in thermos flasks with 30.0 °C 0.9% NaCl solution (with a range between 28.5-31.0 °C at arrival), the ovaries were rinsed and put in a new pre-warmed thermos flask with 38.5 °C 0.9% NaCl solution. COCs were aspirated from 3-8 mm follicles using a 5 ml syringe and an 18 g needle and then put in a pre-warmed container with search medium. Excess follicular fluid was removed and the remaining follicular fluid with the oocytes was put in pre-warmed 60 mm TC dishes with pre-warmed search medium. A light microscope (SteREO Discovery.V8, Carl Zeiss Microscopy GmbH, Jena, Germany) was used for oocyte localization and assessment according to commonly used classification criteria, Grade 1: A light and transparent COC with a compact multilayered cumulus investment and a homogeneous ooplasm. Grade 2: A slightly darker and less transparent COC with a coarse feature and a darker zona. Grade 3: The COC is darker than grade 1 and 2, the cumulus investment is less compact and the ooplasm is irregular with dark clusters. Grade 4: The COC is dark and irregular with an expanded cumulus investment containing cumulus cells in dark clumps. The ooplasm is irregular with dark clusters (Gordon, 2003).

Grade 1-2 COCs were selected for the experiment. The quality of COCs varied between batches, resulting in batches with different proportions of included grade 2 COCs. The COCs were randomly divided into three homogenous groups, washed through search and maturation medium and placed in a 4-well plate, one group per well, with maturation medium containing 20 nM PFOS (P20), 200 nM PFOS (P200) or no PFOS (C). They were then incubated for 22 hours at 38.5 °C in 5% CO_2.

In vitro *fertilization*

Before fertilization the COCs were washed and pipetted in wash-2-medium until 3 to 5 layers of cumulus cells remained. They were then washed through wash-2 medium and fertilization medium and put in a new 4-well with fertilization-medium. Two straws of 250 µl frozen semen from a SRB bull (Sörby) were thawed in 35 °C tap water for 12 seconds. The semen was then placed at the base of 4 pre-warmed plastic tubes with capacitation media for sperm swim up. After incubation in 5% CO_2 for 45 minutes the top layer of capacitation medium (containing the motile sperm) was suspended and placed in a centrifugation tube in a Sorvall ST 8 Centrifuge (ThermoFisher Scientific, Waltham, USA) to spin for 7 minutes at 300 x g. The supernatant was then removed and the remaining sperm pellet was diluted with fertilization medium and concentration determined in a Bürcher chamber. Prepared oocytes were then fertilized *in vitro*, 24 hours post aspiration, with sperm added at a concentration of 1 000 000 sperm/mL and incubated for 22 hours at 38.5°C in 5% CO_2.

In vitro *culture*

After fertilization, all remaining cumulus cells and loosely attached sperm were removed from the zygotes/unfertilized oocytes by pipetting and wash through mSOF medium, before transferring them to a new pre-warmed 4-well plate with mSOF medium. The wells were covered with OVOIL™ (Vitrolife AB, Gothenburg, Sweden) to prevent evaporation and incubated in 38.5 °C in 5% CO_2 and 5% O_2 until day 8 post fertilization.

Cleavage check

The percentage of oocytes cleaved into two cells and above the two cell stage was assessed through light microscope 44 hours post fertilization.

Blastocyst development

On day 7 and 8 post fertilization the batches were evaluated under light microscope regarding blastocyst development. The total number of embryos in each group was counted and the embryos were then divided in early blastocysts, blastocysts consisting of over 50% fluid, expanding and expanded blastocysts, hatching or hatched blastocysts, all according to IETS certification (Robertson & Nelson, 2010). To facilitate statistical analysis, the stages were modified into three groups where stage 1 equals the least developed embryos and stage 3 equals the most developed embryos:

Stage 1: Early blastocyst or blastocyst over 50%. There is no expansion of the embryo, zona pellucida is intact and an antrum is visible. Stage 2: Expanding or expanded blastocysts. There is expansion of the embryo, zona pellucida is thinner than in stage 1 and the antrum is bigger. Stage 3: Hatching or hatched blastocysts. Zona pellucida is ruptured or missing.

The embryo quality was also graded from 1-4 based on morphological characteristics:

Grade 1: Excellent or good. The embryo mass is symmetrical and spherical with individual cells (blastomeres) of the same size, color and density. Zona pellucida is smooth and even. At least 85 % of the cellular material is intact. Grade 2: Fair. There are moderate irregularities in the shape of the embryonic mass or in the size, color and density of the individual blastomeres. At least 50% of the cellular material is an intact, viable embryonic mass. Grade 3: Poor. There are major irregularites in shape of the embryonic mass or in size, color and density of the blastomeres. At least 25 % of the cellular material is an intact, viable embryonic mass. Grade 4: Dead or degenerated. The embryos, oocytes or one-cell embryos are degenerating and nonviable (Robertson & Nelson, 2010).

Nuclear staining

Before nuclear staining the blastocysts were fixated in 2% PFA in PBS with 0.1% PVA (P8136) at 4°C overnight or at room temperature for one hour. They were then washed four times in PBS with 0.1% PVA. BizBenzimide Hoechst 33342 trihydrochloride (B2261) (Hoechst) was used for blue fluorescence staining of DNA, making the nuclei visible. The blastocysts were placed in wells with 4.45 µM Hoechst in PBS with 0.1% PVA protected from light for 20 minutes at room temperature. Before lipid staining the blastocysts were washed once in PBS with 0.1% PVA.

Lipid staining

HCS LipidTOX™ Green Neutral Lipid Stain (H34475) (ThermoFisher Scientific, Waltham, USA) (LipidTOX) was used for green fluorescent staining of intracellular neutral lipid droplets. The blastocysts were placed in drops with LipidTOX mixed with PBS with 0.1% PVA protected from light for 30 minutes at room temperature.

To control the stains, 3 blastocysts were used as negative controls.

Mounting

Directly after lipid staining the blastocysts were transferred from the LipidTOX drops to a black well plate (ThermoFisher Scientific, Waltham, USA). One group of blastocysts in approximately 2 µl of the LipidTOX solution was placed in each well. To preserve the fluorescent stain and prevent bleaching, Vectashield (Vector Laboratories, Burlingame, USA) was added to the wells until they were full. The wells were then covered with coverslips with nailpolish painted around them to prevent them from drying out. The plates were left to dry protected from light at room temperature and when dry at 4 °C until analyzed in the confocal laser scanning microscopy.

Confocal Laser Scanning Microscopy

Zeiss LSM 800

The embryos were analysed using a confocal laser scanning microscope (Zeiss LSM 800, Zeiss, Oberkochen, Germany). The laser lines used were: 405 and 488 nm. All images were taken with a 20X lens and were sectioned in several levels (z-stacks) with a 2 µl interval.

Image analysis

Images captured with the confocal microscope where analysed using Fiji (Schindelin *et al.*, 2012). In each embryo amount of nuclei were recorded as well as amount and volume of lipid droplets. Z-stacks were used for 3D-calculations using the analysis3D plugin adjusted for nucleus and lipid droplet sizes (*Figure 3*).

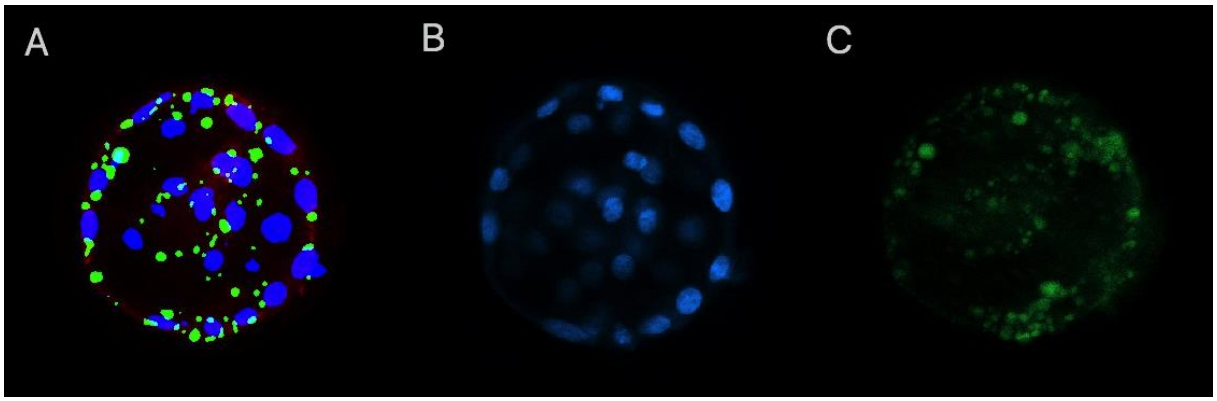

Figure 3. *Nucleus and lipid images. Left to right: A: processed image with identified lipids (green) and nuclei (blue), B: nucleus channel image from the confocal microscope, C: lipid channel image from the confocal microscope.*

Statistics

Mixed effect logistic regression was used to calculate the effect of treatment on the number of cleaved embryos, embryos cleaved above the 2 cell stage and developed blastocysts (glmer model of the lme4 package, R i386, 3.3.1, http://www.r-project.org). Batch was added as a random variable and groups weighted according to size. Categorical and ordinal variables (stage, grade) were analyzed using cumulative link mixed-effect models with multinomial distribution with batch added as random factor and weighted according to group-size (clmm model of the ordinal package, R i386, 3.3.1). To calculate the effect on nuclei count linear mixed effect models were used (lmer model of the lme4 package, Ri386, 3.3.1). The effect of treatment on lipid droplet distribution was made using a linear mixed effect model (lmer model in R) with log-transformed values to assume normal distribution. Batch and individual embryo was added as random effects and the variable stage was included as a fixed effect.

P-values <0.05 were considered to be significant. All results are presented as means (±SEM) unless otherwise specified.

RESULTS

In vitro embryo production

847 oocytes from 13 batches were included in the study *(Table 6).* 162 of them developed into blastocysts. 290 oocytes were included in the control group (C), 287 oocytes in the treatment group exposed to 20 nM PFOS (P20) and 270 oocytes in the treatment group exposed to 200 nM PFOS (P200). A total number of 69 blastocysts from the first 6 batches were stained, including 30 blastocysts from the C group, 22 blastocysts from the P20 group and 17 blastocysts from the P200 group. Some oocytes/blastocysts were lost during the process of IVP, staining or fixation. The quality of the batches varied throughout the study, resulting in a varying proportion of grade 2 blastocysts included in the different batches.

Table 6. *Batch data*

Batch nr	Mean temperature at arrival (°C)	Nr of ovaries	Nr of COCs	Mean nr COCs per ovary
1	31	69	87	1.26
2	29.5	52	66	1.27
3	28.5	53	59	1.11
4	30.25	62	75	1.21

5	28.5	58	60	1.03
6	29.5	71	85	1.20
7	29.5	65	78	1.20
8	29.5	90	123	1.37
9	28.75	48	58	1.21
10+11	29	86	95	1.10
12	29.5	85	63	0.74
13	29	89	75	0.84

Temperature was measured and ovaries were counted at arrival to the laboratory. Number of COCs were counted after selection of suitable ones (based on morphology). The tenth batch (10+11) was divided into two batches due to the high number of COCs.

Cleavage

The percentage of cleaved oocytes 44 hours post fertilization was 88.0 (± 4.9) % in the C group, 85.5 (± 5.5) % in the P20 group and 82.4 (± 8.0) % in the P200 group. Cleavage rate above the 2-cell stage was 66.2 (± 13.5) % in the C group, 63.3 (± 12.4) % in the P20 group and 55.4 (± 14.6) % in the P200 group *(Figure 4).* In the P200 group the mean percentage of both cleaved and cleaved above the 2-cell stage were significantly lower compared to the C group (p=0.02 and 0.04). There was no significant difference between the C group and the P20 group.

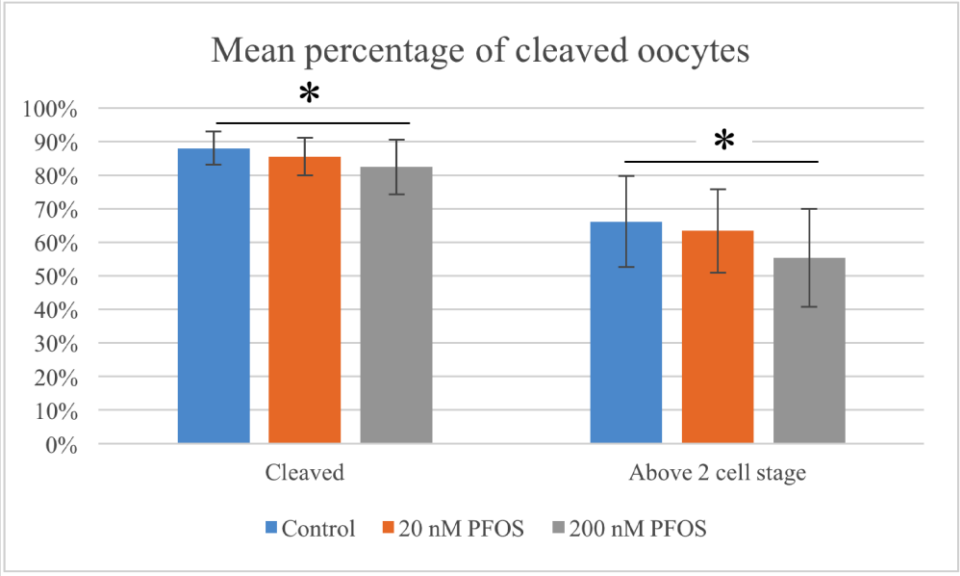

Figure 4. *Mean percentage ±SEM of all cleaved oocytes and oocytes cleaved above the 2 cell stage 44 hours post fertilization. * indicates the significant differences between the C and P200 group.*

Blastocyst development

The percentage of developed blastocysts on day 7 and 8 was 11.4 (± 7.8) % respectively 21.1 (± 6.9) % in group C, 14.8 (± 5.0) % respectively 20.4 (± 10.8) % in group P20 and 8.0 (± 6.0) % respectively 16.4 (± 10.1) % in group P200 (*Figure 5*). No significant differences regarding blastocyst development were found between the C group and the P20 or P200 group on day 7 or 8.

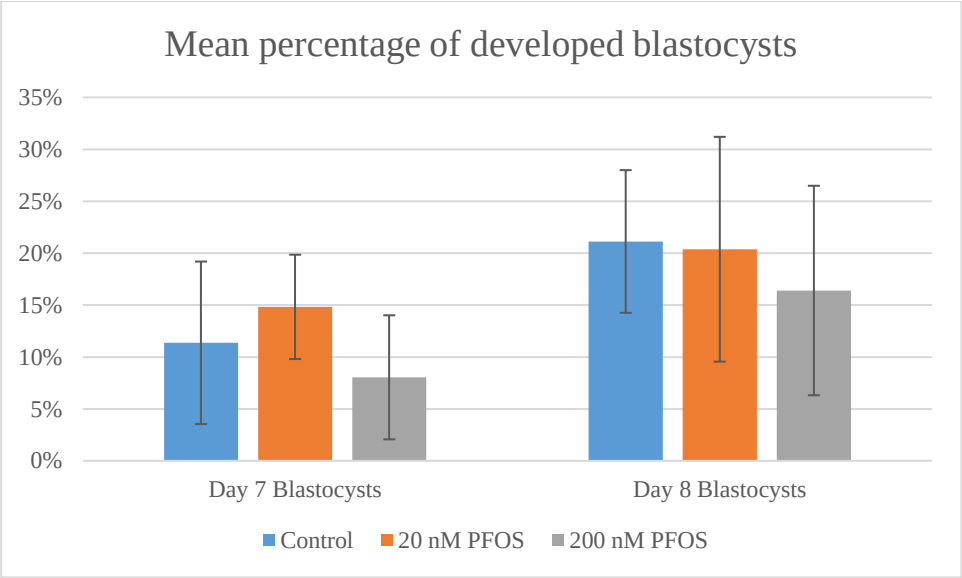

Figure 5. *Mean percentage ±SEM of developed blastocysts on day 7 and 8 post fertilization.*

Nuclei, grade and stage

There was no significant difference between the C group, the P20 group and the P200 group regarding number of nuclei or blastocyst grade *(Table 7).*

Table 7. *Descriptive statistics for blastocyst development*

Parameter	Control	20 nM PFOS	200 nM PFOS	Significance
Number of nuclei[1]	98.3 (±32.6)	92.5 (±30.4)	83.9 (±28.3)	ns
Blastocyst grade (1-4)[2]	1.5 (1-2.5)	1.5 (1-2.5)	1.5 (1-2.5)	ns

ns = not significant. Blastocyst grade 1: excellent, grade 2: fair, grade 3: poor, grade 4: degenerated.

[1]Mean numbers (±SEM) are presented

[2]Median scores with range (min-max) are presented

Compared to the control group, a significantly lower blastocyst stage was found in the P200 group (p=0.02) but not in the P20 group (*Figure 6*).

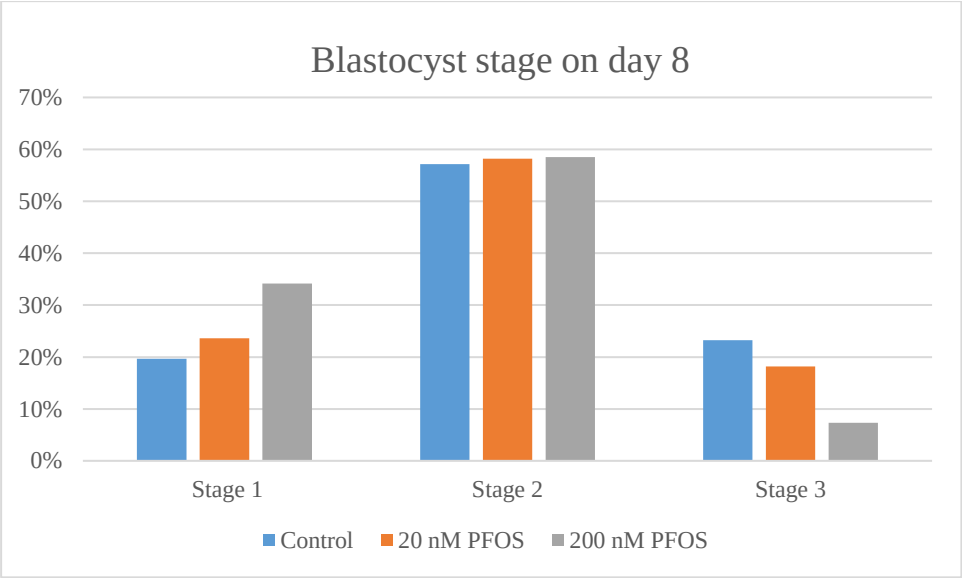

Figure 6. *Distribution of blastocyst stage on day 8 post fertilization.* Stage 1: blastocyst or early blastocyst, stage 2: expanded or expanding blastocyst, stage 3: hatching or hatched blastocyst.

Lipid volume and lipid droplet size

No significant difference was seen regarding total lipid volume, lipid volume per cell or lipid droplet size comparing the P20 and P200 group to the C group ($p>0.05$). There was however a significant difference within the P200 group, where the lipid droplet size was larger at earlier stages compared to later stages ($p<0.01$), an effect not observed in the other groups (*Table 8*).

Table 8. *Mean lipid droplet size (μm^3)*

Stage	Control	20 nM PFOS	200 nM PFOS
1	114.9 (±18.5)	89.4 (±23)	158.3 (±16.7)*
2	111 (±11.7)	77.4 (±14)	73.5 (±18.8)
3	75.8 (±15.9)	95.4 (±16.4)	55.2 (±30.7)

Mean numbers (±SEM) are presented. Stage 1: blastocyst or early blastocyst, stage 2: expanded or expanding blastocyst, stage 3: hatching or hatched blastocyst.
* indicates significant differences compared to stage 2 and stage 3 in the same group (P200).

DISCUSSION

Experimental design

The used PFOS doses in this study were 10 ng/mL (20 nM) and 100 ng/mL (200 nM) and based on the concentrations that Petro *et al.* (2014) found in human follicular fluid (7.5 (0.1-30.4) ng/mL) in their study. Serum levels were also measured in the same women in that study and showed similar results (7.6 (2.8-12.5) ng/mL), suggesting that PFOS serum levels could be closely related to follicular fluid levels. Several other studies have measured median PFOS serum concentrations between 3 and 36 ng/mL (Inoue *et al.*, 2004; Vestergaard *et al.*, 2012; Okada *et al.*, 2013; Toms *et al.*, 2014), but there are also examples of high exposed populations with serum levels up to 1500 ng/mL (Li *et al.*, 2018) which shows that the chosen concentrations in this study were relevant. Compared to the PFOS concentrations used in the study of Domínguez *et al.* (2016) on porcine oocytes our concentrations were over a thousand times lower. In addition, compared to *in vivo* conditions, the time of exposure to PFOS in this study was a lot shorter, since the oocytes were only exposed for 22 hours during *in vitro* maturation. Despite the short exposure time and the low concentrations, several statistical significant effects of PFOS were seen in the P200 group.

There was a large batch variation regarding number of developed blastocysts resulting in large variation in the results between batches. This was possibly due to a variation in ovary quality from the slaughterhouse but also because of varying numbers of observed grade 1 COCs between the batches, resulting in a larger proportion of grade 2 COCs included in the batches with fewer observed grade 1 COCs.

Effects of PFOS

The result that the proportion of cleaved oocytes and the proportion of oocytes cleaved above the 2-cell stage were both significantly lower in the P200 group compared to the control group indicates that PFOS could have a toxic effect on early embryo development *in vitro* which is further supported by the lower median blastocyst developmental stage in the same group. Since no difference could be seen in the P200 group regarding the proportion of day 7 or day 8 developed blastocysts, one possibility could be that PFOS inhibits cleavage of the oocytes that would not have developed into blastocysts anyway, and that the remaining oocytes were more capable and prone to develop into blastocysts, resulting in the same blastocyst proportion as the control group. Another possibility is that PFOS slows down the early developmental process, increasing the time to cleavage which results in a lower cleavage rate and a lower blastocyst stage compared to the control group at the given times of assesment. In women undergoing *in vitro* fertilization and embryo transfer, transferring slow developing blastocysts have been associated with lower pregnancy rates (Shapiro *et al.*, 2001; Poulsen *et al.*, 2017) and lower live birth rates, regardless of the embryo quality (Ferreux *et al.*, 2018).

Although not statistically significant, there was a tendency of an increase in the blastocyst proportion in the P20 group compared to the C group. In contrast, blastocyst proportion decreased in the P200 group (see *Figure 5*). It could be speculated that these results reflect different mechanisms of toxicity. Different mechanisms of toxicity between concentrations and non-monotone dose-response relationships are known actions of endocrine disruptors (Vandenberg *et al.*, 2017). As PFOS can act as an endocrine disruptor (Du *et al.*, 2013; Bull *et al.*, 2014) a non linear effect of PFOS toxicity could be suspected, indicating the possibility of different mechanisms at different concentrations of PFOS. While many traditional toxicants have a linear dose response, endocrine disruptors that interferes with hormones can have U-shaped or inverted U-shaped dose responses, which is why effects at higher doses can not always predict effects at lower doses and vice versa. A non-monotone response also means that completely different mechanisms can be seen at different doses, for example inducing proliferation at one dose while inducing apoptosis at another (Vandenberg *et al.*, 2017).

The larger lipid droplet size of the earlier blastocyst stages seen in the P200 group could be because of a stage-specific effect of PFOS on lipid droplet size. Since the larger lipid droplets was only seen in the early blastocyst stages one possibility could be that the size of the lipid droplets would have decreased when the blastocysts develop into higher stages. Another possibility is that there is something wrong with the blastocysts with larger lipid droplets which inhibits them from developing further. This theory could be supported by the fact that the median blastocyst stage is lower in the P200 group, although it is not possible to determine if the lower stage is due to defective blastocysts or as previously discussed to blastocysts developing more slowly. Regardless of the underlying mechanisms, a suggestion that a larger lipid droplet size affects the blastocyst quality negatively could be made since lipid accumulation in bovine IVP embryos has been shown to cause reduced cryotolerance in several studies (Abe *et al.*, 2002; Sudano *et al.*, 2011), while a decreased lipid accumulation and a high number of small lipid droplets in the embryo improves its resistance to cryopreservation (Pereira *et al.*, 2008). In addition, lipid accumulation due to *in vitro* culture of bovine blastocysts in serum containing media has been associated with an increased expression of genes related to apoptosis and oxidative stress in the blastocysts (Abe *et al.*, 2002; Rizos *et al.*, 2003).

One known mechanism of PFOS, which could be suspected to be responsible for the changes in lipid droplet size we see in this experiment, is its ability to bind to different peroxisome proliferator-activated receptors (PPARs) and interfere with the PPAR signaling pathway which can lead to metabolic disruption (Fang *et al.*, 2012; Lai *et al.*, 2017). The PPAR consists of three different isotypes; PPARα, PPARβ and PPARγ (Huang, 2008), which are nuclear receptor transcription factors that are normally activated by prostaglandins and fatty acids and when activated modulate fatty acid oxidation in different tissues (Dunning *et al.*, 2014). The generation of ATP through fatty acid oxidation is an important energy source for the oocyte. Fatty acid oxidation is induced during maturation *in vivo*, but is not induced to the same extent during *in vitro* conditions which is suspected to affect the oocyte development negatively and to be one of the reasons why less blastocysts are developed under *in vitro* conditions versus *in vivo* (Dunning *et al.*, 2014). PFOS interference with the PPAR signaling pathway has been shown to have different effects depending on the used concentration of PFOS. In a study on fish embryos, PPARα and PPARβ were inhibited at lower doses, while induced at higher doses (Lai *et al.*, 2017). Chicken embryos treated with two environmentally relevant doses of PFOS (0.1 µg respectively 1.0 µg per 1 gram of egg) both resulted in a downregulation of genes involved in fatty acid metabolism (partly due to interference with the PPAR signaling pathway), but interestingly, the downregulation was more evident in the embryos treated with the lower PFOS dose (Jacobsen *et al.*, 2018).

Although it is not possible to identify the mechanisms behind the found effects on embryo development in this study, as earlier discussed, one possibility could be that some kind of endocrine disruption involved. Fetal development together with puberty are the periods that are the most sensitive to exposure of endocrine disruptors. While effects may not be seen at birth or even early in life they can cause permanent changes and lead to defects or diseases later on (WHO/UNEP, 2013). An example of this is a recent study on mice suggesting that prenatal exposure to PFOS may lead to hepatotoxic effects that can cause a liver disorder later in life (Lai *et al.*, 2017). To know what consequences the effects found in this study would have had on later fetal life and life after birth, transferring of the embryos to a live recipient would have been necessary.

The fact that there are subtle but significant effects of PFOS on early embryo development seen in this experiment at clinically relevant concentrations and after an exposure of only 22 hours during *in vitro* maturation is noteworthy and this added knowledge regarding the effects of PFOS is of importance for the general public as well for research concerning similar substances that are used as PFOS replacements. Especially since there are populations that are being exposed to even higher PFOS concentrations than the concentrations used in this study. Despite this, it is not possible to completely simulate the *in vivo* dynamic changes in exposure and environmental conditions in an *in vitro* model (Rizos *et al.*, 2002). During *in vivo* conditions, the oocytes are not only exposed during maturation and

PFOS is not the only substance with possible effects on early embryo development which may add interacting effects between an unknown amount of substances. More studies would be needed to investigate the effect of longer exposure times, interactive effects and the later effects of PFOS on further embryo development and after birth.

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Perfluoroktansulfonsyra (PFOS) är en kemikalie som har tillverkats av människan och använts i stor utsträckning i produkter såsom textil, papper, läder, matförpackningsmaterial och brandskum. Trots att användningen av PFOS nu är förbjuden i många länder, inklusive EU, finns den fortfarande i vår närmiljö och återfinns i kroppsvätskor och organ hos både människor och djur över hela världen. PFOS-molekylen innehåller flertalet mycket starka kol-fluorbindningar som gör den mycket tålig och resistent i naturen. Människor får i sig PFOS främst via mat och vatten. Ett flertal studier från olika delar av världen har uppmätt PFOS-nivåer i blodet med medelvärden kring 3-36 ng/mL. Det finns dock exempel på när betydligt högre halter än så har uppvisats. I Ronneby i Sverige upptäcktes 2013 att en tredjedel av hushållen hade druckit PFOS-kontaminerat dricksvatten sedan 1980-talet. Kontaminationen kom från ett närliggande flygfält där brandskum använts och läckt ut i vattnet under en längre tid. Koncentrationen PFOS hos ett urval av den exponerade populationen uppmättes inom ett spann på 24-1500 ng/mL, med ett medelvärde på 345 ng/mL.

Flera studier på försöksdjur har visat på att PFOS har toxiska effekter, bland annat på reproduktionen, men också att den kan fungera hormonstörande. Ökad abortfrekvens, minskad födelsevikt, påverkan på könshormoner som östrogen och testosteron samt minskad äggcellsöverlevnad är exempel på effekter som har setts vid exponering av PFOS i olika koncentrationer. Få studier har gjorts på människor, men några av dem som gjorts har visat på associationer mellan höga PFOS-värden i blodet och förlängd tid till graviditet hos kvinnor.

I den här studien var syftet att undersöka PFOS effekt på den tidiga embryoutvecklingen på nöt *in vitro*. Då nötkreaturs och människors tidiga embryoutveckling i många avseenden är relativt lika, är kon ett mer passande modelldjur för människan än vad exempelvis möss är, som vanligtvis används som försöksdjur. Genom att använda en modell för *in vitro* produktion av nötembryon där äggcellerna plockas ut ur överblivna äggstockar från redan slaktade kor på väg in i livsmedelskedjan, kunde användningen av försöksdjur undvikas helt.

Under de åtta veckor som försöket pågick kördes 13 försöksomgångar med totalt 847 äggceller. Äggstockarna samlades in på ett slakteri och transporterades sedan till laboratoriet där äggcellerna sögs ut med kanyl och spruta från äggblåsorna. Äggcellerna delades in i tre grupper, två behandlingsgrupper och en kontrollgrupp för att sedan placeras ut i brunnar med mognadslösning. Under mognadsprocessen, som pågick under ca 22 h i en inkubator, fick den ena behandlingsgruppen mogna med tillsats av 0,01 µg/ml PFOS (grupp P20) och den andra med tillsats av 0,1 µg/ml PFOS (grupp P200) i mognadslösningen. Kontrollgruppen (grupp C) fick mogna utan tillsats av PFOS i lösningen. När äggcellerna hade mognat tvättades de och flyttades till brunnar med befruktningslösning där de befruktades med sperma från en tjur med bevisat god fruktsamhet. Efter 22 timmar i inkubator tvättades och förflyttades äggcellerna till ännu en lösning i nya brunnar som täcktes med olja för att förhindra uttorkning. Äggcellerna fick sedan fortsätta sin utveckling i en ny inkubator med optimal temperatur och atmosfär till och med dag åtta efter befruktning. På dag åtta fixerades de äggceller som hade utvecklats till blastocyster, det vill säga mycket tidiga embryon bestående av två olika celltyper, i paraformaldehyd för att sedan färgas in med en kärnfärgning och en fettfärgning innan de monterades på mikroskoperingsglas under täckglas.

Äggcellerna/blastocysterna bedömdes i ett stereomikroskop med hjälp av flertalet parametrar. Antalet äggceller som delat sig en gång och antalet äggceller som delat sig mer än en gång bedömdes 44 timmar efter befruktning. På dag sju och åtta efter befruktning bedömdes både utvecklingsstadium och utseende/kvalité. De färgade blastocysterna bedömdes med hjälp av ett konfokalmikroskop och ett bildanalysprogram avseende antal kärnor, fettvolym samt fettdroppsstorlek.

Av totalt 847 äggceller utvecklades 162 till blastocyster. Efter utförda statistiska analyser visade behandlingsgrupp P200 ett signifikant lägre antal äggceller som hade befruktats och delat sig en gång

och äggceller som hade delat sig flera gånger än vad kontrollgruppen (grupp C) gjorde. P200gruppen visade sig också ha en större andel av blastocyster som inte hade utvecklats lika långt som de i kontrollgruppen hade på dag åtta. Dock sågs ingen signifikant skillnad mellan dessa grupper gällandes den totala andelen utvecklade blastocyster på dag sju eller åtta. Sammantaget visar resultaten på att PFOS troligtvis har en toxisk effekt på den tidiga embryoutvecklingen. Den lägre andelen delade äggceller och den större andelen av lågt utvecklade blastocyster i P200gruppen skulle kunna förklaras av att PFOS gör att blastocystutvecklignen går långsammare. Tidigare studier på kvinnor som genomgår assisterad befruktning med överföring av embryon, visar att överföringen av långsamt utvecklade blastocyster medför en lägre andel graviditeter jämfört med en överföring av normalt utvecklade blastocyster.

Trots avsaknaden av signifikanta skillnader mellan kontrollgruppen och behandlingsgrupperna kunde en tendens till en ökad andel blastocyster i P20-gruppen ses på dag sju samtidigt som tendensen i P200-gruppen var den motsatta, det vill säga en minskad andel blastocyster på dag sju. Att de olika PFOS-koncentrationerna i experimentet gav upphov till motsatta resultat gör att PFOS kan misstänkas ha olika effekter beroende på koncentration. Det är känt sedan innan att det finns hormonstörande substanser som verkar på olika sätt vid olika koncentrationer och som inte följer ett linjärt dos-respons-mönster (det vill säga att de har mer effekter desto högre koncentrationer de förekommer i). PFOS har i tidigare studier visat sig vara hormonstörande och trots att det inte är möjligt att klargöra mekanismerna bakom effekterna i denna studie, skulle PFOS hormonstörande egenskaper eventuellt kunna vara inblandade. Fosterutvecklingstiden tillsammans med puberteten är de perioder som är mest känsliga för hormonella störningar. Även om effekter inte ses vid födsel eller ens tidigt i livet, kan de orsaka permanenta förändringar och leda till defekter eller sjukdomar senare i livet. För att se vad effekterna på embryoutveckling som hittats i denna studie skulle ha betytt för senare fosterutveckling och ett eventuellt liv efter födseln, hade embryona i studien behövt överföras till levande kor för att kunna utvecklas vidare.

I resultatet sågs även en skillnad på fettdroppsstorlek mellan de olika blastocyststadierna i P200-gruppen. De lägsta blastocyststadierna hade större fettdroppar än de högre blastocyststadierna. Denna skillnad kunde inte ses i de andra två grupperna (P20 och C). Resultatet stämmer överens med tidigare studier där PFOS har visat sig ha egenskaper som kan störa fettmetabolismen. Dock verkar PFOS effekt på fettmetabolism både skilja sig mellan olika studier och mellan olika koncentrationer. Det finns också studier som tyder på att embryon med ett högre fettinnehåll är sämre rustade för exempelvis nedfrysning, och därmed fungerar sämre att använda vid metoder för assisterad befruktning.

Att denna studie visar på effekter av PFOS på den tidiga embryoutveckling hos nöt under så pass låga koncentrationer och efter endast 22 timmars exponering är anmärkningsvärt och viktig information eftersom vi vet så lite om PFOS och de ämnen som nu introduceras för att ersätta PFOS. Det är dock viktigt att komma ihåg att det inte är möjligt att fullt jämföra laboratoriestudier med de dynamiska förändringar i exponeringsmängd och omgivande miljöfaktorer som finns i det levande djuret. I verkligheten är dessutom PFOS inte den enda substansen med möjliga effekter på embryoutveckling, vilket adderar interagerande effekter från ett okänt antal substanser hos det enskilda djuret eller den enskilda människan. Ytterligare studier behövs för att undersöka PFOS effekt på den tidiga embryoutvecklingen hos nöt, PFOS effekt vid längre exponeringstider samt dess effekter på senare embryoutveckling och livet efter födseln.

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