Perfluorononanoic acid (PFNA) alters lipid accumulation in bovine blastocysts after oocyte exposure during *in vitro* maturation

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ABSTRACT

Perfluorononanoic acid (PFNA) is one of the perfluoroalkyl acids present in human tissues. In this study, effects on early embryo development after PFNA exposure were investigated using the bovine *in vitro* production system. Oocytes were exposed to PFNA during maturation *in vitro* (10 µg mL^{-1} and 0.1 µg mL^{-1}), and then fertilized and cultured in parallel with control groups. Developmental parameters (cleavage, blastocyst formation) were followed and embryo quality evaluated (stage, grade). Embryos developed after exposure to 0.1 µg mL^{-1} were stained to distinguish nuclei, active mitochondria and neutral lipids. 10 µg mL^{-1} of PFNA had a severe negative effect on blastocyst formation (OR: 0.27 $p<0.05$), an effect not observed at 0.1 µg mL^{-1}. However, lipid droplet distribution was significantly altered in embryos exposed to 0.1 µg mL^{-1}, suggesting a disturbance of lipid metabolism after exposure to sublethal levels of PFNA during oocyte maturation *in vitro*.

KEYWORDS

Bovine, IVP, PFNA, lipid droplet, image analysis, developmental toxicology, endocrine disruption

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1. INTRODUCTION

Per- and polyfluoroalkyl substances are highly fluorinated aliphatic chemicals characterized by their extremely strong and stable C-F bond [1]. The subgroup perfluoroalkyl acids (PFAAs) contains compounds that, because of their durability and hydrophobicity, have been used widely since the 1950s in water and stain repellent materials, cookware, food packaging, insulating agents, and flame-retardants [1-3]. As a consequence of this widespread use and their stability, PFAAs can be found in both nature and wildlife, accumulating at the top of the food chain [1, 4, 5]. Because these chemicals are known to be toxic, there is legislation restricting their use (for examples, see Buck et al. [1]). Environmental decrease in PFAA levels is expected to be slow because of their persistence.

In humans, PFAAs can be detected in human tissue and have been found to pass through the placenta, exposing the growing embryo [6, 7]. The PFAAs usually studied are the sulphonic acid perfluorooctane sulfonic acid (PFOS, detected in the highest concentrations in human tissue) and the carboxylic acid perfluorooctanoic acid (PFOA, the second most concentrated in human tissue). Perfluorononanoic acid (PFNA) has also been frequently observed [3, 8-11] and, in contrast to PFOS and PFOA, PFNA levels have been increasing in humans in the first decade of this century [9, 10, 12]. PFNA is detected in human serum at mean concentrations of 0.8-1.49 ng mL^{-1}, with demographic, geographic and temporal differences [3, 6-8, 11]. For instance, remarkably higher levels are displayed in certain regions of Japan (mean 3.54-6.7 ng mL^{-1}) [13, 14]. PFNA has also been detected in human placenta, foetal organs [7] and follicular fluid (0.2-2.1 ng mL^{-1} [6]) indicating exposure to the maturing oocyte.

The developmental toxicity of PFAAs has been demonstrated in animal studies (reviewed by Lau et al. [15]), with severity varying between species and compounds. Generally, the perfluorinated sulphonic acids and the chemicals with longer carbon chains have higher toxicity compared to the carboxylic acids [16-18].

PFAAs have been studied in relation to subfecundity or infertility, although with contradictory results [19-24]. In some studies, PFNA (but not PFOS and PFOA) were associated with pregnancy loss and increased time to pregnancy [25, 26].

Safety assessment and toxicity testing of chemicals, and investigations of reproductive toxicity, include *in vivo* tests, most commonly using small rodents as model organisms. With the European REACH Legislation [27], the use of experimental animals has increased [28]. Even though the *in vitro* models of today can not completely replace the *in vivo* models used, the further improvement of *in vitro* techniques is essential for minimizing the need for experimental animals [28, 29] in line with the 3Rs and the scientific concept.

For female reproductive disorders, no validated laboratory *in vitro* tests are available [30]; however, early embryo development in cows resembles human early embryo development more than the commonly used rodent models (reviewed by Santos et al. [28]), suggesting that bovine *in vitro* embryo production might be a useful complement for assessment of reproductive toxicity (with the added advantage of not relying on experimental animals). Further, the technique enables the study of certain windows of exposure including the late stages of oogenesis, a specialized process where many critical events occur [28]. The half-lives of PFAS in human serum takes years [31] compared to rodents that process the compounds faster, resulting in an elimination half-life of PFNA in mice serum of 26-69 days [32]. The pharmacokinetics is less understood in other species such as monkeys and bovines but is slower compared to rodents [33, 34].

The aim of this study was to investigate the effect of PFNA exposure during oocyte maturation on the development of early-stage bovine embryos *in vitro.* Bovine oocytes were exposed during

maturation *in vitro*, and the bovine in vitro produced (IVP) embryos were used as a model for assessing reproductive toxicity.

2. MATERIALS AND METHODS

2.1 Experimental design

Production of bovine embryos through maturation, fertilization, and culture *in vitro* were conducted using cumulus-oocyte-complexes (COCs) from bovine ovaries. Two experiments were run exposing COCs (n=846) to PFNA during maturation *in vitro* in two different concentrations. Following COC selection, experimental and control groups were kept separate during all subsequent *in vitro* procedures. In the first experiment, COCs were exposed to 10 µg mL^{-1} PFNA (PFNA 10, n=200) cultured in parallel with a control with no addition of PFNA (C 10, n=201), and in the second experiment, the COCs were exposed to 0.1 µg mL^{-1} PFNA (PFNA 0.1, n=223) and cultured in parallel with a control (C 0.1, n=222). The experiment was run in n=6 replicates for PFNA 10 and n=8 replicates for PFNA 0.1.

Embryo development was evaluated (see below, section 2.4) after the primary cleavage divisions at 44h post fertilization (pf), and at days 7 and 8 pf. At day 8 pf, embryo morphology and stage of development (grade, stage) were evaluated using a stereomicroscope. For the lower PFNA concentration, day 8 pf blastocysts were fixed and stained for nuclei, neutral lipids, and active mitochondria and further analyzed morphologically using confocal microscopy (Fig. 1).

2.2 Media and reagents

Chemicals and reagents were obtained from Sigma Chemical Company (Sigma Aldrich, Stockholm, Sweden) if not stated otherwise. Media were produced at the IVF laboratory at the Department of Clinical Sciences (Swedish University of Agricultural Sciences, Uppsala, Sweden) as described by

Gordon, 1994 [35]. Completed media were adjusted for pH and osmolality, filtered through a 0.2 µm filter unit, stored at 4°C and used within one week according to protocols. Before use, media were pre-heated or equilibrated in a humidified atmosphere of 5% CO_2 at 38.5°C for at least one hour. Incubations were conducted in 500 µL wells of medium in a humidified atmosphere of 5% CO_2 at 38.5°C if not stated otherwise.

Search-medium for selection of COCs consisted of pre-heated HEPES-buffered tissue culture medium 199 (TCM199, M7528) supplemented with 0.2 % w/v bovine serum albumin (BSA), Fraction V (A3311) and 50 µg mL^{-1} gentamicin sulphate (G1264). For maturation *in vitro*, maturation-medium consisting of bicarbonate-buffered TCM 199 (M2154) supplemented with 0.68 mM L-glutamine (G8540), 50 µg mL^{-1} gentamicin sulphate (G1264), 0.4 % w/v BSA, Fraction V (A3311), 0.1 µg mL^{-1} FSH and 0.03 µg mL^{-1} LH (Stimufol, PARTNAR Animal Health, Stoumont, Belgium) were used. PFNA (perfluorononanoic acid 97%, 394459-5G) was dissolved in TCM 199 without HEPES (TCM199, M2154) and added in the maturation-medium of the treatment groups until final concentrations of 10 µg mL^{-1} and 0.1 µg mL^{-1} respectively were reached.

Maturation of COCs were recorded and matured oocytes were prepared for fertilization in modified HEPES-buffered Tyrode's medium without glucose [35], with addition of 0.3 % w/v BSA, Fraction V (A3311) and 50µg mL^{-1} gentamicin sulphate (G1264) (wash-medium) and sperm prepared in modified Ca^{2+}-free Tyrode's medium [35] with 6.9 mM glucose (G6152), 16 mM Sodium DL-lactate (L7900) and an addition of 50 µg mL^{-1} gentamicin sulphate (G1264) (capacitation medium). Fertilizations were conducted in modified HEPES-buffered glucose-free Tyrode's medium [35] with 16 mM Sodium DL-lactate (L7900) complemented with 50µg mL^{-1} gentamicin sulphate (G1264), 3 µg mL^{-1} heparin (H3184), 3 µg mL^{-1} penicillamine, 3 µg mL^{-1} epinephrine and 1.1 µg mL^{-1} hypotaurine (pencillamine-hypotaurine-epinephrine (PHE)-solution [35]) (fertilization-medium). Final cultures were conducted in modified synthetic oviductal fluid (mSOF [35]) with addition of 0.4 % w/v fatty acid free BSA (A7030), 50 µg mL^{-1} gentamicin sulphate (G1264), 20 µl mL^{-1} BME amino acids solution

(50x) (B6766) and 10 µl mL^{-1} MEM non-essential amino acids solution (100x) (M7145), wells covered in OVOIL™ (Vitrolife, Göteborg, Sweden).

2.3 Oocyte collection and *in vitro* maturation (IVM)

Abattoir-derived ovaries from non-stimulated heifers and cows were collected after the animals had been killed. As the animals were not sacrificed for the sake of these experiments, no ethical permission was needed according to Swedish legislation. Ovaries were transported approximately 3h to the IVF laboratory at the Department of Clinical Sciences (Swedish University of Agricultural Sciences, Uppsala, Sweden) in insulated containers with sterile 0.9% saline-solution, with an arrival temperature of about 30°C (range 28-33°C). COCs were aspirated using a 5 mL syringe and a 1.8-gauge needle from 3-8 mm diameter follicles. In search-medium, the COCs were selected according to Gordon's [29] morphological criteria. COCs with compact multilayer cumulus investment and homogeneous ooplasm were selected for the experiments and divided randomly and evenly into two groups (group size range; 20-40), treated experimentally, and cultured in parallel along with control groups. For *in vitro* maturation, COCs were incubated in maturation-medium for 22h with the addition of PFNA for the treated groups.

2.4 In vitro fertilization and culture

After maturation *in vitro* the COCs were transferred to pre-heated wash-medium. Oocytes were prepared for fertilization through manual removal of excessive cumulus cells until 3-5 layers remained surrounding the oocyte. Semen from a single bull of Swedish red dairy breed with proven field and *in vitro* fertility was used (3-1716 Sörby). Motile sperm were selected after 45 min swim-up in capacitation medium and added to the oocytes at a concentration of 1 x 10^6 spermatozoa mL^{-1}, and sperm and oocytes were incubated in fertilization media for 22h to facilitate fertilization. After fertilization, the remaining cumulus cells and sperm were removed through manual pipetting and

the presumed zygotes were cultured until day 8 pf in mSOF, covered in OVOIL^{TM} to prevent extensive evaporation and oxidation.

Evaluation of embryos

Developing embryos were first evaluated 44h pf using a stereomicroscope (SteREO Discovery.V8, Carl Zeiss Microscopy GmbH, Jena, Germany) for primary cleavage division, and the cleavage level (i.e. the proportion of oocytes cleaved at the 2-cell stage or higher) was determined. At days 7 and 8 pf, the proportions of oocytes that had developed into embryos were recorded, and embryo morphologies and developmental stages were further assessed. Developmental stages were scored according to IETS classification with modification into 3 stages (Fig. 2): early blastocysts or blastocysts (stage 1), expanding or expanded blastocysts where the zona pellucida are intact but thinner due to expansion of the embryo (stage 2), and hatching or hatched embryos (stage 3) [36]. The quality of each embryos (grade 1-4) was assessed according to IETS classification, where grade 1 (excellent/good) refers to a spherical and symmetrical embryo mass consistent with the stage of development with blastomeres uniform in size, shape and color and only minor irregularities; grade 2 (fair) refers to embryos with at least 50% of the embryo showing an intact, viable embryonic mass with moderate irregularities in shape, mass or size; grade 3 (poor) refers to embryos with at least 25% of the embryo showing an intact, viable embryonic mass but with severe irregularities in shape, mass or size of the embryo; and grade 4 (dead or degenerating) in the classification for non-viable embryos or oocytes [36, 37].

Staining and image analysis

Staining and confocal microscopy for further analysis of the embryos were applied to pf day 8 blastocysts after treatment with 0.1 µg mL^{-1} PFNA during maturation *in vitro,* a method previously used to detect deviations during early embryo development [38, 39]. Fluorescent labeling for visualization of active mitochondria was conducted through incubation with 200nM MTO (Mitotracker® Orange CMTMRos, ThermoFisher Scientific, Waltham, USA). Fixation followed

incubating the embryos in 2% paraformaldehyde (PFA) in phosphate-buffered saline with 0.1% polyvinyl alcohol (PBS-PVA) overnight at 4°C or at room temperature (RT) for one hour, followed by rinsing in PBS-PVA. For visualization of nuclei, embryos were incubated with 5 µM DRAQ5® (Deep red Anthraquinone 5, 4084S, BioNordika, Stockholm, Sweden) for 20 minutes at RT. After rinsing in PBS-PVA, embryos were stained for neutral lipids using LipidTOX™ (HCS LipidTOX™ Green Neutral Lipid Stain H34475, ThermoFisher Scientific, Waltham, USA) for 30 min at RT according to the manufacturer's instructions.

Stained blastocysts were mounted in approximately 2µl of fluid on a well of a microscope slide (ER-201B-CE24, Thermo Fisher Scientific, Portsmouth, NH) in Vectashield (Vector Laboratories, Burlingame, CA, USA). Images were captured using an LSM 510 laser scanning microscope (Zeiss LSM 510, Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with He/Ne 543, He/Ne 633 and Ar 450-530 nm lasers. Each embryo was scanned using standard magnification (20x) in single scans and seven levels of sectioned scans (z-stack).

Images taken as single channel images were converted to overlay images and saved in TIFF format. Only z-stack levels 2, 4 and 6 were used for image analysis to prevent nuclei and lipid droplets being counted twice. Image analysis was performed using CellProfiler (Cell Profiler 2.2.0 (rev ac0529e), http://cellprofiler.org/, accessed at 2017-02-06, [40]) and manually.

The blastocysts were identified by segmentation using a pixelwise classifier trained in Ilastik [41] and confirmed manually, and non-blastocysts were excluded. Nuclei and lipid droplets were identified using the IdentifyPrimaryObjects module in CellProfiler where an initial Maximum Correlation Threshold [42] was applied followed by Watershed separation [43]. Cell outlines were estimated by expanding the nuclei by a maximum of 50 pixels. For image analysis, 88 embryos were included in 264 images. Embryos for which one or more image showing poor quality regarding fluorescent labeling for nuclei (n=31) and lipids (n=8) were excluded from the analysis. Lipid droplet (n=32975) sizes were measured at maximum diameter (Fig. 3). Two researchers independently assessed

mitochondria distribution based on 3D images of the z-stack by using scores of 1 through 3 (1: even distribution without distinct aggregations of mitochondria, 2: mild asymmetry in distribution with some aggregations, 3: uneven distribution with pronounced aggregations, see [38]) (Fig. 4). Blastocysts were excluded from the analysis (n=9) if the assessments deviated or if image quality was inadequate for making a determination.

Statistics

Mixed-effect logistic regression was performed to calculate the odds ratio (OR) for the effect of treatment on developmental competence at three time points (cleaved, cleaved higher than the 2-cell stage level 44h pf, and pf day 8 blastocysts). OR results are presented in terms of the treatment effect compared to the relevant control, where OR < 1 indicates a negative effect of treatment on developmental competence. Replicates were added as a random factor and weighted depending on group-size. Data on embryo development on blastocysts day 7 and 8 pf were treated as repeated measurements (glmer model of the lme4 package, R i386, 3.3.1, http://www.r-project.org). Categorical variables (stage, grade, and mitochondrial distribution) were analyzed using cumulative link mixed-effect models with multinomial distribution (clmm model of the ordinal package, R i386, 3.3.1) with replicate as a random effect. Dose group (PFNA 0.1 or 10) was initially added as a random effect to the models due to significant differences in developmental parameters between the two experiments (lm model, R i386), but was removed based on AIC and likelihood-ratio tests between models ($p>0.05$) indicating that the variation was accounted for through the variable replicates and individual controls. No differences were found regarding parameters associated with laboratory work between the different concentrations (time for aspiration, maturation and amount of ovaries, $p>0.05$).

Linear mixed effect models was performed to calculate the effect of PFNA 0.1 on nuclei and lipid droplet size (lmer model of the lme4 package, R i386, 3.3.1) with replicate as a random factor. For lipid droplet size, log-transformed values were used to assume normal distribution of the

response variable. The difference in lipid droplet size between groups was significant but did not show a monotone distribution (Fig. 5). Thus, assuming the normal distribution was insufficient to explain changes between the treatments, bin values were added and mixed effect logistic regression with lipid droplet size as a response variable was used according to cut-off value (<3 µm, 3-6 µm, 6-9 µm, 9-12 µm >12 µm in diameter).

P-values <0.05 were considered to be significant. Data are presented as mean ± standard deviation (SD) if not otherwise stated.

3. Results

3.1 Effect of PFNA on developmental competence of immature oocytes *in vitro*

Table 1. Result of PFNA exposure during maturation *in vitro* on developmental competence of the immature oocytes.

	PFNA 0.1^ *n=223*	C 0.1^ *n=217*	PFNA 10^ *n=199*	C 10^ *n=200*
Cleaved[a]	0.81 ± 0.09	0.80 ± 0.07	0.75 ± 0.10*	0.85 ± 0.03
Cleaved above 2[a]	0.59 ± 0.10	0.60 ± 0.05	0.60 ± 0.14	0.71 ± 0.07
Day 7 blastocysts[b]	0.12 ± 0.10	0.11 ± 0.05	0.05 ± 0.03*	0.15 ± 0.06
Day 8 blastocysts[b]	0.20 ± 0.09	0.19 ± 0.05	0.08 ± 0.05*	0.26 ± 0.08

*^Results presented as mean of batch results ± SD after exposure with 0.1 µg mL-1 in PFNA 0.1 and 10 µg mL-1 in PFNA 10 and relevant control groups (C0.1 and C10), [a]Cleaved oocytes and cleaved above the 2-cell stage of cultured immature oocytes 44h post fertilization (pf), [b]proportion of blastocysts days 7 and 8 pf, *Indicates a significant difference between treatment and control.*

Treatment with 10 µg mL^{-1} PFNA (PFNA10) during oocyte maturation had a negative effect on developmental competence. The effect could be seen after maturation *in vitro* where the cumulus cloud of the treated group did not expand as it did in the control (Fig. 6). The risk of impaired development was increased with PFNA exposure, both regarding the proportion of cleaved oocytes 44h pf (OR 0.59, p=0.01) and the proportion of blastocysts developed from cultured immature oocytes (OR 0.27, p<0.001). No significant effect was seen on cleaved oocytes more advanced than the 2-cell stage at 44h pf (p=0.09) (Table 1). This developmental toxicity at 10 µg mL^{-1} PFNA was not observed at 0.1 µg mL^{-1} PFNA exposure during oocyte maturation *in vitro*. Results of logistic regression analyses are presented in Table 2 and developmental parameters are presented in Table 1.

Table 2. Logistic regression of the effect of PFNA treatment during maturation *in vitro* on developmental parameters presented as odds ratio (confidence intervals) compared to controls.

	PFNA 0.1		PFNA 10	
Variable	Odds ratio (CI)	P-value	Odds Ratio (CI)	P-value
Cleaved 44h pf[a]	0.87 (0.57-1.37)	0.54	0.59 (0.39-0.90)	0.01
Cleaved above 2 cells 44h pf[a]	0.73 (0.51-1.05)	0.09	0.72 (0.49-1.04)	0.09
Blastocyst rate[b]	0.85 (0.53-1.37)	0.51	0.27 (0.14-0.53)	0.0009

[a] Cleaved embryos and Cleaved above 2 cell stage of cultured immature oocytes 44h pf, [b] blastocyst rate after 8 days pf.

3.2 Effect of PFNA exposure during maturation *in vitro* on blastocyst morphology day 8 pf

Developed blastocysts after treatment with PFNA during *in vitro* maturation in the 0.1 µg mL^{-1} group (PFNA 0.1: n=35, C 0.1: n=34) and 10 µg mL^{-1} (PFNA 10: n=16, C 10: n=50) were morphologically assessed for stage score (1-3) and grade classification (1-4). In these experiments, no risks of changes in stage score in developed blastocysts 8 days pf were observed after PFNA exposure during oocyte

maturation *in vitro* either at 0.1 µg mL^{-1} PFNA (1.26 (-0.19-2.71), p=0.54) or 10 µg mL^{-1} PFNA (1.43 (-0.28-3.14), p=0.51). No changes in grade classifications of formed blastocysts 8 days pf were observed (PFNA 0.1: 2.04 (0.47-3.61), p=0.11, PFNA 10: 2.19 (0.44-3.94), p=0.16).

3.3 Effect of 0.1 µg mL^{-1} PFNA exposure on nuclei count, mitochondria, and lipid distribution

There was no observed difference in number of nuclei in formed blastocysts 8 days pf in PFNA0.1 during maturation *in vitro* compared to the control group (PFNA 0.1: 91.7±33, C 0.1: 99.2±40, p=0.40). In addition, PFNA 0.1 did not affect the distribution of mitochondria within the blastocysts (OR (CI): 1.15 (0.52-2.59), p=0.73, median grade (range): PFNA 0.1: 2 (1-3), control: 2 (1-3)).

However, there was a significant difference in lipid droplet distribution in PFNA 0.1 during oocyte maturation, with a higher proportion of larger lipid droplets in this treatment compared to the control (Fig. 5, p=0.04). Blastocysts in this group also had a higher proportion of very large lipid droplets (diameter >12 µm, p<0.0001) but a lower proportion of small lipid droplets (diameter ≤9 µm) compared to control (Table 3). In contrast, no statistical difference was found in lipid droplets with a diameter of 9-12 µm (p=0.09, Table 3).

Table 3. Logistic regression of the effect of exposure to 0.1 µg mL-1 PFNA (PFNA0.1) during maturation in vitro on the distribution of lipid droplet size in day eight blastocysts after fertilization

Lipid droplet size[c]	C 0.1	PFNA 0.1	Odds ratio (CI)	p-value
Sum[k]	395.51 (154.23)	431,57 (223.82)	1.13 (1.00-1.27)	0.04
<3 µm[d]	83.93 (44.46)	90.16 (88.24)	0.95 (0.89-1.00)	0.04
3-6 µm[d]	95.56 (43.78)	89.43 (58.67)	0.82 (0.78-0.87)	2.28e-12
6-9 µm[d]	68.09 (32.07)	64.62 (26.92)	0.87 (0.82-0.93)	9.55 e -06
9-12 µm[d]	51.86 (25.28)	58.19 (23.23)	0.94 (0.99-1.13)	0.09
>12 µm[d]	96.07 (85.19)	129.16 (80.19)	1.32 (1.25-1.39)	<2.0e-16

[c]Lipid droplet (n=32975) size measured as maximum diameter in day eight embryos (n=88) in PFNA exposed group (PFNA0.1) and control-group (C0.1), [k]Sum (SD) and overall effect of PFNA0.1 on distribution of lipid droplet size, [d]Effect of PFNA0.1 on the proportion of lipid-droplets of size <3, 3-6, 6-9, 9-12 and >12 µm in maximum diameter

4. DISCUSSION

4.1 Effect of PFNA on developmental competence of maturing bovine oocytes *in vitro*

Previous studies have investigated the developmental toxicity of PFNA using laboratory animals like rodents and zebrafish [16-18, 44-47]. In this study, we have examined endpoints of developmental toxicity using the bovine IVP as a model, exposing oocytes during 22h maturation *in vitro* to either 10 µg mL^{-1} PFNA (PFNA 10) or 0.1 µg mL^{-1} PFNA (PFNA 0.1).

PFNA 10 exposure was based on previous *in vitro* studies on zebrafish [16, 18, 47] and levels measured in mouse serum after oral exposure while PFNA 0.1 was based on levels measured in human follicular fluid [6] multiplied 50-500x to compensate for the short exposure time. No comparable studies using the IVP model are available. Exposure in PFNA 0.1 is consistent with low-dose *in vitro* exposure [17] and significantly lower than the dose measured in mouse serum after oral administration [44, 46, 48].

Signs of severe developmental toxicity, including a decreased proportion of cleaved embryos at 44h pf and impaired blastocyst development at 7 and 8 days pf, could be seen after exposure to 10 µg mL^{-1} PFNA during *in vitro* maturation, exceeding the effects observed in zebrafish [16, 18, 47]. In mice, similar maternal serum concentrations did not affect litter size, pup weight or live pups/litter [46, 48]. This toxicity difference could be due to different species responses to PFAAs [15] (specifically, the lower chemo-permeability of the zebrafish chorion might have reduced uptake [49] and different metabolism of PFAAs in mice [32]). A similar level of toxicity was not observed in embryos exposed to 0.1 µg mL^{-1} PFNA where no difference could be seen in the proportion of embryos cleaved or developed into day 7 or 8 pf blastocysts.

Embryos were assessed morphologically after 7 and 8 days pf by evaluating endpoints of developmental toxicity, such as impaired development, malformations, or degradation of the early embryo (stage, grade). No differences were observed between controls and PFNA exposed groups.

However, the extensive toxic effects of PFNA 10 resulted in a very small number of embryos available for evaluation (day 8 pf blastocysts, n=16).

4.2 Effects of 0.1 µg mL^{-1} PFNA on nuclei count, mitochondria, and lipid distribution

Bovine oocytes exposed to PFNA 0.1 µg mL^{-1} (PFNA 0.1) did not show any signs of developmental toxicity when assessed using non-invasive morphological criteria (stage, grade). These findings were confirmed through staining procedures for visualizing nuclei and mitochondria, which is not surprising because nuclei count is a measure of the number of cell-divisions, and changes in mitochondria distribution can be a sign of embryo degradation or cell death [50, 51].

Lipid accumulation has previously been evaluated in bovine embryos produced by IVP and is known to affect the viability or the susceptibility of cryopreservation in bovines [52, 53]. Different lipid storage in the early embryo is associated with changes in lipid metabolism in bovines, seen as differences in lipid content between breeds [54]. In this study, lipid droplet sizes were studied as an endpoint for lipid metabolism [54-56]. A change in the size distribution of lipid droplets in PFNA 0.1 was observed, suggesting a disturbance of lipid metabolism during the early embryo development. This result is supported by the mode of action of PFNA, which disrupts endocrine activation of a peroxisome proliferator-activated receptor (PPAR). PPARs are nuclear ligand-activated transcription-factors associated with lipid metabolism and involved in catabolism and oxidation of fatty acids [57, 58]. Specifically, PFNA has been shown to disrupt activation of PPAR-alpha (PPARα), demonstrated by both by direct effects on the receptor [46] and the lack of effect in knock-out mice [48]; other PFAAs have shown similar activation of PPARα [59]. Lipid metabolization through fatty acid oxidation is important for the oocyte meiotic maturation and early embryonic development [57, 60], and changes in lipid metabolism during this period might be especially problematic for a developing blastocyst [60].

Lipid metabolism has also been investigated in human cohorts, and epidemiological studies suggest that exposure to PFAAs during embryo development may cause adverse outcomes later in life [61, 62]. However, the results are contradictory. An increase in BMI of female offspring has been associated with *in utero* PFNA exposure [61], and *in utero* exposure to PFOS, PFOA, and PFNA has been positively associated with higher levels of total and non-high density cholesterol [62]. Other results associate obesity in children with PFNA exposure [63]. Finding possible negative effects of PFAAs on human health is difficult, because the effects might be present only in the context of certain metabolic conditions [64].

4.3 The usefulness of bovine IVP as a model for developmental toxicity

The IVP setup enables developmental-specific windows of exposures including the final stages of the oocyte maturation. The oogenesis is an especially sensitive period where many critical events take place and mimicking this period with exposure during the *in vitro* maturation might result in changes visible first later during the embryo development [28] without confounders of exposure of the male gamete. Further, the system might be a useful tool investigating concerns raised from results connecting exposure of the female and impaired results after IVF-treatment [65], especially within the group of couples with unknown infertility reasons.

Species differences in toxicological sensitivity suggest that models used for risk-assessment of developmental toxicity should resemble the target species as closely as possible. Bovine early embryo development is more similar to human embryo development than are small rodents or zebrafish, so mechanistic studies of developmental toxicity using bovine IVP may provide valuable information for human risk assessment [28]. Without resorting to the use of experimental animals, we have demonstrated developmental toxicity of PFNA to bovine embryos, and also demonstrated differences in lipid metabolism that appear to have been caused by disturbances to the same endocrine pathway demonstrated in other studies [46, 48].

Although it is unlikely that *in vitro* tests will soon be able to completely replace *in vivo* models, clearly further *in vitro* models are needed to assess potential adverse effects to human fertility [30]. The bovine IVP system seems to be a potentially very useful *in vitro* model for studying the adverse effects of PFNA and possibly other per- and polyfluoroalkyl substances as well. Although, consideration is needed regarding the period of exposure during oocyte maturation *in vitro* as the first meiotic divisions of the gametes *in vivo* are conducted during fetal development and thus this *in vitro* method cannot handle effects of life-long exposure.

4.4 Conclusion

In this study, we investigated the effect of PFNA during oocyte maturation *in vitro* using bovine IVP as a model for reproductive toxicity. We observed acute toxicity after exposure to 10 µg mL^{-1} PFNA as well as changes in lipid droplet distribution in embryos exposed to 0.1 µg mL^{-1} PFNA during maturation *in vitro*. More studies exploring long-term effects and lower-dose exposures are needed for further health risk assessment.

Conflict of interest

The authors declare no conflict of interest.

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FIGURES

Figure 1:

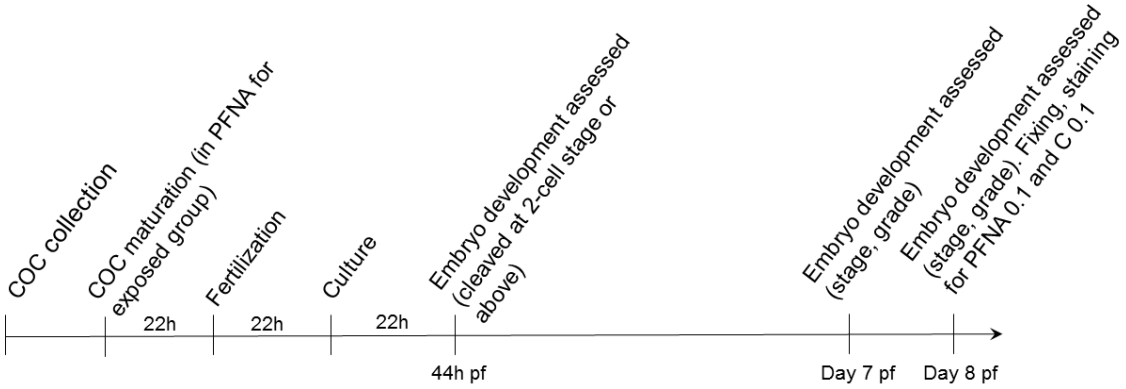

Figure 1. Experimental design.

Bovine cumulus oocyte complexes (COCs) were selected for the experiment and exposed to PFNA in two concentrations during maturation *in vitro.* Embryo development was evaluated after the primary cleavage divisions at 44h pf and at days 7 and 8 pf. For the lower concentration (PFNA 0.1), day 8 blastocysts were stained for nuclei, neutral lipids and active mitochondria.

Figure 2:

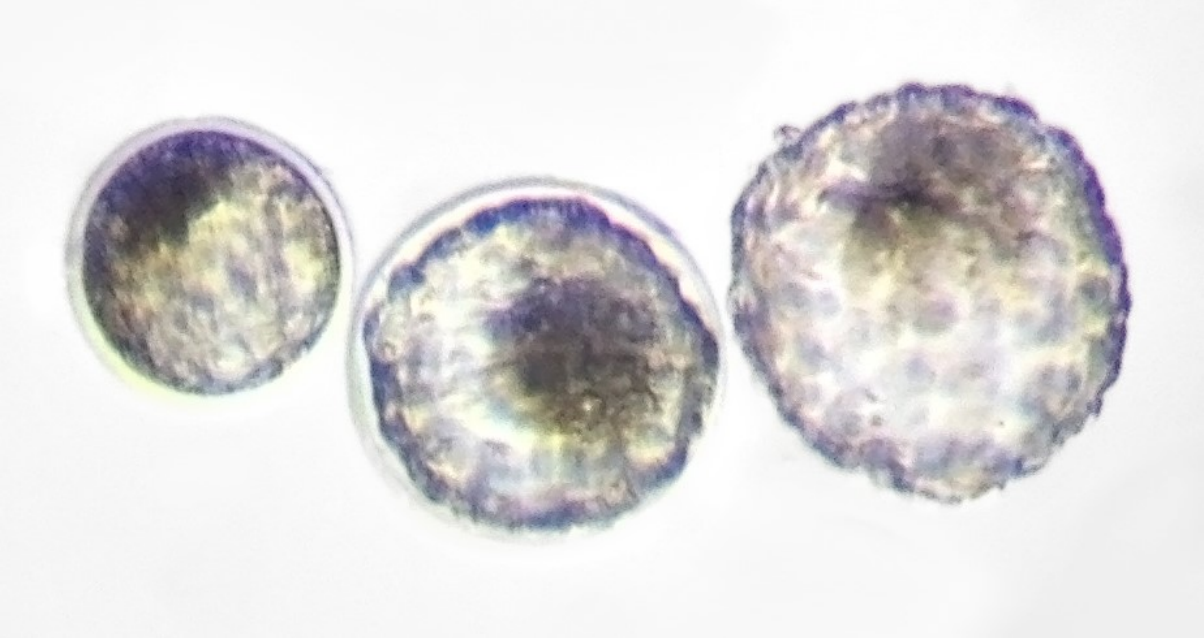

Figure 2. Classification of blastocysts 8 days pf for stage and grade.

Left: blastocyst of excellent/good quality (stage 1, grade 1); Middle: expanding blastocyst with intact but thinner zona pellucida than stage 1, excellent/good quality (stage 2, grade 1); Right: hatched blastocyst of excellent/good quality (stage 3, grade 1).

Figure 3:

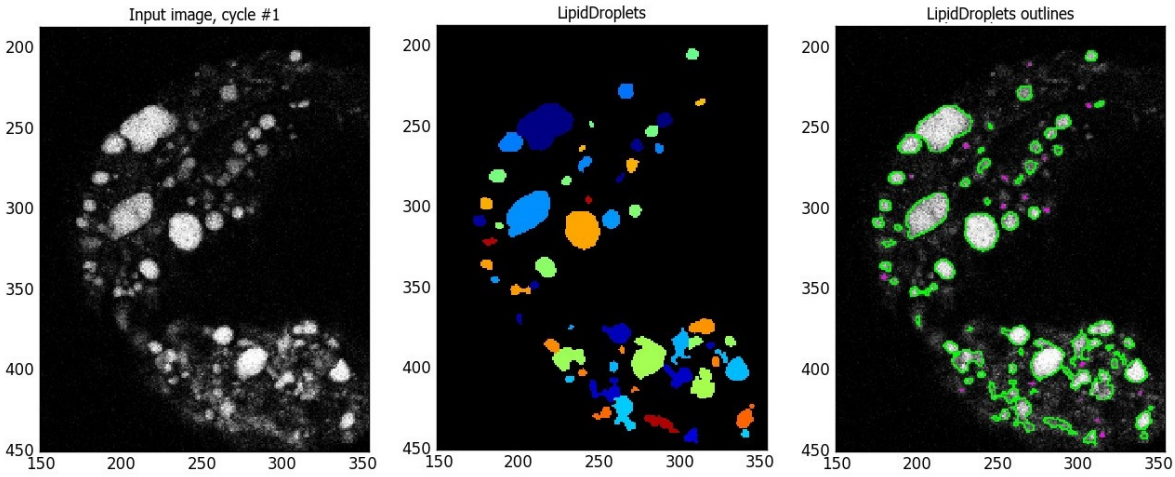

Figure 3: Lipid droplet identification in confocal images of day 8 pf blastocysts.

Fluorescent labeling for visualization of neutral lipids were applied and images captured in sectioned scans in seven levels (z-stack) using a confocal microscopy. Images taken as single channel-images (left) from z-stack levels 2, 4 and 6 were used for lipid-identification. Lipid droplets were identified using CellProfiler using Maximum Correlation Threshold followed by Watershed separation (middle, right). Lipid droplet (n=32975) sizes were measured at maximum diameter.

Figure 4.

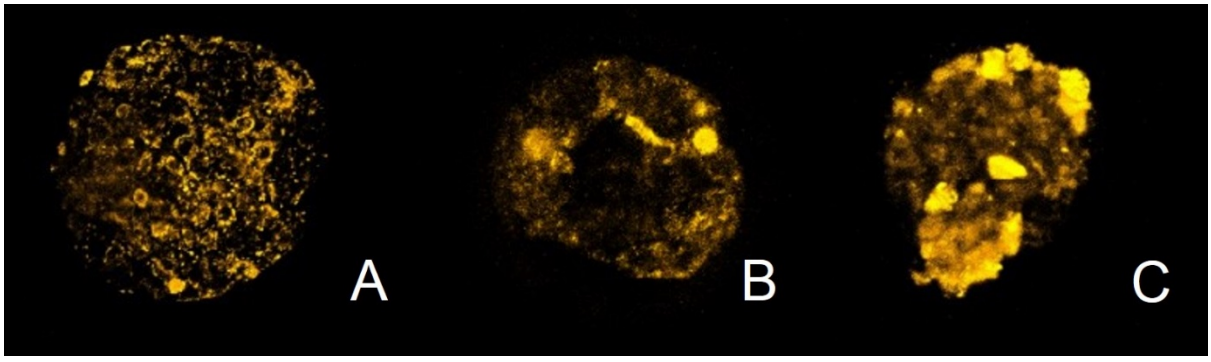

Figure 4. Grading of distribution of active mitochondria.

A: Grade 1, even distribution without distinct aggregations of mitochondria; B: grade 2, mild asymmetry in mitochondria distribution with some aggregations; C: grade 3, uneven mitochondria distribution with pronounced aggregations.

Figure 5.

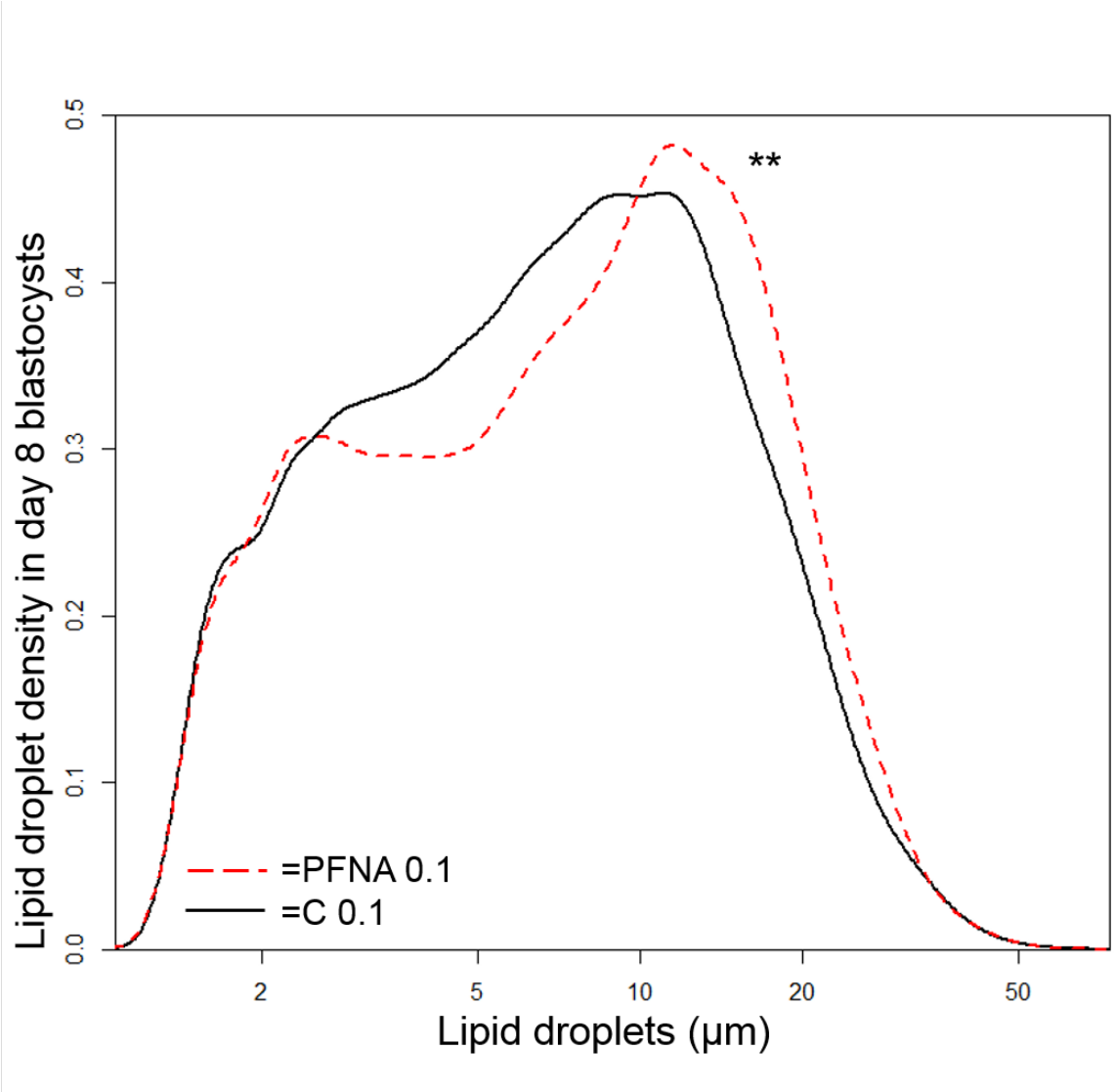

Figure 5. Densities of lipid droplets in day 8 pf blastocysts.

Lipid droplets of different size (diameter) in treatment (PFNA0.1) and control (C 0.1) showing a non-monotonic distribution. ** indicates a significant difference (p=0.04).

Figure 6.

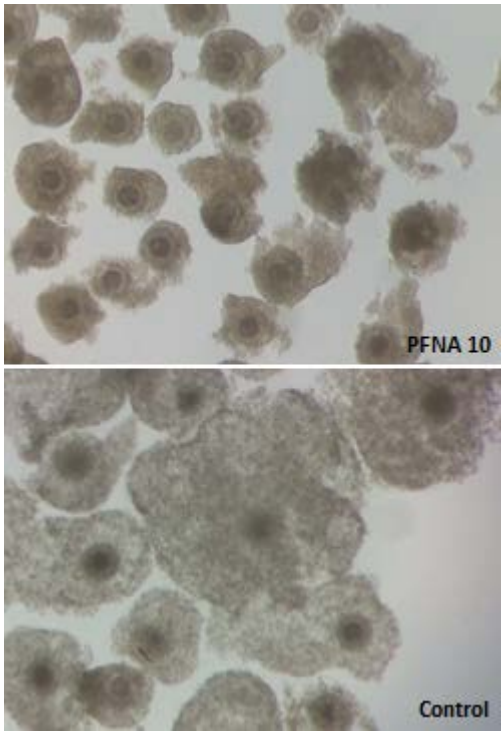

Figure 6. Cumulus oocyte-complexes after maturation *in vitro* treated with 10 µg/mL PFNA and Control. Note the change in expansion of the cumulus cloud between treated group (PFNA 10) and control.