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Effect of perfluorononanoic acid (PFNA) on bovine early embryo development *in vitro*

Effekten av perfluorononansyra (PFNA) på tidig embryonal utveckling *in vitro* hos nötkreatur

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SUMMARY

Perfluoroalkyl and polyfluoroalkyl substances (PFASs) is a large family of highly fluorinated chemicals, used in different commercial products (*e.g.* in clothing, shampoos, kitchenware and fire-fighting foams) utilizing their different properties, and have been found in wildlife, humans and in the environment. According to several temporal trend studies, PFNA (perfluorononanoic acid) seems to increase in concentration in the general population and in the environment. PFNA is closely related to the more well-known PFOA (perfluorooctanoic acid), a substance with known reproductive toxicity, but studies regarding PFNAs full toxicological potential effects, especially developmental toxicity, are limited. The aim of this study was to examine the early effects of PFNA exposure on oocytes during *in vitro* maturation. Using an *in vitro* model, a controlled environment is established where individual chemicals can be tested without the usage of laboratory animals. Regarding oocyte maturation and initial embryo development, the bovine model is a better model for humans, compared to the murine model.

In this study a bovine *in vitro* embryo production model was used. Abattoir-derived bovine ovaries were used to collect cumulus oocyte complexes (n = 440). Half of these were matured *in vitro* under PFNA exposure (0.1 µg/ml) followed by *in vitro* fertilization and culture, as for the control group. The used concentration was based on a previous study where PFNA was measured in follicle fluid of women (0.4 (0.2-2.1) ng/ml). A margin of safety and the short time of exposure in this study are the reasons for the raised concentration compared to the measured values in the follicle fluid.

Oocyte and embryo development were assessed regarding cumulus expansion, cleavage rate 44h post fertilization and day 7 and 8 blastocyst development and morphology. Day 8 blastocysts were stained with Mitotracker Orange (visualizes active mitochondria) and fixed in paraformaldehyde, followed by additional staining with nuclear stain DRAQ5 and lipid stain LipidTOX. For analysis of the embryos a confocal laser scanning microscope was used. The embryos were evaluated concerning number of nuclei, distribution of mitochondria, lipid droplet morphology and lipid pixel intensity. Statistical analyses were performed by linear mixed models and generalized linear mixed models, with replicate as random factor and observations on day 7 and 8 as repeated measures.

A total of 440 oocytes from 8 batches were included to *in vitro* maturation, resulting in 88 blastocysts. No significant difference between treated and control group was seen regarding cumulus expansion, cleavage rate, blastocyst development day 7 and 8, quality grade of blastocysts, stage of blastocysts, number of nuclei, mitochondrial distribution scoring or mean lipid pixel intensity. However, there was a significant difference in lipid droplet distribution score between treated and control group ($p = 0.048$), suggesting an increase in blastocyst lipid content due to the PFNA exposure. It is difficult to know exactly what effect this change in lipid expression has on further embryo development and more studies are needed to investigate this.

SAMMANFATTNING

Poly- och perfluorerade alkylsyror (PFASs) är en stor familj bestående av högfluorerade kemikalier som används i en mängd olika kommersiella produkter (t.ex. i kläder, schampo, köksredskap och brandskum) där deras olika egenskaper utnyttjas. Dessa syntetiska ämnen har kunnat uppmätas i människa, djur, natur och i vår vardagsmiljö. PFNA (perfluorononansyra) är en substans vars förekomst och koncentration i den genomgående populationen och miljön tycks öka enligt tidstrend studier. PFNA är närbesläktad till PFOA (perfluoroktansyra) som är en mer välkänd och studerad substans med känd reproduktionstoxicitet. Det finns mycket få studier om PFNAs fulla potentiella toxikologiska effekter, särskilt på fosterutvecklingen. Syftet med denna studie var att undersöka vilka effekter exponering av PFNA under oocytmognaden *in vitro* har på tidig embryoutveckling. Användandet av en *in vitro* modell möjliggör en kontrollerad miljö där enskilda kemikalier kan testas utan att en stor mängd försöksdjur behöver nyttjas. Nötmodellen, till skillnad från musmodellen, är en bättre modell för människor avseende oocytmognad och tidig embryonal utveckling.

I denna studie användes en *in vitro*-modell av nöt för framställning av embryon. Nötäggstockar från slakteri användes för att samla in cumulus-oocyt-komplex (n = 440). Hälften av dessa fick mogna *in vitro* med exponering av PFNA (0.1 µg/ml) följt av *in vitro* fertilisering och odling, precis som kontrollgruppen. Den använda koncentrationen av PFNA baserades på uppmätta koncentrationer i follikelvätska hos kvinnor (0.4 (0.2-2.1) ng/ml) i en studie av Petro *et al.* (2014). En pålagd säkerhetsmarginal samt den korta exponeringstiden i denna studie är anledningen till att den använda dosen är högre jämfört med de uppmätta värdena i follikelvätskan.

Oocyternas och embryonas utveckling utvärderades avseende expandering av cumuluscellerna, klyvningskvot 44h efter fertilisering samt blastocystutveckling och morfologi på dag 7 och 8. Dag 8-blastocyster färgades med Mitotracker Orange (färgar in aktiva mitokondrier) och fixerades i paraformaldehyd följt av ytterligare färgning med kärnfärgen DRAQ5 och lipidfärgen LipidTOX. Analysering av embryona gjordes med hjälp av konfokal laser scanning mikroskop. Embryona utvärderades med avseende på kärnantal, fördelning av mitokondrier, morfologi på lipiddroppar och lipidfärgens pixelintensitet. Statistiska analyser utfördes med linjära mixade modeller och generaliserade mixade modeller, med replikat som slumpmässig faktor och observationer från dag 7 och 8 som upprepade mätningar.

Totalt 440 oocyter från 8 batcher genomgick *in vitro* mognad vilket resulterade i 88 blastocyster. Ingen signifikant skillnad mellan behandlad grupp och kontrollgrupp kunde ses avseende expandering av cumulusceller, klyvningskvot, blastocystutveckling dag 7 och dag 8, kvalitetsgradering på blastocysterna, blastocyststadie, kärnantal, gradering av mitokondriefördelning eller medelpixelintensitet på lipider. Gällande storleksfördelning på lipiddropparna var det en signifikant skillnad mellan behandlad och kontrollgrupp ($p = 0.048$) vilket tyder på en möjlig ökning av lipidinnehållet i blastocyster efter exponering för PFNA. Det är svårt att veta exakt vilken effekt denna ändring i lipidstatus har på embryots fortsatta utveckling och ytterligare studier behövs för att utreda detta.

CONTENT

ABBREVIATIONS

ATP	Adenosine triphosphate
BSA	Bovine Serum Albumine
C	Control group, no treatment during maturation *in vitro*
CO_2	Carbon dioxide
COCs	Cumulus oocyte complexes
DRAQ5	DRAQ5® (Deep Red Anthraquinone 5) – a far-red fluorescent DNA stain
EDC	Endocrine disrupting chemical
FSH	Follicle stimulating hormone
FTOs	Fluorotelomer olefins
HeNe	Helium-neon
ICM	Inner cell mass
IETS	International Embryo Transfer Society
IVF	*In vitro* embryo fertilization
IVP	*In vitro* embryo production
LH	Luteinizing hormone
LipidTOX	HCS LipidTOX™ Green Neutral Lipid Stain – green fluorescent lipid stain
LSM 510	Confocal laser scanning microscope from Zeiss, Oberkochen, Germany
mM	Millimolar
mOsmo	Milliosmolality
mSOF	Modified synthetic oviductal fluid
MTO	MitoTracker® Orange CMTMRos – orange fluorescent mitochondrial stain
mW	Milliwatt
n:2 FTIs	Fluorotelomer iodides
n:2 FTOHs	Fluorotelomer alcohols
N_2	Nitrogen

nM	Nanomolar
nm	Nanometer
O_2	Oxygen
PASFs	Perfluoroalkane sulfonyl fluorides
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PFAAs	Perfluoroalkyl acids
PFAIs	Perfluoroalkyl iodides
PFASs	Perfluoroalkyl and polyfluoroalkyl substances
PFBS	Perfluorobutane sulfonic acid
PFCAs	Perfluoroalkyl carboxylic acids
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonic acid
PFPAs	Perfluoroalkyl phosphonic acids
PFPIAs	Perfluoroalkyl phosphinic acids
PFSAs	Perfluoroalkane/-alkyl sulfonic acids
PFSIAs	Perfluoroalkane/-alkyl sulfinic acids
PVA	Polyvinyl alcohol
T	Group treated with 0.1 µg/ml PFNA during maturation *in vitro*
UNEP	United Nations Environment Programme
WHO	World Health Organization

INTRODUCTION

According to the World Health Organization/United Nations Environment Programme report in 2013, there are close to 800 chemicals (year 2012) which are known or suspected to be able to interfere with endocrine functions. Only a few of these have been studied and investigated, and its effect determined *in vivo*. The greater part of the chemicals available on the market today have not been submitted to any tests at all. This makes it very unclear what the risk is when we are exposed to multiple chemicals that could possibly disturb the reproductive system. Several laboratory studies reveal that chemical exposures can induce endocrine disorders both in humans and wildlife. The most susceptible periods of exposure to endocrine disrupting chemicals (EDCs) are during pre-fetal and fetal development and during puberty. Exposures during these periods does not necessarily lead to changes that are visible as birth defects but can contribute to changes that could come to increase the incidence of diseases and reproductive disturbance or malfunction throughout life or in subsequent generations (WHO/UNEP 2013).

A large family of man-made highly fluorinated chemicals called perfluoroalkyl and polyfluoroalkyl substances (PFASs) are being used in commercial products such as food packaging, fire-fighting foams and water and oil repellent products, and they have been found in the environment, wildlife and humans (KemI, 2015; Borg & Håkansson, 2012; Buck *et al.* 2011). Some of these chemicals are known EDCs (Jensen & Leffers, 2008). Several temporal trend studies made in Sweden reports that perfluorononanoic acid (PFNA) is a substance that seem to increase in the general population and in the environment (Borg & Håkansson, 2012) but studies regarding its full toxicological potential effects, especially developmental toxicity, are limited (Abbott, 2015; Bull *et al.*, 2014).

In vitro embryo production is a well-known and established reproduction biotechnology and method in bovine breeding, which is frequently used throughout the world. In the bovine, this technique is well developed and in the last years, the amount of transferred *in vivo* and *in vitro* embryos has increased, and the proportion of embryos produced *in vitro* has increased considerably. In 2014, 10.5 % of all registered embryos transferred in Europe were IVP embryos (AETE, 2015). In reproductive toxicology studies, an *in vitro* model enable a controlled setting where testing of individual chemicals is possible but without the usage of a very large amount of laboratory animals (Santos et al., 2014; Ménézo & Hérubel, 2002). Unlike the murine model, the bovine *in vitro* model is a more suited model for humans regarding oocyte maturation and early embryo development (Ménézo & Hérubel, 2002).

The aim of this study was to proceed on an unpublished pilot study on the effect of PFNA on bovine oocyte maturation *in vitro*, this time conducted by lowering the concentration of exposure and examine if effects were still seen. The used PFNA concentration was based on the study by Petro *et al.* (2014), where measured levels of PFNA in follicle fluid of women were 0.4 (0.2-2.1) ng/ml (median concentration with range, min-max), making the concentration used in this study nearly 50-500 times higher - an outcome of added margin of safety and the short time of exposure used in this study. Evaluations of embryo development were done by morphology assessment and staining with mitochondrial, nuclear and lipid stain. By using *in vitro* bovine embryo production as a model to study the effects of exposure, no experimental animals were necessary.

LITERATURE REVIEW

EDCs

WHO/UNEP defines EDCs as: “an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations”. Environmental contamination leads to exposure to these chemicals which can interfere with hormone synthesis, storage, function and/or metabolism. EDCs has been found in human follicular fluid and could through its direct contact to the oocyte and surrounding cells have an association with the increasing incidence of human subfertility (Petro *et al.*, 2012). Chronic exposure to EDCs of environmentally relevant concentrations may generate the same or even more toxic effects than an acute exposure with high concentrations (Petro *et al.*, 2012). It has been seen that effects on fertilization- and blastocyst rate *in vitro* occurs already at EDC-concentrations that are lower than the *in vivo* levels (Petro *et al.*, 2012).

Perfluoroalkyl and polyfluoroalkyl substances (PFASs)

PFASs are organic and nonorganic substances that all have in common that they consist of a carbon chain where one or several hydrogen atoms are replaced by fluorine (Buck *et al.*, 2011). The binding between carbon and fluorine is very strong which makes PFASs extremely persistent in the environment (KemI, 2016; Smart, 1994). In addition, numerous PFASs are mobile in soil and could therefore pose a problem to water resources (KemI, 2016).

On the global market there are over 2000 different PFASs with a great variety of biological, chemical and physical attributes. Ninety-three of them belong to the perfluoroalkyl carboxylic acids (PFCAs) family where PFNA is included (KemI, 2015; Buck *et al.*, 2011). Figure 1 shows a range of selected PFASs families and their relationships.

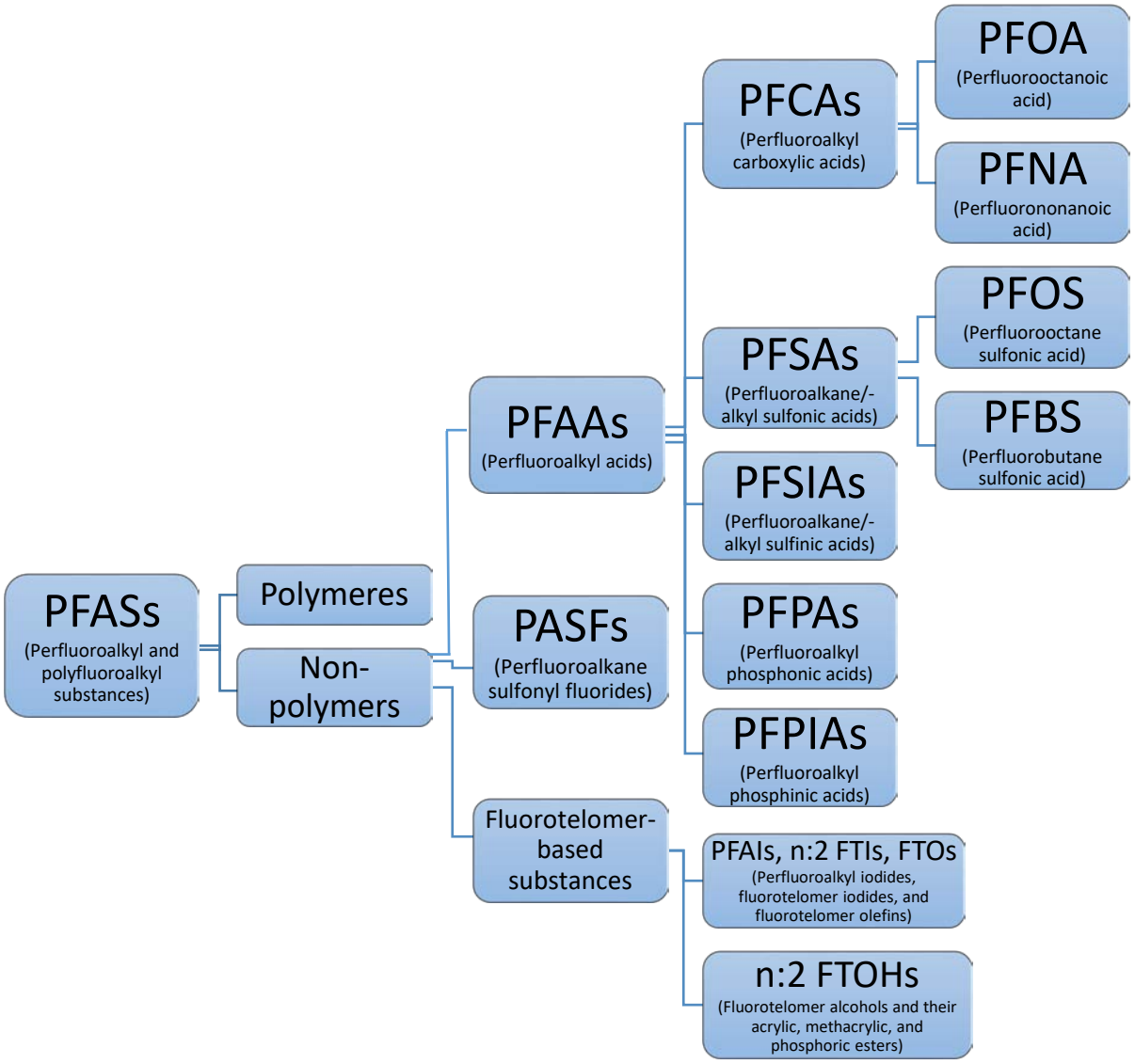

Figure 1: *PFASs families.* A range of selected PFASs families in hierarchical order according to the classification by Buck *et al.* (2011).

Since the 1950s, PFASs have been frequently used in industries throughout the world as surfactants, emulsifiers and surface protectors (Borg & Håkansson, 2012; Slama & Cordier, 2010). The substances can be found in water and stain/oil repellent products like carpets, clothing, furniture, cleaning products, shampoos, food packaging, non-stick kitchenware and fire-fighting foams (Slama & Cordier, 2010; OECD, 2002).

Sources of PFASs in the environment are for example release from industrial use, training areas for fire departments, leakage from waste disposals and landfill sites, sewage plants and from customer products (KemI, 2015; WHO/UNEP 2013; Borg & Håkansson, 2012). As early as 1976, perfluorooctanoic acid (PFOA) was isolated in pooled human plasma in the United States (Waldbott & Yiamouyiannis, 1977). Since then there have been numerous reports worldwide that have detected PFASs in human, wildlife and the environment, for example umbilical cord serum, breast milk, follicle fluid, amniotic fluid, urine, mammal and aquatic animal organs such as liver and kidneys, environs like surface and drinking water, sludge, air and ice caps (Bull *et al.*, 2014; Lau *et al.*, 2007). Human are exposed to PFASs mainly through food and drinking

water, but also through usage of chemical products and inhalation of dust (Berglund *et al.*, 2013). Placental and lactational transfer in humans have been observed (Kim *et al.*, 2011). Dietary exposure is thought to be the dominating source, with seafood being the largest contributor (Haug *et al.*, 2010). In the same study, they observed that with age the serum concentration of PFASs increased, suggesting bioaccumulation. Several studies also suggest a biomagnification capacity, as the highest concentrations of PFASs were found in fish-eating top predators (Lau *et al.*, 2007).

There is a high oral uptake of PFASs and they accumulate primarily in the liver and in the blood, due to high affinity to proteins (Berglund *et al.*, 2013; Borg & Håkansson, 2012). Generally, no metabolism of substances with a carboxylic or sulfonic group has been seen (Borg & Håkansson, 2012) and renal elimination has been observed (Bull *et al.* 2014) although the overall elimination is poor (Lau *et al.*, 2007). In one biomonitoring study of PFCAs in humans it was shown that as the carbon chain length increased up to C9-C10 the efficiency of renal clearance decreased (Zhang *et al.*, 2013).

PFOA and perfluorooctane sulfonic acid (PFOS) have in screenings been the most frequently measured PFASs, and they are also the most toxicologically investigated substances in this group (Borg & Håkansson, 2012; Buck *et al*., 2011). Examples of their toxic effects from the review by EFSA (Bull *et al.*, 2014) are effects on endocrine hormones, decreased sperm quality and concentration, hepatomegaly, effects on pro-inflammatory and anti-inflammatory cytokines, reduction of total thymocytes and splenocytes, reduction of pup survival in mice, neurodevelopmental effects in form of affected behavior in mice pups, delayed mammary gland development, increased risk of cardiovascular problems, effects on lipid metabolism, increased time-to-pregnancy, fetal abnormalities and an increase of adenomas in liver and thyroid gland. The majority of these studies were performed on mice and the most common way of exposure was oral intake.

The length of the carbon chain seems to be related to the toxicity of the individual substance amongst the perfluoroalkyl acids (PFAAs) family (Zheng *et al.*, 2012; Hagenaars *et al.*, 2011). PFAAs with longer chain length (≥ C8) tend to have greater toxic potential than those with shorter chain length (Zheng *et al.*, 2012; Hagenaars *et al.*, 2011) and studies also show that the bioaccumulating ability increases with increasing chain length (Stevenson *et al.*, 2006; Martin *et al.*, 2003). A tendency towards longer half-lives the longer the carbon chain can be seen in an assemblage of data by Borg & Håkansson (2012) of a handful different PFASs, reaching from days to several years in human serum. The properties of the individual substance also depends on what functional group it contains; PFASs with a sulfonate group have generally a higher toxic and bioaccumulative potential than PFASs with a carboxyl group (Martin *et al.*, 2003; Hagenaars *et al.*, 2011).

Since 2006 there have been restrictions in the usage of PFOS and PFOS-related compounds as the European Union established a directive that stated a phase-out and regulation of PFOS as a substance or constituent in products and articles manufactured by the member states (European Parliament and Council Directive 2006/122/ECOF). The directive also mentions that PFOA should be kept under survey on the continuous risk assessments and that alternatives should be used because of the suspicion that PFOA has a similar risk profile as PFOS. Replacement

substances with similar properties have been developed. They are not covered by the restrictions, which has led to a distribution of numerous substances that are not well known and lacking a complete risk assessment (Berglund *et al.*, 2013; Borg & Håkansson, 2012). PFNA is such a substance.

PFNA

Perfluorononanoic acid (PFNA) (*Figure 2*) belongs to the group of perfluoroalkyl carboxylic acids (PFCAs) just like PFOA (*Figure 1*). PFNA has in several zebrafish embryo studies been found to be more toxic than PFOA (Ulhaq *et al.*, 2013; Zheng *et al.*, 2012; Hagenaars *et al.*, 2011). According to these studies, the recurring toxicologic ranking order of PFNA in relation to the two more well-known PFAAs, PFOS and PFOA, is PFOS>PFNA>PFOA. The order is of a concentration-dependent aspect.

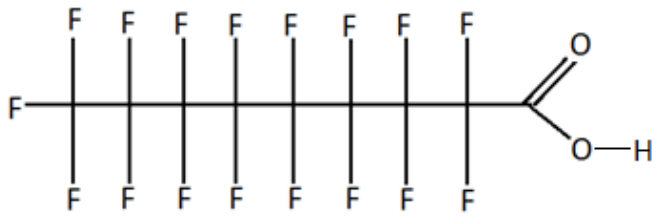

Figure 2: *Structure of PFNA.* Perfluorononanoic acid (PFNA), $C_9HF_{17}O_2$. (Adopted from Borg & Håkansson, 2012 and Buck *et al.*, 2011).

PFNA has been manufactured and used since the mid-1970s, mostly as a component in the manufacturing of fluoropolymers and as a fluorinated surfactant (Buck *et al.*, 2011).

In the United States PFNA has been found in over 98 % of the human population (WHO/UNEP 2013). PFNA was among the four most frequently observed PFASs in human serum in a temporal study in the U.S. (Kato *et al.*, 2011). It was revealed that PFNA concentration showed an upward trend between 1999 and 2008 and the concentration increased with age. There are substances that can degrade into PFNA, for example 8-2 fluorotelomer alcohol (FTOH) that could contribute to the increased concentrations both in organisms and nature (Henderson & Smith, 2006). In addition to serum, PFNA has been detected in for example umbilical cord serum, human milk, wildlife tissue, artic ice, water samples and soil (Bull *et al.*, 2014).

In mice and rats, PFNA is stored primarily in the liver, and the half-lives in serum and liver are longer than the half-life of PFOA (Tatum-Gibbs *et al.*, 2011). In the same study, there was a large sex difference in serum elimination after a single oral dose of PFNA, with females having a faster elimination time (estimated half-life of 1.4 days for female rats and 30.6 days for males). Biological half-life of PFNA in humans was estimated to approximately 2-4 years in a study by Zhang *et al*. (2013).

In vitro and *in vivo* studies on rat cells and rats, respectively, showed that PFNA exposure changed the gene expression of genes involved in lipid metabolism and inflammation (Fang *et al.*, 2012). PFAAs are similar to fatty acids in their structure with the hydrophobic perfluorocarbon tail and polar functional group which promote interactions with proteins and interference with different fatty-acid binding proteins (Bischel *et al.*, 2010). Due to this resemblance, PFASs are often considered as fatty acids by the body, like they are being transported by albumin in the blood (Borg & Håkansson, 2012). In the study by Bishel *et al.* (2010), using bovine serum albumin (BSA) as protein model, > 99 % of the PFNA were bound

to BSA. The closely related substance PFOA has been shown to be a potent activator of PPARα (peroxisome proliferator-activated receptors-alpha) and PPARγ, which normally are activated by fatty acids and prostaglandins (Dunning *et al.*, 2014; Vanden Heuvel *et al.*, 2006). PPARs are nuclear hormone receptors and involved in the regulation of lipid and glucose metabolism, inflammation and also cell proliferation and differentiation (Wolf *et al.*, 2010). Activation of PPARs lead to proliferation of peroxisomes, generating catabolism and a reduction of fatty acids and cholesterol in serum and a lipid accumulation in the liver (Borg & Håkansson, 2012). However, in epidemiological studies there was a link between PFOA and increased cholesterol levels in serum in human from highly exposed districts (Watkins *et al.*, 2015). PPARs play a role in development and their expression is needed for the developmental toxicity effects of PFNA to happen (Wolf *et al.*, 2010).

PFNA has been found to be cytotoxic and hepatotoxic in *in vitro* studies and immunotoxic *in vivo* (Wolf *et al.*, 2010). In mice, exposure during pregnancy lead to decreased pup survival, postponed eye opening and puberty, decreased body weight and increased weight of the liver (Wolf *et al.*, 2010). Apart from this, studies on reproductive toxicology and PFNA are scarce.

Embryo development

The ovaries are the female gonads which are producing oocytes and female sex hormones. In the ovaries there are a great amount of follicles, each one enclosing an evolving egg, an oocyte. The development to a matured oocyte starts during fetal stage with primordial follicles which during the estrous cycles progress into primary follicles followed by secondary follicles. The primordial follicle is surrounded by a thin single layer of epithelial cells. These cells proliferate and constitute of several layers and two cell types in the secondary follicle, granulosa cells and thecal cells, each separated by a basal lamina. The granulosa cells are closest to the oocyte, nourishing and protecting it by generating the zona pellucida, a layer of glycoproteins which separates the oocyte from the granulosa cells. The granulosa cells start to secrete a fluid into the follicular cavity as the secondary follicle have obtained a certain size, forming a fluid-filled antral follicle. The fluid contains nutrients needed for further oocyte and follicular maturation and enzymes needed for the ovulation. Increasing plasma concentrations of follicle-stimulating hormone (FSH) makes one or a few follicles grow into being a dominant preovulatory follicle (Sjaastad *et al.*, 2010).

During the oocyte and follicle maturation the granulosa cells differentiates into two subpopulations, mural granulosa cells and cumulus cells. The mural granulosa cells stays in connection with the basal lamina while the cumulus cells are in close contact with the oocyte, enclosing it. Together with the oocyte, the cumulus cells form a cumulus oocyte complex (COC). A preovulatory surge of luteinizing hormone (LH) increase the concentration of LH in plasma, initiates final maturation, involving cumulus expansion and ovulation of the dominant follicle, releasing the COC into the oviduct (Gordon, 2003).

For the fertilization to take place, the sperm must be transported through the cervix and the uterus and undergo capacitation, a maturing process for the spermatozoa. Fertilization, which occur in the oviduct, happens when a capacitated sperm binds and penetrates the zona pellucida and fuses with the oocyte (Sjaastad *et al.*, 2010).

The fusion results in a zygote and cell divisions kickoff as soon as it is formed, initially once every 24 hours. In bovine, a morula is formed 5-7 days post fertilization, which consists of an undifferentiated cell mass. After another 1-2 days the blastocyst is developed consisting of approximately 50 to 200 cells of two specific populations, an inner cell mass (ICM) that will evolve into the embryo, and an outer layer of cells (trophoblasts) that will form the fetal membranes. The zona pellucida is intact throughout the movement through the oviduct and into the uterus. It is thinning due to embryo expansion and ruptures 7-10 days post fertilization, allowing the blastocyst to hatch (Sjaastad *et al.*, 2010).

IVP – in vitro embryo production

In vitro embryo production in bovine starts off by collecting immature oocytes and attending cumulus cells – COCs, prior to the LH-surge, by aspirating follicles in the ovaries after slaughter or through ultrasound guided ovum pick up through the vaginal wall in the living cow or heifer. The following steps are *in vitro* maturation, *in vitro* fertilization and finally *in vitro* culturing until day 8 post fertilization. In the bovine, oocyte maturation usually proceeds for 22-24 hours, and the ideal incubation time for oocytes with addition of sperm for *in vitro* fertilization is 24 hours to obtain maximum proportion of fertilized oocytes (Gordon, 2003). However, it is not possible to completely simulate the *in vivo* conditions with its dynamic changes using *in vitro* environment. Compared to *in vivo*, the number of oocytes that are developed into blastocyst *in vitro* is reduced and limited to approximately 40 % (Rizos *et al.*, 2002).

Investigating the reproductive toxicology of chemicals using *in vivo* tests require great quantities of laboratory animals. In addition, mice are not the optimal model animal for humans, in particular when it comes to oocyte maturation and fertilization (Ménézo & Hérubel, 2002). Bovine oocyte maturation and initial embryo development are on the other hand quite similar to that of humans, enabling more authentic and complex screening methods (Santos *et al.*, 2014). Using a suitable *in vitro* fertilization (IVF) model is key to recognize the risks and the mechanisms of action of an endocrine disrupting chemical (EDC) (Santos *et al.*, 2014).

The gold standard for good oocyte quality is its ability to result in a living descendant, which includes the ability of the oocyte to be fertilized, develop into a blastocyst and create a pregnancy after transfer into a live recipient. While working with IVP, other parameters are used to assess the quality which are more practical, but these cannot replace the gold standard. Parameters used are for example the developmental capacity of the oocyte, which is measured as the timing of the very first cleavage of the zygote, the morphological appearance of the cumulus cells and oocyte cytoplasm, lipid content and ultrastructural assessment of the nuclear stage and cytoplasm (Leroy *et al.*, 2008).

MATERIAL AND METHODS

Experimental design

The ovaries that were used came from both heifers and cows and were collected immediately after slaughter at an abattoir. The collection went on during a maximum of 1 hour before transportation back to the laboratory. In the laboratory COCs were aspirated from selected

follicles, followed by assessment and election of suitable ones. Approved COCs were then randomly divided into two groups: control group (C) or treatment group (T) with supplement of 0.1 µg/ml PFNA, that from here on were handled separately from each other throughout the trial. According to standard protocols the oocytes were then submitted to *in vitro* maturation, fertilization and culture. The semen used for fertilization came from one individual, a bull of SRB breed, with previously proven *in vivo* and *in vitro* fertility. During six weeks eight batches were run with an outcome of groups with 21-34 COCs in each batch. The total number of COCs submitted to *in vitro* maturation in the trial was 440. Set parameters for assessment of oocyte maturation and embryo development were registration of cumulus expansion, cleavage rate 44h post fertilization and day 7 and 8 blastocyst development and morphology. Day 8 blastocysts were stained with mitochondrial stain and fixed in paraformaldehyde, followed by additional staining with nuclear stain and lipid stain. For analysis of the embryos confocal laser scanning microscope was used. The status of exposure in the blastocysts was known in all assessments.

Media

All of the chemicals used were received from (Sigma Aldrich, Stockholm, Sweden) if not otherwise mentioned. Completed media were all filtered sterile through 0.2 µm filter unit and stored at 4 °C. At least 1 hour before usage the media was put to equilibrate in 5 % CO_2 incubator at 38.5 °C, or pre-warmed in a heat-box (Search and Wash2 Medium).

Search Medium

Every week fresh Search Medium was made. After aspiration COCs were preserved and washed in Search Medium consisted of TCM 199 with hepes (modified buffer system) (M7528) with supplement of 0.2 % w/v Albumin, Bovine 96-99 %, BSA (Fraction V) (A3311), and 50 µg/ml Gentamicin-sulphate (G1264). pH was measured and corrected if needed to 7.3 – 7.4 and milliosmolality (mOsmo) to 290 – 300 mOsmo.

Maturation Medium

Each day of ovary collection fresh Maturation Medium was made for *in vitro* maturation. The Maturation medium consisted of TCM 199 without hepes (M2154) with supplement of 0.68 mM L-Glutamine (G8540), 50 µg/ml Gentamicin-sulphate (G1264), 0.4 % w/v Albumin, Bovine 96-99 %, BSA (Fraction V) (A3311), 50 µg/ml FSH and 10 µg/ml LH (Stimufol, Partnar Animal Health). pH was measured and corrected if needed to 7.3 – 7.4 and milliosmolality to 280 – 300 mOsmo. In treated groups *in vitro* maturation occurred in Maturation Media with added 0.1 µg/ml PFNA dissolved in TCM 199 (M2154).

Fertilization Media

Three media were used for *in vitro* fertilization, made up weekly. Firstly, oocytes were washed and handled in Wash2 Medium which consisted of 0.3 % w/v Albumin, Bovine 96-99 %, BSA (Fraction V) (A3311), 2 mM Calcium Chloride Dihydrate ($MgCl_2$ $2H_2O$) (C7902), 50µg/ml Gentamicin-sulphate (G1264), 10 mM HEPES (H3375), 0.49 mM Magnesium Chloride Hexahydrate ($MgCl_2$ $6H_2O$) (M2393), 1 µl/ml Phenol red (P0290), 3.19 mM Potassium Chloride (KCl) (P5405), 2 mM Sodium Bicarbonate ($NaHCO^3$) (S5761), 114 mM Sodium

Chloride (NaCl) (S5886), 21 mM Sodium DL-lactate (L7900), 0.40 mM Sodium Phosphate Monobasic (NaH_2PO_4) (S5011) and 0.5 mM Sodium Pyruvate (P4562). pH was measured and corrected if needed to 7.3 – 7.4 and milliosmolality to 270 – 290 mOsmo.

Capacitation Medium, used for the selection of motile sperm, consisted of 0.6 % w/v Albumin, Bovine 96-99 %, BSA (Fraction V) (A3311), 50µg/ml Gentamicin-sulphate (G1264), 6.9 mM Glucose (G6152), 5 mM HEPES (H3375), 0.49 mM Magnesium Chloride Hexahydrate ($MgCl_2$ $6H_2O$) (M2393), 2.68 mM Potassium Chloride (KCl) (P5405), 25 mM Sodium Bicarbonate ($NaHCO^3$) (S5761), 110 mM Sodium Chloride (NaCl) (S5886), 21 mM Sodium DL-lactate (L7900), 0.40 mM Sodium Phosphate Monobasic (NaH_2PO_4) (S5011) and 1 mM Sodium Pyruvate (P4562). pH was measured and corrected if needed to 7.3 – 7.4 and milliosmolality to 290 – 310 mOsmo.

Fertilization Medium for *in vitro* fertilization consisted of 0.6 % w/v Albumin, Bovine Serum, Fatty Acid Free (A7030), 2 mM Calcium Chloride Dihydrate ($MgCl_2$ $2H_2O$) (C7902), 50 µg/ml Gentamicin-sulphate (G1264), 10 mM HEPES (H3375), 0.49 mM Magnesium Chloride Hexahydrate ($MgCl_2$ $6H_2O$) (M2393), 1 µl/ml Phenol red (P0290), 3.19 mM Potassium Chloride (KCl) (P5405), 24.88 mM Sodium Bicarbonate ($NaHCO^3$) (S5761), 114 mM Sodium Chloride (NaCl) (S5886), 21 mM Sodium DL-lactate (L7900), 0.40 mM Sodium Phosphate Monobasic (NaH_2PO_4) (S5011), 0.5 mM Sodium Pyruvate (P4562), with addition of 3 µg/ml Heparin (H3149) and PHE (consisting of 1.5 µM Epinephrine (E1635), 10 µM Hypotaurine (H1384), 20 µM Pencillamine (P4875), 1.8 mM Sodium Chloride (NaCl) (S5886), 10.5 mM Sodium DL-lactate (L7900), and 42 µM Sodium Metabisulphite (S9000)). pH was measured and corrected if needed to 7.8 and milliosmolality to 290 – 310 mOsmo.

Culture Medium

Modified synthetic oviductal fluid (mSOF Medium) was used for *in vitro* culture of the oocytes and embryos, and was made up weekly. This medium consisted of 0.4 % w/v Albumin, Bovine Serum, Fatty acid free (A7030), 1.71 mM Calcium Chloride Dihydrate ($CaCl_2$ $2H_2O$) (C7902), 50 µg/ml Gentamicin-sulphate (G1264), 1.5 mM Glucose (G6152), 1 mM L-glutamine (G8540), 0.49 mM Magnesium Chloride Hexahydrate ($MgCl_2$ $6H_2O$) (M2393), 0.03 µl/ml Phenol red (P0290), 7.2mM Potassium Chloride (KCl) (P5405), 1.19mM Potassium Phosphate Monobasic (KH_2 PO_4) (P5655), 0.33 mM Pyruvic Acid Sodium Salt (P4562), 25 mM Sodium Bicarbonate ($NaHCO_3$) (S5761), 0.11 M Sodium Chloride (NaCl) (S5886), 9.9 mM Sodium DL-lactate (L7900), with addition of BME Amino Acids Solution (50x)(B6766) and MEM Non-essential Amino Acids Solution (100x) (M7145). pH was measured and corrected if needed to 7.4 and milliosmolality to 270 – 280 mOsmo.

Procedure of *in vitro* embryo production

Maturation

After collection at the abattoir the ovaries were transported for 3-4 hours to the laboratory in thermos flasks in 30.0-33.0 °C 0.9 % NaCl solution. At arrival, the ovaries were rinsed in a sieve with 38.5 °C 0.9 % NaCl solution and then put into a clean pre-warmed thermos flask with fresh 38.5 °C 0.9 % NaCl solution. With a 5 ml syringe and 18 gauge needle COCs were

aspirated from 3-8 mm follicles and kept in pre-warmed 25 ml Universal Containers with approximately 2 ml of Search Medium. These were then brought into the main laboratory where excess follicular fluid was removed from the containers leaving the COCs on the bottom. This remaining oocyte-containing follicular fluid was distributed between a couple of pre-warmed 60 mm TC dishes and mixed with Search Medium for oocyte localization through light microscope. Collected COCs were through light microscope assessed of morphological appearance according to classification criteria commonly used, Grade 1: Compact multilayered cumulus covering with a homogenous ooplasm and an overall light and transparent COC. Grade 2: As same as grade 1 but with a rough feature and a darker zona at the outer edge of the oocyte, the overall COC is a little darker and less transparent. Grade 3: The cumulus covering is less compact with an irregular ooplasm containing dark clusters, the overall appearance is darker than grade 1 and 2. Grade 4: Expanded cumulus covering with cumulus cells dispersed in dark clumps, irregular ooplasm containing dark clusters, the overall COC is dark and irregular (Gordon, 2003). Approved COCs were then randomly divided into two groups that from here on were handled separately from each other throughout the trial. The COCs were then washed through two pre-warmed 30 mm TC dishes containing 2 ml of Search Medium followed by two pre-warmed 30 mm TC dishes of equilibrated Maturation Medium. The COCs were then placed in a 4-well plate in groups of 20-35 oocytes with 500 µl of Maturation Media per well and incubated for maturation *in vitro* at 38.5 °C in 5 % CO_2 for 24 hours. Two homogenous groups were randomly made by each batch treated with PFNA (T) or no treatment (C). Treated groups had their own special Maturation Medium (see under “Media”).

Fertilization

Twenty-four hours post aspiration of COCs were completed the oocytes were fertilized. During maturation, the cumulus surrounding the oocytes had expanded and a subjective manual assessment of the expansion was done as a first step. Before fertilization COCs were transferred into Wash2 Medium where most of the cumulus surrounding the zona was removed by pipetting, leaving 3 to 5 layers of cumulus cells. All oocytes were then rinsed through Wash2 Medium three times followed by one time in Fertilization Medium before being transferred into 480 µl of Fertilization Medium in a 4-well plate, one group per well.

Two straws of 250 µl frozen bovine semen from a bull of SRB breed (3-1716 Sörby) were used. These were thawed at 35 °C for 12 seconds using tap water and then emptied into a pre-warmed 5 ml bore plastic tube. The semen was then distributed into another 4 pre-warmed small bore plastic tubes filled with 1 ml of Capacitation Media, where 100 µl semen was layered at the base of each tube. The tubes were incubated into 5 % CO_2 for sperm swim up for 45 minutes. Motility of the sperm were assessed through light microscope before and after swim up.

Thereafter the top layer of Capacitation Medium in the tubes were pooled and transferred into a pre-warmed centrifuge tube, set to spin for 300 x g for 7 minutes in a Sorvall ST 8 Centrifuge (ThermoFisher Scientific, Waltham, USA). The supernatant was then removed and the remaining pellet was diluted with Fertilization Medium. The number of spermatozoa per ml was determined using a Bürcher chamber. The spermatozoa were added to the prepared oocytes creating a final concentration of 1 x 10^6 spermatozoa/ml. The oocytes and spermatozoa were thereafter incubated for 22 hours at 38.5 °C in 5 % CO_2.

Transfer to culture

Twenty-two hours post fertilization the zygotes and unfertilized oocytes were prepared for a transfer to SOF Medium. First excess sperm and remaining cumulus cells, as well as remaining Fertilization Medium, were removed by pipetting and wash through 4 wells of SOF Medium leaving the zygotes/unfertilized oocytes as clean as possible. Then they were transferred into a well with 500 µl of SOF Medium followed by 300 µl of pre-equilibrated paraffin-oil, Ovoil (Vitrolife AB, Gothenburg, Sweden) distributed on top of the media to prevent evaporation while incubated at 38.5 °C in 5 % CO_2 and 5 % O_2 until day 8 post fertilization.

Cleavage check

Forty-four hours post fertilization the oocytes were assessed through light microscope regarding cleavage stage. In each group, they were assessed concerning percentage of oocytes cleaved into 2 cells and percentage of oocytes cleaved above the 2-cell stage.

Blastocyst development

An evaluation of blastocyst development was made on day 7 and 8 (168 and 192 hours post fertilization). The batches were assessed under light microscope concerning the total number of embryos per total fertilized oocytes. These were then divided according to IETS certification (Robertson & Nelson, 2010) in early blastocyst, blastocyst consisting of over 50 % fluid, expanding and expanded blastocyst, hatching or hatched blastocyst. Evaluation regarding stage of development and embryo quality was done according to IETS standards but stage was modified into three groups to facilitate statistical analysis:

Stage of development was graded from 1-3 where 3 is the highest stage and the most developed embryos. Stage 1: Early blastocysts or blastocysts over 50 %. The zona pellucida is intact, an antrum starting to portrait but no expansion of the embryo. Stage 2: Expanding or expanded blastocysts. Embryo has started to expand with bigger antrum and zona pellucida is thinner than in stage 1. Stage 3: Hatching or hatched blastocysts. Zona pellucida has ruptured or is completely missing.

Embryo quality was based on morphological characteristics, grade 1-4. Grade 1: Excellent or good. The embryo mass is symmetrical and round with individual cells (blastomeres) homogenous in size, density and color. The zona pellucida should be smooth also be round and even. Any irregularities should be insignificant. Grade 2: Fair. Individual blastomeres have moderate irregularities in size, color and density or in the shape of the embryonic mass. At least 50 % of the ICM need to be an intact, viable embryonic mass. Grade 3: Poor. Major irregularities concerning the shape of the embryonic mass, or in size, color and density of individual blastomeres. At least 25 % of the ICM need to be an intact, viable embryonic mass. Grade 4: Dead or degenerating, oocytes and one-cell embryos included (Robertson & Nelson, 2010).

Fixation

Day 8 blastocysts were stained with a mitochondrial staining, Mitotracker Orange, followed by fixation in 2 % paraformaldehyde in PBS with 0.1 % polyvinyl alcohol (P8136). The fixation

occurred overnight at 4 °C or at room temperature for one hour. Excess PFA was then washed of in PBS with 1 % PVA (P8136) before additional lipid and nuclear staining.

Staining

Mitotracker Orange

The orange-fluorescent stain MitoTracker® Orange CMTMRos (M7510) (ThermoFisher Scientific, Waltham, USA) (MTO) stains active mitochondria in live cells. Blastocysts were put in SOF Medium with 200 nM MTO and set to incubate for 30 minutes in 5 % CO_2, 5 % O_2 at 38.5 °C and thereafter washed three times in SOF Medium. Fluorescence excitation and emission spectra maxima 554 nm and 576 nm respectively.

DRAQ5

DRAQ5® (Deep Red Anthraquinone 5) (4084S) (BioNordika, Stockholm, Sweden) (DRAQ5) is a far-red fluorescent stain that stains DNA making nuclei visible. The blastocysts were put in 1 µl/ml of DRAQ5 solution mixed with PBS with 0.1 % PVA (P8136) (final concentration of 5 µM) for 20 minutes in room temperature protected from light followed by one wash in PBS with 0.1 % PVA (P8136). Fluorescence excitation and emission spectra maxima 647 nm and 681 nm respectively.

LipidTOX

HCS LipidTOX™ Green Neutral Lipid Stain (H34475) (ThermoFisher Scientific, Waltham, USA) (LipidTOX) is a green fluorescent stain that stains intracellular neutral lipids. It has a high affinity for neutral lipid droplets and is used for steatosis detection. The blastocysts were put into a solution of LipidTOX, prepared according to manufacturer instructions, and used in a concentration of 1:100 mixed with PBS with 0.1 % PVA (P8136) for 30 minutes at room temperature, protected from light. Fluorescence excitation and emission spectra maxima 495 nm and 505 nm respectively.

A total number of 10 embryos from 2 different batches were used as negative controls to test the stains.

Confocal Laser Scanning Microscopy

Mounting

In preparation for the confocal laser scanning the embryos were transferred on to a black well plate (ThermoFisher Scientific, Waltham, USA). Approximately 2 µl of the LipidTOX stain solution was transferred with the embryos per well. 1 µl of Vectashield (Vector Laboratories, Burlingame, USA) was added as help to stabilize the stains and prevent bleaching. A coverslip was attached over the wells and the plate was left to dry protected from light. Until analyzed the plates were kept at 4 °C in a dark box.

Zeiss LSM 510 and image analysis

Zeiss LSM 510 Confocal Microscope (Zeiss, Oberkochen, Germany) was used for embryo analysis. With a confocal microscope cells can be optically sectioned in three dimensions, and using laser scanning undesirable light from out-of-focus field can be excluded as the depth of

focus can be controlled (Ankerhold & Zimmermann, 2002). The cell structures earlier labeled with fluorophors are now made visible through light excitation using laser beams.

Three different laser lines were used, each working in different wavelength spectral composition: Tunable Argon laser (488 nm at 30 mW), Helium-neon (HeNe) laser (543 nm at 1 mW) HeNe laser (633 nm at 5 mW). Images taken were single scan and sectioned scan in seven levels (z-stack), and for LipidTOX an additional camera image using AxioCam MRm Zeiss (Zeiss, Oberkochen, Germany) with the same settings for all images. All images were taken with 20X lens.

The resulting images were assessed and evaluated in the image handling software program Zen2009 (Carl Zeiss Microscopy GmbH, Oberkochen, Germany). This program displayed the confocal images, enabling the manual assessment. The embryos were evaluated concerning number of nuclei where the nuclei were manually counted in the z-stack image. Images of level 2, 4 and 6 *(Figure 4)* were used to prevent a single nucleus to be counted twice. Mitochondrial staining was evaluated with regards to distribution on a scale 1-3 where 1 is the best grade (even distribution) and 3 is the worst grade (very uneven distribution with areas devoid of mitochondria or distinct aggregations of mitochondria) *(Figure 3)*. Neutral lipid staining was evaluated with regards to dominating size of lipid droplets (1-3 where 1 equals a majority for small droplets (≤ 3 µm in diameter) and 3 equals a majority of large droplets (> 3 µm), 2 equals unclear majority) *(Figure 5)*. LipidTOX was in addition evaluated regarding pixel intensity as a possible measure of the total amount of lipid content in the blastocyst. Pixel intensity was measured in two different images of the same embryo (one camera image with the same settings for all embryos throughout, and one 3D image based on the z-stack image) using a measuring function in the software program ImageJ. All images were assessed by the same person.

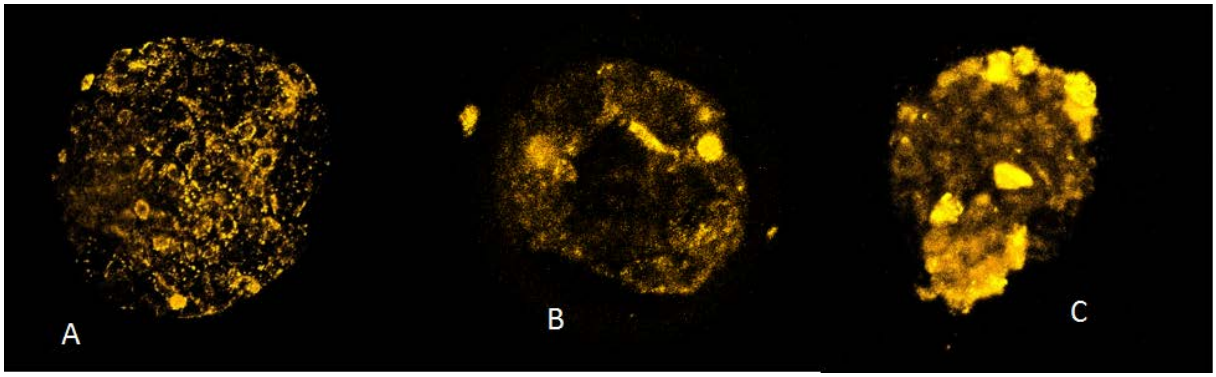

Figure 3: *Mitochondrial distribution scoring*. Images received from the confocal microscope were evaluated concerning mitochondrial distribution, using mitochondrial staining. A scale 1-3 where 1 is the best grade (even distribution) and 3 is the worst grade (very uneven distribution) was used; score 1 (A), score 2 (B), score 3 (C).

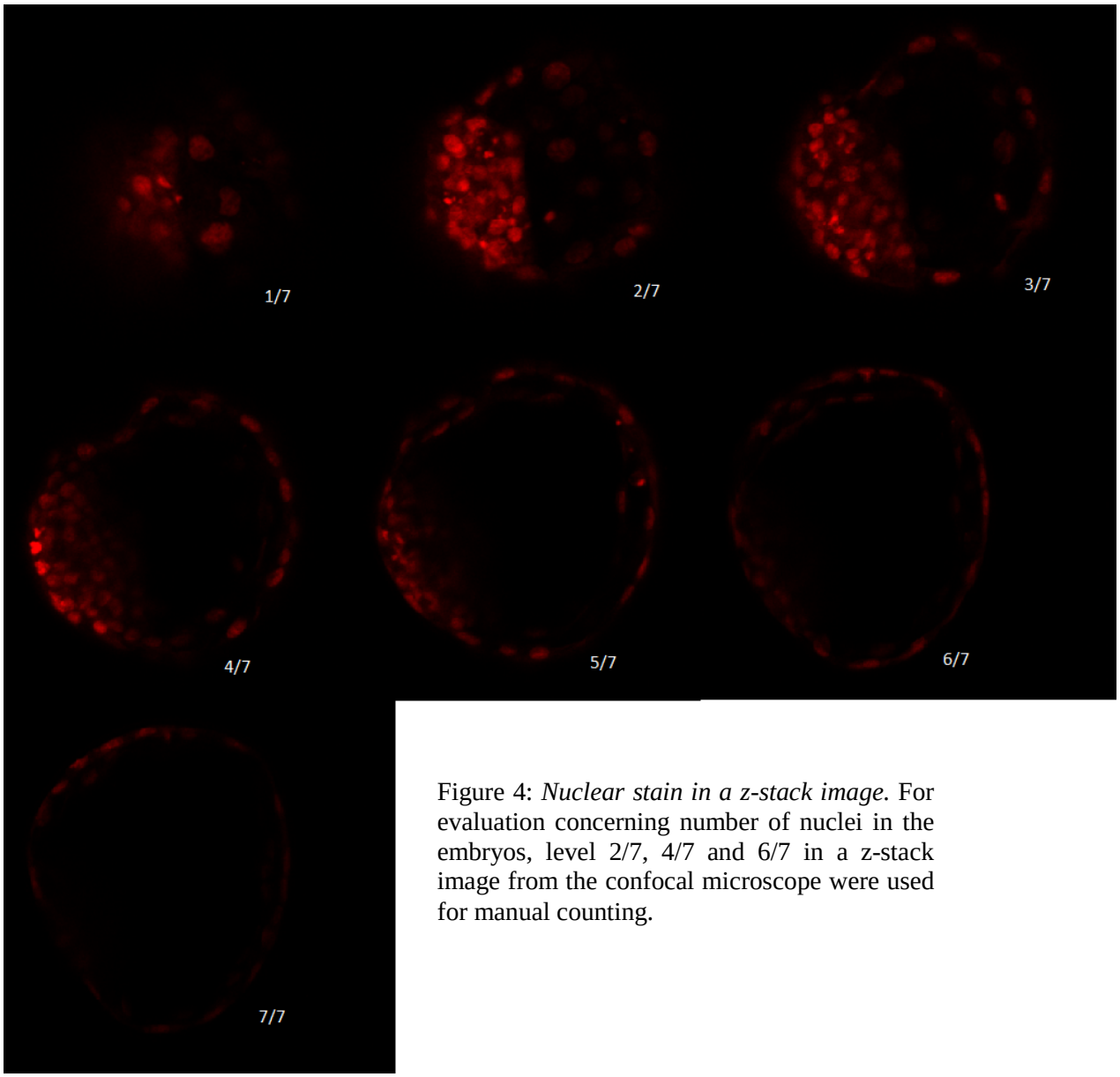

Figure 4: *Nuclear stain in a z-stack image.* For evaluation concerning number of nuclei in the embryos, level 2/7, 4/7 and 6/7 in a z-stack image from the confocal microscope were used for manual counting.

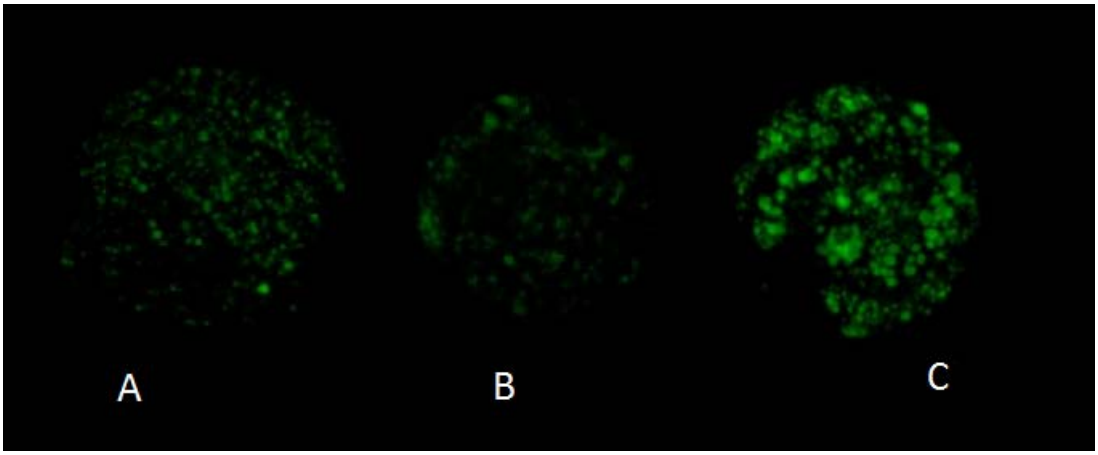

Figure 5: *Lipid droplet size scoring.* Images of neutral lipids, made visible by lipid stain, was evaluated and scored regarding the dominating size of the lipid droplets. A scale 1-3, where 1 equals a majority of small droplets (≤ 3 µm), 3 equals a majority of large droplets (> 3 µm) and 2 equals an unclear majority, was used; score 1 (A), score 2 (B), score 3 (C).

Statistics

For statistical analyses of the effect of treatment on number of cleavage and blastocyst formation, logistic regression (proc glimmix with binominal distribution, SAS 9.3, Milltown, USA) were performed, with replicate as random factor and observations on day 7 and 8 as repeated measures. For statistical analysis of continuous data collected by image analysis, i.e. number of nuclei, area and mean pixel intensity, linear mixed models with replicate as a random factor were used. Effect of treatment on ordinal variables (stage, grade, lipid droplet size, and mitochondrial distribution) was analyzed using the glimmix procedure with multinomial distribution and cumulative logit as function link (a proportional-odds cumulative logit model), again with replicate as random factor. P-values less than 0.05 were considered to be significant. Data are presented as mean values ± standard deviation (SD) if nothing else is stated.

RESULTS

In this study 440 oocytes from 8 batches were included to *in vitro* maturation, resulting in 88 blastocysts. 223 oocytes were in the treated group, exposed to 0.1 µg/ml PFNA during maturation, and 217 oocytes were in the control group, without PFNA added.

During the different steps in IVP, staining and fixation in this experiment, a couple of oocytes/blastocysts were lost in the process. These were eliminated from the starting number since they did not go through the whole trial. Some (n = 16) blastocysts failed to properly absorb dye and (n = 42) blastocysts could not be evaluated regarding at least one variable. The quality of the batches (the percentage of developed blastocysts) varied throughout the trial with a couple of them having under 10 % blastocyst development on day 8, which was considered a poor outcome.

In vitro embryo production

Assessment of the cumulus expansion after *in vitro* maturation showed no difference between groups or batches.

Cleavage rate calculated on oocytes in culture 44 hours post fertilization was 80.96 (± 8.75) % for treated group and 79.95 (± 9.16) % for control group (*Figure 6*). Cleavage rate above 2-cells was 59.00 (± 10.19) % for treated group and 59.98 (± 6.75) % for control group. For these two variables, there was no significant difference between the treated group and the control group.

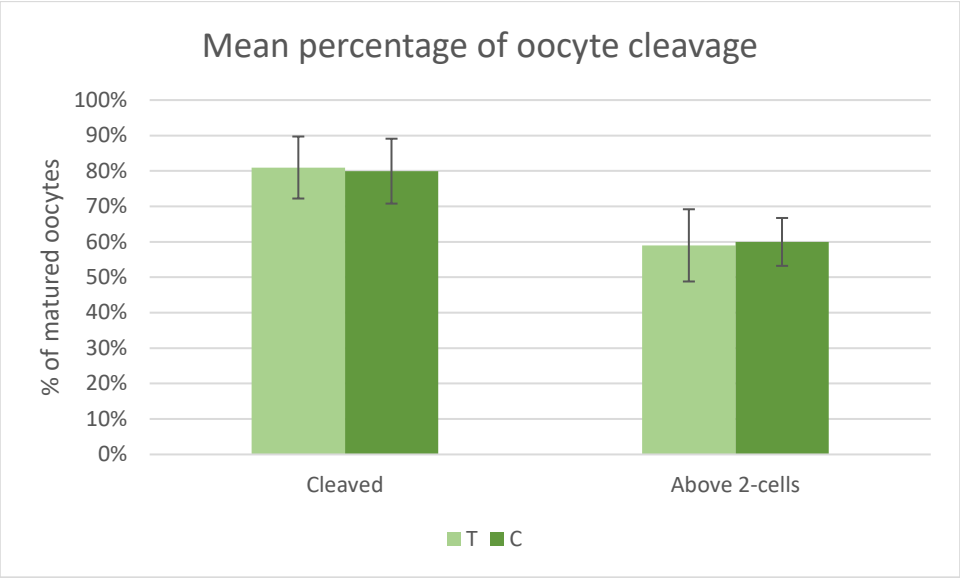

Figure 6: *Mean percentage of oocyte cleavage.* Assessed 44 hours post *in vitro* maturation and fertilization, calculated from the number of oocytes matured.

Blastocyst development

From the evaluation of blastocyst development made on day 7 and 8, no significant difference between the groups in number of blastocysts developed, quality grade or developmental stages was obtained. In addition, no significant difference was found when analyzing day 7 and 8 as repeated measures *(Table 1).*

Table 1. *Descriptive statistics for blastocyst development – blastocyst development parameters*

Parameter	Treated	Control	Significance
Day 7 blastocyst development[a]	11.94 (± 10.49) %	10.53 (± 5.22) %	ns
Day 8 blastocyst development[a]	19.92 (± 8.92) %	19.28 (± 5.15) %	ns
Stage of blastocysts (score 1-3)[b]	2 (1-3)	2 (1-3)	ns
Grade of blastocysts (grade 1-4)[b]	1.5 (1-2.5)	1 (1-3)	ns

ns = not significant. Embryo development and evaluation on day 7 and day 8 blastocysts. Blastocyst percentage was calculated on number of oocytes to *in vitro* maturation. Stage of blastocyst development was scored from 1-3 where 3 is the highest stage and the most developed embryos. Embryo quality was based on morphological characteristics and graded from 1-4 where grade 1 equals excellent or good and grade 4 equals dead or degenerating embryo.

[a]Mean percentage values (± SD) are presented

[b]Median values with range (min-max) are presented

Staining

The day 8 blastocysts were stained, fixed and analyzed concerning number of nuclei, mitochondrial distribution and lipid droplet size. With the nuclear staining used in this study there was no significant difference in the number of nuclei in the blastocysts between the treated (84.59 ± 36.10) and the control group (95.30 ± 39.26) *(Table 2)*.

The distribution pattern of stained mitochondria in the blastocysts were graded from 1-3. In the control group (n = 43), 11 blastocysts were scored having an even distribution of mitochondria (score 1), 19 blastocysts had a fair distribution (score 2) and 13 blastocysts had a very uneven distribution with areas devoid of mitochondria or distinct aggregations of mitochondria (score 3). In the treated group (n = 45) 6 had score 1, 27 had score 2 and 12 had score 3. There was no significant difference in the scoring between the groups *(Table 2)*.

In the neutral lipid staining the blastocysts were scored from 1-3 regarding the dominating size of the lipid droplets. In the control group (n = 41), 7 blastocysts were scored having a majority of small droplets (≤ 3 µm; score 1), 17 blastocysts had no dominating size of droplets and 17 had a majority of large droplets (> 3 µm; score 3). In the treated group (n = 40), the large droplets were more abundant (17 had score 2 and 23 had score 3) and no blastocyst were found to have a majority of small droplets (score 1) (*Figure 7*). There was a significant difference in distribution of lipid droplet size between treated and control group ($p = 0.048$) *(Table 2)*.

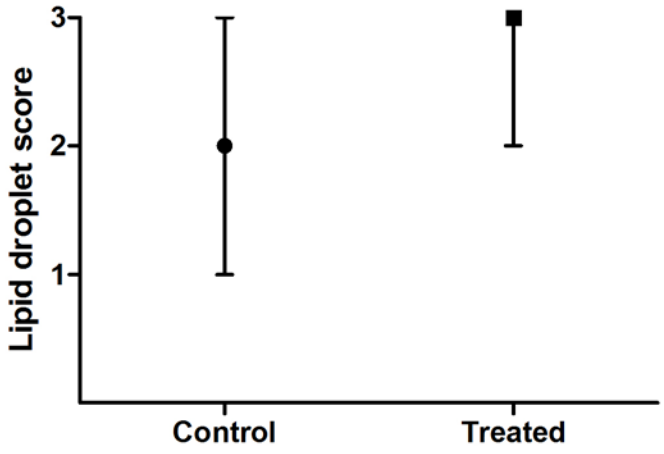

Figure 7: *Lipid droplet score*. Median lipid droplet size score and range in the treated group and the control group. The score was significantly different between the two groups ($p = 0.048$).

No significant difference between groups in mean pixel intensity for camera images (799.19 ± 522.37 for treated group and 833.50 ± 484.48 for control group) and 3D images (2.88 ± 3.10 for treated group and 3.67 ± 3.07 for control group) *(Table 2)*.

Table 2. *Descriptive statistics for blastocyst development – blastocyst image analysis*

Parameter	Treated	Control	Significance
Number of nuclei[a]	84.59 (± 36.10)	95.30 (± 39.26)	ns
Mitochondrial distribution scoring (score 1-3)[b]	2 (1-3)	2 (1-3)	ns
Lipid droplet size scoring (score 1-3)[b]	3 (2-3)	2 (1-3)	$p = 0.048$
LipidTOX pixel intensity cam[c]	799.19 (± 522.37)	833.50 (± 484.48)	ns
LipidTOX pixel intensity 3D[c]	2.88 (± 3.10)	3.67 (± 3.07)	ns

ns = not significant. Embryo development and evaluation on day 8 blastocysts regarding number of nuclei, distribution of mitochondrial, lipid droplet size scoring and pixel intensity in the treated group and in the control group. The distribution pattern of stained mitochondria in the blastocysts were graded from 1-3 where 1 equals even distribution and 3 very uneven distribution. In the lipid staining the blastocysts were scored from 1-3 regarding the dominating size of the lipid droplets where 1 equals a majority of small droplets (≤ 3 µm) and 3 equals a majority of large droplets (> 3 µm). The lipid stain was in addition evaluated regarding pixel intensity in two different images of the same embryo (one camera image and one 3D image).

[a]Total numbers (± SD) are presented

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

[c]Mean values (± SD) are presented

DISCUSSION

This study was designed to investigate if the presence of PFNA during oocyte maturation *in vitro* would cause any effects on maturation, further development, mitochondrial activity, neutral lipid morphology and generation of blastocysts on to day 8 after fertilization (day -1 equals onset of maturation). The concentration of PFNA used for exposure was 0.1 µg/ml (= 2.15 µM).

Experimental design

Time of exposure was very short in this study compared to *in vivo* conditions. The oocytes were exposed during *in vitro* maturation only, which continues for 22-24 hours in a strictly controlled environment, compared to constant exposure and fluctuating conditions *in vivo* (Gordon, 2003). The used concentration was based on a previous study where the measured concentration of PFNA in follicle fluid of women was 0.4 (0.2-2.1) ng/ml (median concentration with range, min-max) (Petro *et al*, 2014). This means that the concentration used in this study was approximately 50-500 times higher than the measured concentrations in women. Despite the short exposure time, effects were seen in the developed blastocysts of the exposed group nine days post exposure.

For reproductive toxicology studies, *in vitro* models enable a controlled environment where individual chemicals can be tested without usage of great quantities of laboratory animals (Santos *et al.*, 2014; Ménézo & Hérubel, 2002). The bovine model, unlike the murine model, is a better model for humans regarding oocyte maturation and initial embryo development (Ménézo & Hérubel, 2002). To be remembered though, it is not possible to fully mimic all the

in vivo conditions in laboratory set ups (Rizos *et al.*, 2002) and therefore all extrapolation of conclusions from *in vitro* results to *in vivo* conditions should be made with care. In addition, screening of individuals often reveal levels of more than one EDC which raise questions of potential cocktail effects (WHO/UNEP 2013).

With a subjective scoring as a method of evaluation, which is used in this study, there is an increased risk of bias. To overcome that, the measures could have been done blindly and evaluated by more than one person. Preferably the images would have been analyzed objectively, but without functioning image analyses that was not possible here.

The variation between batches were counteracted by a complete randomization of the oocytes between the groups before treatment.

PFNA toxicity

There are few developmental toxicity studies of interest available on PFNA where comparison with execution and results (or lack of results) found in this study, was possible to do. Therefore, parallels are drawn with the closely related compound PFOA and its causative effects instead. PFOA is more investigated and well-known than PFNA, and developmental toxicity so far found on PFNA is similar to that of PFOA (Bull *et al.*, 2014) making an extrapolation of developmental effects by PFOA to PFNA possible. It must be remembered though despite their resemblance these are two distinct individual chemical substances with their own characteristics.

Zheng *et al.* (2012) found that PFNA delayed hatching of the blastocyst in zebrafish with an EC_{50} concentration of 214 mg/L. No such effect was seen in this study.

Lipid droplets are organelles in cells where neutral lipids are stored (Greenberg *et al.*, 2011). The outcome of this study was that significant result was seen in the LipidTOX neutral lipid staining where treated groups had higher scoring in the dominating size of lipid droplets in the blastocysts. This suggests a possible increase in lipid content, as the size of lipid droplets seem to correlate with increased lipid volume (Suzuki *et al.*, 2011), due to PFNA exposure. Accumulation of lipid droplet in mice mammary alveolar and epithelial cells was seen after PPARα activation during pregnancy (Yang *et al.*, 2006), suggesting disruption of the lipid metabolism. This corresponds with what Wolf *et al.* (2010) describes about the expression of PPARs and their need for activation for the developmental toxicity of PFNA to occur. In rat liver, gene expression studies with PFOA demonstrated that genes involved in lipid metabolism and lipid transport were the most activated genes (Lau *et al.*, 2007).

In bovine, PPARγ is expressed in the granulosa cells and oocytes and has been found in early stages of growing follicles with an increase as the development and maturation progresses but a downregulation after the LH-surge. Activation of this PPAR isotype seem to control the production of steroid hormones in the granulosa cells, and therefore PPARγ is suggested to be involved in oocyte maturation. An interruption of the PPARγ gene has actually been seen to cause female subfertility (Huang, 2007). Studies using PPARα knockout mouse models reveal that developmental toxicity effects, such as reduced body weight, neonatal mortality and

delayed eye opening, after PFOA and PFNA *in utero* exposure are dependent on PPARα expression (Borg & Håkansson, 2012; Wolf *et al.*, 2010).

Disturbance in the neutral lipid metabolism, with its active enzymes, and lipid droplet anomaly has in mammalian cells been shown to be strongly related to development of diseases such as obesity, diabetes, atherosclerosis, neuropathy, cardiomyopathy and different forms of lipid storage diseases in human (Athenstaedt & Daum, 2006). Perhaps the increase in lipid content of exposed blastocysts in this study is not due to an actual increase but a failure in the degradation and utilization of the lipids compared to non-exposed blastocysts. However, this is in contrast to the fact that activation of PPARα, -γ and -δ can stimulate lipid droplet formation (Greenberg *et al.*, 2011).

During oocyte maturation, stored lipids in the oocyte serves as an energy source but during embryo development this source is not used in the same degree (Santos *et al.*, 2014) which correlates with the downregulation of, at least, PPARγ after the LH-surge (Huang, 2007). The question is how this lipid accumulation seen in this study affects the embryo and further development.

Intracellular lipid droplets are often positioned side by side with mitochondria for interaction and enabling transfer of fatty acids to mitochondria for β-oxidation (Suzuki *et al.*, 2011). Therefore, an impact on mitochondria could be assumed in this study as an effect was seen on the lipid droplets. In this study, no significant difference was seen in the mitochondrial distribution pattern in the blastocysts between the treated group and controls. The reason for this could be that the sample size of oocytes was too small, or that PFNA and its mechanism of action does not correlate with that of neutral lipids, giving no relevant effect on embryos regarding mitochondrial activity.

Effects of PFOA on mitochondria seen in previous studies are an increase in the nonselective permeability of the membranes (Starkov & Wallace, 2002). This leads to an interruption in the transmembrane potential which modifies the osmolarity and can lead to apoptosis (Hagenaars *et al.*, 2013). The increased permeability and the following movement of ions disturb the proton gradient, the oxidative phosphorylation and the essential mitochondrial production of Adenosine triphosphate (ATP) which also can lead to apoptosis (Hagenaars *et al.*, 2013).

There was no correlation in mean pixel intensity value between the camera image and 3D picture, suggesting a comparison in lipid content based on these two methods of measure would not correspond within the same embryo. In a comparative study, where the amount of lipid droplets was compared with fluorescence intensity technique in oocytes and blastocysts, both methods were highly correlated, suggesting that the use of fluorescence technique or a quantification method of measuring lipid content and accumulation are equivalent (Barceló-Fimbres & Seidel, 2011), a result that was not seen in this study. However, there were several images (n = 19) in this study that could not be evaluated on this parameter which probably could have had an impact on the statistical outcome. Another explanation could be that the image settings was individually adjusted in every z-stack scan made, and differed from the settings in the camera images, or that the two different methods simply does not correlate despite findings in the previously study.

In summary, several parameters were assessed in this study but the only significant difference was seen in the distribution of neutral lipids in the blastocysts where the treated group had a higher scoring regarding size of the lipid droplets. This suggests an increase in blastocyst lipid content due to PFNA exposure. The physiological relevance of this finding on further embryo development remains to be established. Follow up studies are also needed to investigate effects of longer exposure time and exposure with lower concentrations of PFNA and find a level where no effects are seen, in order to form an idea of a safety margin of exposure concentrations.

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