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Bovine oocyte exposure to perfluorohexane sulfonate (PFHxS) induces phenotypic, transcriptomic, and DNA methylation changes in resulting embryos in vitro

Ida Hallberg [a,*,1], Sara Persson [a,b,2], Matts Olovsson [c,3], Mikaela Moberg [a], Petter Ranefall [d,4], Denise Laskowski [a,5], Pauliina Damdimopoulou [e,6], Marc-Andre Sirard [f,7], Joëlle Rüegg [g,8], Ylva C.B. Sjunnesson [a,9]

[a] Department of Clinical Sciences, Division of Reproduction, The Centre for Reproductive Biology in Uppsala, Swedish University of Agricultural Sciences, SE-750 07 Uppsala, Sweden
[b] Environmental research and monitoring, Swedish Museum of Natural History, P.O Box 50007, SE-104 05 Stockholm, Sweden
[c] Department of Women's and Children's Health, Uppsala University, 751SE-751 85 Uppsala, Sweden and The Centre for Reproductive Biology in Uppsala, SE-752 37 Uppsala, Sweden
[d] Department of Information Technology, Division of Visual Information and Interaction, and SciLifeLab BioImage Informatics Facility, Uppsala University, Uppsala, Sweden
[e] Division of Obstetrics and Gynecology, Department of Clinical Science, Intervention and Technology, Karolinska Institutet and Karolinska University Hospital, SE-141 86 Stockholm, Sweden
[f] Department of Animal Sciences, Laval University, Qubec G1V 0A6, QC, Canada
[g] Department of Organismal Biology, Environmental Toxicology, The Centre for Reproductive Biology in Uppsala, SE-752 36 Uppsala, Sweden

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ABSTRACT

Knowledge on the effects of perfluorohexane sulfonate (PFHxS) on ovarian function is limited. In the current study, we investigated the sensitivity of oocytes to PFHxS during in vitro maturation (IVM), including consequences on embryo development at the morphological, transcriptomic, and epigenomic levels. Bovine cumulus-oocyte complexes (COCs) were exposed to PFHxS during 22 h IVM. Following fertilisation, developmental competence was recorded until day 8 of culture. Two experiments were conducted: 1) exposure of COCs to 0.01 μg mL^{-1} – 100 μg mL^{-1} PFHxS followed by confocal imaging to detect neutral lipids and nuclei, and 2) exposure of COCs to 0.1 μg mL^{-1} PFHxS followed by analysis of transcriptomic and DNA methylation changes in blastocysts.

Abbreviations: aRNA, antisense RNA; ATM, serine/theonine kinase; BSA, bovine serum albumin; CI, confidence interval; COC, cumulus-oocyte-complex; DEG, differently expressed gene; DMR, differently methylated region; dpf, days post fertilisation; ER, oestrogen receptor; FDR, false discovery rate; gDNA, genomic DNA; hpf, hours post fertilisation; IPA, ingenuity pathway analysis; IQR, inter-quartile range; IVF, in vitro fertilisation; IVM, in vitro maturation; NFKB1, nuclear factor kabba B subunit 1; NS, not significant; OR, odds ratio; PBS, phosphate buffered saline; PFAS, Per- and polyfluoroalkyl substances; PFHxS, Perfluorohexane sulfonate; PFNA, perfluorononanoic acid; PFOS, perfluorooctane sulfonate; PPAR, peroxisome proliferator activated receptor; PVA, polyvinyl alcohol; ROS, reactive oxygen species; TGF, transforming growth factor; TP53, tumour protein 53.

* Correspondence to: Division of Reproduction, Department of Clinical Sciences, Swedish University of Agricultural Sciences, The Centre for Reproductive Biology in Uppsala, SE-750 07 Uppsala, Sweden.
E-mail addresses: ida.hallberg@slu.se (I. Hallberg), sara.persson@slu.se (S. Persson), matts.olovsson@kbh.uu.se (M. Olovsson), mikan.moberg@hotmail.com (M. Moberg), petter.ranefall@it.uu.se (P. Ranefall), denise.laskowski@slu.se (D. Laskowski), pauliina.damdimopoulou@ki.se (P. Damdimopoulou), marc-andre.sirard@fsaa.ulaval.ca (M.-A. Sirard), joelle.ruegg@ebc.uu.se (J. Rüegg), ylva.sjunnesson@slu.se (Y.C.B. Sjunnesson).
[1] ORCID: 0000-0001-5395-050X
[2] ORCID: 0000-0003-4700-0553
[3] ORCID: 0000-0001-5497-9013
[4] ORCID: 0000-0002-6699-4015
[5] ORCID: 0000-0001-6775-3080
[6] ORCID: 0000-0001-8458-0855
[7] ORCID: 0000-0001-8667-6682
[8] ORCID: 0000-0002-6580-9201
[9] ORCID: 0000-0003-4500-5123

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Decreased oocyte developmental competence was observed upon exposure to ≥ 40 μg mL^{-1} PFHxS and altered lipid distribution was observed in the blastocysts upon exposure to 1–10 μg mL^{-1} PFHxS (not observed at lower or higher concentrations). Transcriptomic data showed that genes affected by 0.1 μg mL^{-1} PFHxS were enriched for pathways related to increased synthesis and production of reactive oxygen species. Enrichment for peroxisome proliferator-activated receptor-γ and oestrogen pathways was also observed. Genes linked to DNA methylation changes were enriched for similar pathways. In conclusion, exposure of the bovine oocyte to PFHxS during the narrow window of IVM affected subsequent embryonic development, as reflected by morphological and molecular changes. This suggests that PFHxS interferes with the final nuclear and cytoplasmic maturation of the oocyte leading to decreased developmental competence to blastocyst stage.

1. Introduction

Birth rates in humans are declining, and while this may be mainly attributed to increased female education and the use of contraceptives [1], infertility in humans is common and estimated to affect up to one in six couples globally [2–5]. In females, fertility is dependent on a mature oocyte capable of driving early embryo development. Oocyte developmental competence is acquired during oocyte maturation in the ovarian follicle. During this time, the oocyte is surrounded by follicular fluid. The follicular fluid has been found to contain exogenous compounds such as industrial chemicals, plasticisers, pesticides, and flame-retardants [6–10], which could influence the process of oocyte maturation and consequently the subsequent embryo and placental development.

Per- and polyfluoroalkyl substances (PFASs) are a large group of synthetic chemicals widely used because of their water- stain and oil-repellent properties in a range of consumer products such as non-stick surfaces, protective clothing, and in fire-fighting foam [11]. Because of their extreme stability, they are resistant to degradation and persist in the environment, bioaccumulating in wildlife and in humans [12,13]. PFASs are found to pass the blood-follicle barrier, exposing the maturing oocyte [10] and the developing foetus in utero [14]. Perfluorohexane sulfonate (PFHxS) was increasingly used after the phase-out of perfluorooctane sulfonate (PFOS), but since 2017 has been included on the REACH legislation's Candidate List of Substances of Very High Concern, and marked for progressive replacement [15]. This has led to indications of decreased serum concentrations in recent years within the EU [16,17] and in the US [18]. However, due to its resistance to degradation, environmental and human exposure will continue for decades to come.

Information on effects of PFHxS on ovarian function is scarce and there are ambiguous results regarding associations between exposure and fertility or reproductive outcomes [19]. PFHxS has been negatively associated with outcomes related to ovarian functions in humans, such as ovarian aging [20], alterations in the menstrual cycle [21] and inversely correlated to baseline follicle count [22]. On the other hand, there are studies suggesting no association between exposure and sex hormone levels [23,24]. Recently, PFHxS exposure was shown to cause cytotoxicity and inhibit maturation in porcine oocytes in vitro [25]. Mechanisms underlying PFHxS' proposed effects on ovarian function have not been identified. However, more generally, activation of peroxisome proliferator-activated receptors (PPARs) is implicated in PFHxS toxicity [26,27]. This is the proposed mechanistic explanation for, e.g., lipid accumulation observed in zebrafish livers upon PFAS exposure [28].

Developmental chemical exposure has previously been linked to changes in the epigenetic landscape [29,30]. Epigenetic changes are defined as alterations that alter gene functions without changing the DNA sequence. PFAS exposure has been associated with one type of epigenetic modification (altered microRNA) in women [31], but the potential of PFHxS to disrupt the epigenetic landscape in embryos upon oocyte exposure is not known. The epigenome is extensively re-programmed in the germ line during early development, both during oocyte final maturation inside the ovaries and during primordial germ cell development in early embryos. Disruption of this reprogramming might permanently affect development and health later in life [32].

There are currently no studies on PFHxS effects on bovine oocytes, their subsequent developmental competence, or the potential of PFHxS to alter the epigenome and transcriptome in embryos upon oocyte exposure. We have previously shown that perfluorononanoic acid (PFNA) and PFOS alters lipid distribution in blastocysts when exposure takes place during final oocyte maturation in the bovine model [33,34]. In the current study, we investigated oocyte sensitivity to PFHxS during in vitro maturation (IVM) and the effects on subsequent embryo survival and development. Bovine cumulus oocyte complexes (COCs) were exposed to PFHxS during IVM and after fertilisation and culture, embryo development, morphology, lipid profile, gene expression, and DNA methylation were investigated.

2. Materials and methods

2.1. Experimental design

Bovine COCs were exposed to PFHxS during IVM (Fig. 1). After IVM, oocytes were fertilised and cultured until 8 days post fertilisation (dpf) under serum-free conditions. Developmental competence was recorded as proportion of embryos cleaved, embryos cleaved beyond two cell stage, blastocysts rate at 7 and 8 dpf, as well as morphology of developed embryos (stage, grade). Initially, COCs (n = 386) in groups of ten were exposed to PFHxS in concentrations of 0.01, 0.1, 1.0, 10, 20, 40 and 100 μg mL^{-1} in six replicates and developmental competence followed until 8 dpf. At 8 dpf, blastomere counts in the blastocysts (n = 39) were recorded and lipid distribution studied (total volume lipid, average lipid droplet volume and average lipid/cell) using confocal microscopy. Based on the results, another set of COCs (n = 1316) was cultured with exposure to a concentration where no embryo lethality was observed. COCs were exposed to 0.1 μg mL^{-1} PFHxS (n = 674) or to vehicle only (controls, n = 642) to generate blastocysts for microarray studies. Developmental competence was recorded until 8 dpf and embryos were individually snap frozen (n = 80) for RNA/DNA extraction of groups of ten embryos of equivalent grade and stage. Gene expression profile and methylation pattern in the blastocysts were analysed and compared between control and treated groups.

2.2. Media and reagents

Media were prepared at the in vitro fertilisation (IVF) facilities of the Department of Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden. All chemicals were purchased from Sigma Aldrich (Sigma Aldrich, Stockholm, Sweden) if not stated otherwise. Media were prepared according to previous set protocols [35] with slight modifications as described in previous related projects from our facilities [36–38]. Details on media and regents can be found in Table S1 of the Supporting Information (SI).

2.3. Exposure solutions and validation of concentrations

For the experiment, perfluorohexane sulfonate (PFHxS, Cas no. 108427–53–8, 50 929, potassium salt, ≥98.0%) was dissolved in

molecular grade water and diluted in stock solutions so that the same volume could be added to the maturation media for all exposure groups. Stock solutions were stored at 4 °C protected from light. Due to persistence of the compound, we did not expect significant degradation during the course of the experiments. For the control, the same volume of vehicle (molecular grade water) was added to the maturation media.

Three of the stock solutions containing PFHxS were sent for quantification to SGS Analytics, Linköping, Sweden. Samples were diluted in molecular grade water. Quantification was performed by a liquid chromatography system (1290 Infinity II, Agilent, CA, US) coupled to a triple quadrupole mass spectrometry (LC-MS/MS, 6495 A, Agilent, CA, US) according to standard protocols (DIN 38407–42 mod.). Results were adjusted to blank signal and internal standard recoveries used to compensate for potential loss during processing. The results from the quantification were considered as validated exposure, whereas the exposure concentrations were calculated based on the confirmed results for the remaining exposure groups (Table 1).

Table 1
Concentrations of PFHxS exposure during in vitro maturation (IVM).

Nominal concentration, μg mL^{-1} PFHxS	Validated concentration, μg mL^{-1}	Calculated exposure, μg mL^{-1}[a]	Calculated exposure, μM
.01 μg mL^{-1}	0.008	–	0.02
0.10 μg mL^{-1}	–	0.08	0.19
1 μg mL^{-1}	–	0.76	1.89
10 μg mL^{-1}	7.96	–	20.0
20 μg mL^{-1}	–	15.93	39.8
40 μg mL^{-1}	29.23	–	73.1
100 μg mL^{-1}	–	73.01	182.7

[a] all stock solutions were acquired by diluting the original stock solution. Three of the seven stock-solutions were sent for validation of exposure concentration. For the remaining four stock-solutions, the exposure concentration was calculated based on the results of the analysed stocks

2.4. In vitro maturation and exposure of COCs

Bovine ovaries from non-stimulated heifers and cows were collected from the slaughterhouse after the animals had been killed. The animals were not killed for the sake of this experiment, and hence no ethical permission was needed according to Swedish legislation. The ovaries were transported to the IVF facilities directly after collection in a pre-heated container filled with saline solution. The temperature upon arrival at the laboratory was on average 29 °C (range 27–31). COCs with compact multilayer cumulus cloud and homogenous ooplasm (acceptable COCs according to criteria described by Gordon [39]) were selected for the experiment. In the first experiment, COCs were randomly and equally divided into groups of 10 (range 9–10) between control and treated groups. In the second experiment, on average 38 (range 31–46) COCs per group were used. In both experiments, COCs were subjected to IVM in bicarbonate buffered tissue culture medium 199 (TCM199) with the addition of 0.4% w/v bovine serum albumin (BSA) Fraction V, 0.68 mM L-Glutamine, 0.5 μg mL^{-1} FSH, 0.1 μg mL^{-1} LH (Stimufol, PARTNAR Animal Health, Stoumont, Belgium) and 50 μg mL^{-1} gentamicin sulphate for 22 h. PFHxS dissolved in molecular grade water was added to the maturation media in a volume of 25 μL mL^{-1} to reach exposure concentrations of 0.01, 0.1, 1.0, 10, 20, 40 and 100 μL mL^{-1} respectively. In all replicates a control was present and the treatments randomly assigned to the available groups. For the first experiment, there were not enough groups to include all treatments in all replicates. Subsequently, this resulted in a larger set of COC treated as control (exposed to only vehicle) compared to exposed groups (Table 2). Treated groups were incubated for IVM in parallel with a control and subsequently kept separate for all following procedures.

2.5. In vitro embryo production

After IVM, excessive cumulus layers were removed by pipetting until 3–5 layers remained. Presumed MII oocytes were then washed three times in media for preparation of zygotes (Table S1, SI) before co-incubated with thawed frozen spermatozoa at a concentration of 10^6 mL^{-1} using semen selected by swim-up procedure. The same ejaculate from the same bull with proven in vivo as well as in vitro fertility was used. Spermatozoa and oocytes were incubated for 22 h in modified HEPES-buffered glucose free medium [35] with the addition of 3 μg mL^{-1} of pencillamine, 1 μg mL^{-1} hypotaurine and 0.3 μg mL^{-1} epinephrine (PHE), 16 mM sodium DL-lactate, 50 μg mL^{-1} gentamicin sulphate and 3 μg mL^{-1} heparin. Following fertilisation, remaining cumulus cells and sperm were removed by pipetting in culture media. The groups of 10 COCs were covered in paraffin oil (OVOIL™, Vitrolife, Göteborg, Sweden) and cultured in drops. The larger groups of COCs in the second experiment were cultured without oil in wells. After in vitro fertilisation, presumed zygotes were washed to remove remaining spermatozoa and cumulus-cells. They were subsequently cultured in modified synthetic oviductal fluid (mSOF) with the addition of 0.4% w/v fatty acid free BSA, 20 μg mL^{-1} BME amino acids solution (50 ×) and 10 μg mL^{-1} MEM non-essential amino acids solution (100 ×), in drops or wells covered in OVOIL™ until 8 dpf.

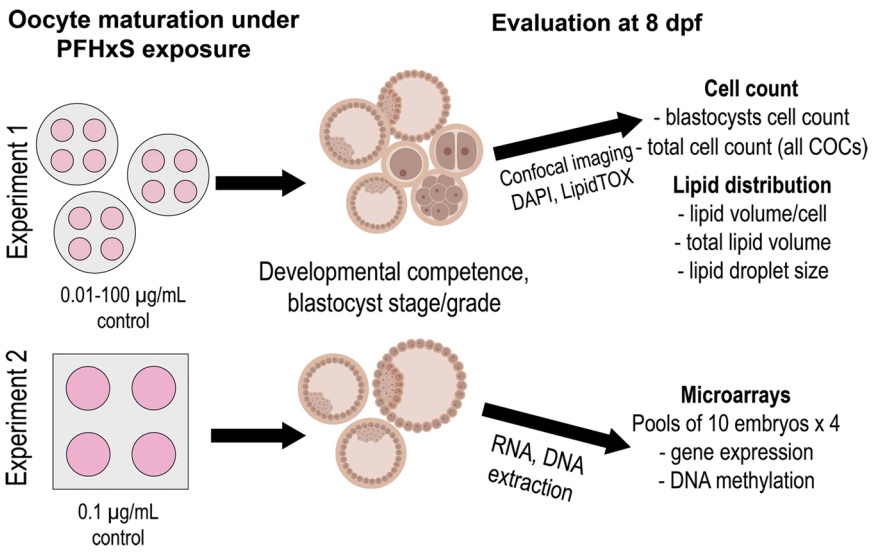

Fig. 1. Experimental design. Cumulus oocyte complexes (COCs) retrieved from the slaughterhouse were exposed to PFHxS during in vitro maturation in two different experiments. First, groups of 10 COCs were exposed at seven concentrations ranging from 0.01 to 100 μg mL-1 and for the second experiment COCs were exposed to 0.1 μg mL-1. For both experiments, exposed groups were cultured in parallel with a control with noexposure to PFHxS. Oocyte developmental competence was assessed during culture until 8 days post fertilisation (dpf) when all cultured COCs were either stained for confocal microscopy (first experiment) or subjected to extraction of RNA/DNA from blastocysts that were used for gene expression and methylation analysis using microarrays (second experiment).

Table 2
Oocyte developmental competence upon PFHxS exposure during IVM presented as proportion of developed embryos and results from logistic regression of development parameters.

Treatment, PFHxS	COCs	Embryos cleaved 44 hpf[a]			Embryos cleaved beyond two-cell stage 44 hpf[a]			Blastocysts 7 dpf[b]	Blastocysts 8 dpf[b]	Blastocyst 7 and 8 dpf[d]	
μg mL^{-1}	n	% (SEM)	OR (CI)[c]	p[e]	% (SEM)	OR (CI)	p[e]	% (SEM)	% (SEM)	OR (CI)	p[e]
.00	89	78 (6)	Ref	–	59 (5)	Ref	–	17 (3)	21 (2)	Ref	–
0.01	40	62 (9)	0.59 (0.24–1.43)	.24	48 (12)	0.72 (0.32–2.21)	.22	8 (5)	12 (8)	0.52 (0.16–1.44)	.23
0.10	40	65 (6)	0.73 (0.30–1.81)	.49	42 (4)	0.64 (0.27–1.53)	.41	8 (2)	10 (4)	0.41 (0.11–1.18)	.17
1	48	73 (3)	0.92 (0.39–2.21)	.84	56 (4)	1.03 (0.48–1.53)	.93	16 (4)	19 (3)	0.85 (0.33–2.02)	.52
10	40	45 (9)	0.26 (0.11–0.59) *	.001	35 (13)	0.42 (0.18–0.95) *	.04	13 (5)	12 (5)	0.53 (0.16–1.44)	.58
20	30	67 (7)	0.75 (0.28–0.87)	.57	37 (3)	0.48 (0.19–1.21)	.12	13 (7)	20 (10)	0.92 (0.31–2.47)	.33
40	40	53 (14)	0.37 (0.16–0.87) *	.02	25 (5)	0.26 (0.10–0.62) *	.003	0 (NA)	8 (3)	0.30 (0.07–0.95)	.05[c]
100	59	61 (7)	0.48 (0.22–1.01)	.05	35 (5)	0.42 (0.20–0.86) *	.01	1 (4)	3 (3)	0.13 (0.02–0.47) *	.003

[a] cleaved and cleaved beyond two-cell stage calculated from cultured COCs, [b]days post fertilisation, [c]odds ratio (OR) (confidence interval (CI)) from logistic regression on blastocyst development [d]blastocyst rate at day 7 and day 8 were considered repeated measurements resulting in one regression model for blastocyst development [e]non-adjusted p-values from logistic regression compared to control (0.00, reference level), asterisks denote significant values of $p < .05$.

2.6. Evaluation of oocyte developmental competence

Embryo development was evaluated during in vitro embryo production (IVP) as previously described [33]. At 44 h post fertilisation (hpf), the proportions of embryos cleaved and cleaved beyond the two-cell stage were recorded. At 7 and 8 dpf, the proportion of embryos developing into blastocysts and subsequent morphology of developed blastocysts was assessed (stage, grade). The blastocyst stage and quality grade were assessed by the same person within every replicate following the IETS grading scheme with slight modification: blastocyst stage was modified into three stages, namely i) early blastocyst/blastocysts, ii) expanding/expanded blastocysts where the zona pellucida was slightly thinned due to blastocyst expansion, and iii) hatching/hatched blastocyst. Blastocysts were graded from 1 to 4, where grade 1 corresponds to a top-quality embryo [40].

2.7. Fixation, fluorescent labelling, and confocal imaging (experiment 1)

At 8 dpf, the whole group of COCs (i.e., including oocytes, embryos halted before blastocyst stage and blastocysts) were fixed in 2% paraformaldehyde at 4 °C overnight, followed by rinsing in phosphate buffered saline with 0.1% polyvinyl alcohol (PBS-PVA). For evaluation of neutral lipids, fluorescent labelling with LipidTOX™ (HCS LipidTOX™ Green Neutral Lipid Stain H34475, Thermo Fisher Scientific, Waltham, MA, USA) was used by incubation for 30 min at room temperature according to manufacturer's instruction. Embryos were mounted on a microscope slide (ER-201B-CE24, Thermo Fisher Scientific) with the addition of approximately 2 μL Vectashield containing DAPI (Vector Laboratories Inc., CA, US), which enabled DNA visualisation and blastomere identification.

Blastocysts (n = 39) and the rest of the group (n = 291, including oocytes, embryos halted or degraded) were scanned together with negative controls to ensure the absence of background fluorescence using standard magnification (20 ×). A confocal microscope (Zeiss LSM 800) equipped with He/Ne 543 and Ar 450/530 lasers was used. Negative controls did not show fluorescence. To evaluate the number of cell divisions that embryos had progressed through, the cell counts were manually evaluated (total cell count). For blastocysts, scans were captured in sections of 2 μm interval (z-stack) and images further analysed using Fiji for ImageJ software (www.imagej.net/fiji) (Fig. 2).

2.8. Image analysis (experiment 1)

Blastocysts (n = 39) were subjected to digital image analysis. The basis for the segmentation was an iterative 3D version of the Per Object Ellipsefit (POE) algorithm. The original POE method computes local threshold levels for each object, where the threshold level is set to optimise the ellipse fit of that object, given that it fulfils input criteria of minimum and maximum diameters. The method was originally presented as a 2D segmentation method [41], but was also available in a 3D version [42]. However, there might be a drawback to this method if a slightly darker object touches a brighter object. Then the threshold level

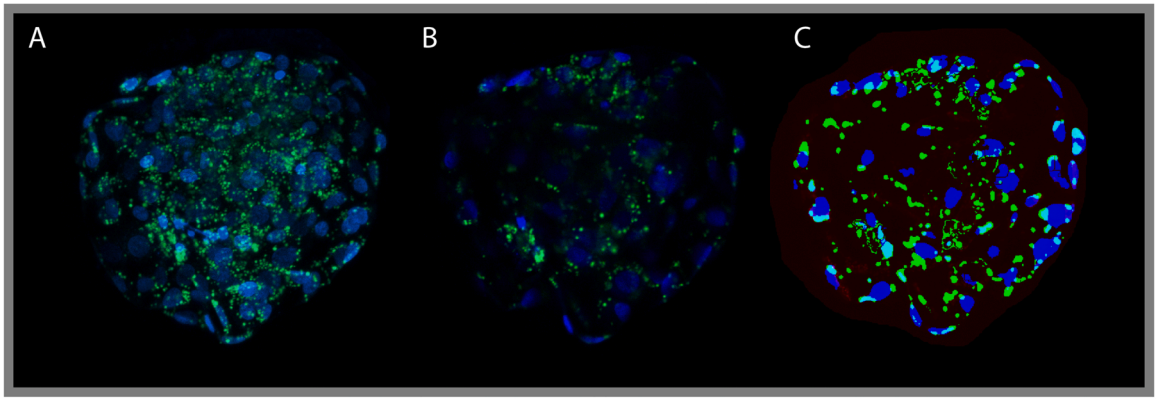

Fig. 2. Confocal imaging and computed image analysis. Bovine blastocyst day 8 post fertilisation stained with fluorophores to detect neutral lipids and nuclei, all images show the same embryo. A: overlay image of all z-planes combined, B: one z-plane showing one section of the embryo, and C: corresponding image from image analysis output.

might be set to optimise the ellipse fit for the brighter object, and the darker object will not be included at all. This could have been the case in this application, and we therefore developed an iterative version of the 3D POE method. This iterative version was set up as follows: first, objects were segmented using 3D POE. The resulting mask and a "seed" image were kept, which contained the segmented objects but eroded two voxels. Second, the segmented objects were masked from the raw image by making the corresponding voxels black. After this step, 3D POE was used to segment objects in the masked image. The resulting mask was merged with the previously segmented objects and the "seed" image was merged with the previous "seed" image. These steps were repeated until no more objects were segmented from the masked image. The objects in the resulting mask were separated using the 3D Watershed Split function in ImageJ [43], with the combined mask as input, and the combined "seeds" as seeds.

This segmentation method was used for segmentation of both blastomeres and lipids, but with different parameters for expected object min. and max. diameters and with different colour channels as input. To acquire the most favourable settings, a set of blastocysts (n = 5) blinded for treatment were manually validated using different cut-offs until the most coherent results between manual validation and automated image analysis was achieved (Fig. 2). The same settings were used for the control and the treated groups. After analysis, each blastocyst was blindly validated manually to reveal embryos in which computed analyses had failed and nuclei and/or lipids were significantly over- or underestimated. This resulted in 2 embryos being excluded from analysis. The number of blastomeres was recorded in each embryo (blastocyst cell count, n). The lipid distribution was evaluated by approximating the average total volume of lipids in the embryos (total volume lipid, μm^3), the average lipid droplet size within each embryo (average lipid droplet size, μm^3), and average amount of lipid per blastomere (lipid volume/cell, μm^3). Fig. 2 show a confocal image of a blastocyst and corresponding output from automated image analysis.

2.9. Statistical analysis

For statistical comparison between control and treated groups, RStudio for R (i386, 4.0.5, r-project.org) was used. The effect of PFHxS treatment on developmental competence (proportion of embryos cleaved, cleaved beyond two-cell stage) was calculated using mixed effect logistic regression with binary distribution, with replicate added as random factor and weighted for group size (glmer model of the package lme4). That is, the resultant models for developmental competence were variable/cultured COCs ~ treatment + replicate and weighted for COC count. Blastocyst rate at 7 and 8 dpf were treated as repeated measurements resulting in one model for blastocyst development (glmmSQL model of the MASS package), i.e., blastocysts/COCs ~ treatment + replicate + correlation structure of day of blastocyst assessment. Upon exposure to 40 μg mL^{-1} PFHxS, there were no blastocysts at 7 dpf, but some blastocysts had developed at 8 dpf. Therefore, logistic regression to calculate OR was based only on observations at 8 dpf for this concentration. For the first experiment ordinal variables (stage, grade) were presented as descriptive statistics as this experiment was not designed to allow calculation of effect on ordinal variables in several levels. However, in the second experiment, where 0.1 μg mL^{-1} PFHxS was compared to the control, enough data was obtained and the effect of treatment was calculated. This comparison was assessed using cumulative link mixed effect models with multinomial distribution (MCMCglmm model of the CRAN package) with replicate as a random factor (stage/grade ~ treatment + replicate). To assess whether PFHxS treatment affected cell count (i.e., amount of cell divisions reached at 8 dpf), the individuals were divided into two groups. First, the fate of all COCs subjected to culture was calculated, i.e., the cells were counted at 8 dpf in all oocytes, zygotes, embryos halted/degraded or developing into blastocysts (referred herein as "total cell count"). As the reasons for halted development before blastocyst stage can be several, the cell count in only the blastocysts at 8 dpf was compared between the treated groups and control (referred to as "blastocyst cell count") as a second step. The effect of treatment on blastocyst and total cell count was calculated using linear mixed effect models (lmer of the lme4 package) adding replicate as random factor. The same package was used with replicate as random factor to assess lipid distribution, evaluating the following endpoints: total volume lipids, average lipid droplet size, and average lipid/cell in the blastocysts. Log transformed values for average lipid/cell and average lipid droplet size were used to reduce skewness. To account for possible different lipid distribution in different blastocyst stages, cell count and the interaction treatment*cell count was added to the models but later removed based on AIC and *p*-values. For blastocyst evaluation of staining parameters (blastocysts cell count and lipid parameters), the treatment group of 100 μg mL^{-1} had to be excluded due to insufficient observations (2). *P*-values <[T 0.05ere considered statistically significant.

2.10. Parallel DNA and RNA extraction (experiment 2)

Individual embryos from the second experiment, where COCs were exposed to 0.1 μg mL^{-1} PFHxS or vehicle, were individually snap frozen in a fixed volume of 2 μL PBS-PVA. The blastocysts were pooled in groups of 10 in four replicates for each control and PFHxS-treated group. Blastocysts from nine replicates were matched for grade and stage to account for different embryo characteristics (see Table S2 in the SI for details on group composition) [44]. Genomic (g)DNA and total RNA was extracted in parallel using the AllPrep DNA/RNA micro kit (Qiagen Cat no. 80284) according to manufacturer's instructions with slight modifications as described previously [45]. DNA was washed and eluted in elution buffer and subsequently evaluated using the NanoDrop technology (ND 1000, NanoDrop Technologies, Wilmington, DE, USA) and stored frozen before further use. The RNA was eluted in 15 μL RNAse-free water and evaluated using for purity, quality, and concentration (Agilent Bioanalyser 2100, Agilent Technologies Inc., Santa Clara CA, USA). All samples had an RNA integrity number (RIN) indicating sufficient quality (>8, range 8.1–9.0).

2.11. RNA amplification and hybridisation (experiment 2)

To obtain enough antisense (a)RNA from the pools of embryos for microarray hybridisation, a two-round application kit was used according to manufacturer's instructions (2-round RiboAmp® HS^{PLUS} RNA Amplification kit, Applied Biosystems, Foster City, California, USA). Amplified aRNA was quantified using the NanoDrop technology and labelled with Universal Linkage System Fluorescent Labelling Kit (ULS™, Leica Wetzlar, Germany) with Cy3 and Cy5 in a dye-swap design with four arrays. For hybridisation, 825 ng aRNA was used and hybridised for 17 h in 65 °C in 10 × blocking agent and 25 × fragmentation buffer provided by Agilent. After hybridisation, the slide was washed and scanned using a PowerScanner (Tecan Group Ltd., Mannedorf, Switzerland). Features were extracted using Array-pro 6.3 software (Media Cybernetics, Rockville, Maryland, USA).

2.12. Microarray based analysis of gene expression profile in day 8 blastocysts (experiment 2)

The microarrays used for gene expression studies were adapted for an EmbryoGENE related project and have been previously described [46]. In the microarray, 42,242 probes are covered including reference genes and control probes [46]. Before comparison between treatments, the background signal was subtracted from the raw fluorescence intensity. Relative transcript abundance was analysed using Flexarray [47]. Loess and quantile normalisation were used for normalisation within the fluorophores (Cy3/Cy5) and between the arrays (four replicates). Comparison between the treatments (0.1 μg mL^{-1} PFHxS and control) was performed with the limma algorithm, where each probe

was attributed a probability to fold change between treated/control. Differently expressed genes (DEGs) were defined as significant ($p < .05$) probes with fold change > 1.5. When applying fold change or false-discovery rate (FDR) by Benjamini Hochberg correction (0.25), not enough transcripts were generated for pathway analysis. In order to obtain a general overview of pathways affected, all significant transcripts ($p < .05$ considering also small fold changes) were analysed by Ingenuity Pathway analysis software (IPA, QIAGEN). This tool considered all transcripts connected with a pathway as described by the scientific literature. In this way, several transcripts associated with the same direction in the same pathway could be identified to represent more important information at a cellular level.

2.13. gDNA fragmentation and hybridization (experiment 2)

The same pool of embryos used for the gene expression analysis were subjected to methylation studies using a microarray-based approach adapted for EmbryoGENE related projects as previously described [48]. The gDNA was amplified and fragmented using *Mse*I enzyme (NEB-R0525S) to enable standardised fragmentation regardless of methylation status. Following fragmentation, the gDNA was treated with methylation-sensitive enzymes to enable fragmentation according to methylation status (thus being able to detect differences based on methylated regions in the genome). After enzymatic digestion, samples were amplified by ligation-mediated PCR resulting in exponential amplification of methylated regions. Samples were labelled (Cy3/5) and processed using the same four-array dye swap design as for the transcriptomic microarray. Hybridisation was performed for 40 h in 65 °C in 100 × blocking agent and 2 × hybridization buffer followed by rinsing in solutions provided by Agilent. Hybridized samples were then scanned using a PowerScanner and features analysed using Array Pro.

2.14. Microarray-based analysis of methylation pattern in day-8 blastocysts (experiment 2)

The microarray platform was developed for EmbryoGENE related projects and described previously [48,49]. The microarray contains methylation sites in the bovine genome without topological bias, surveying genomic regions and CpG Islands and included controls (total 2.5% of total probe number) [48]. A previously described pipeline for data-processing was used [48]. Quantile inter-array scale normalisation was followed by loess intra-array normalisation (limma-package). For comparison between treated group and control, differently methylated regions (DMRs) were defined when $p < .05$ and fold change > 1.5. However, as FDR adjustment or fold change did not generate enough DMRs for pathway analysis using IPA, all significant probes ($p < .05$) were used in IPA analysis, also considering small fold changes between the groups.

3. Results

3.1. Validation of exposure concentration

Of the seven concentrations of PFHxS prepared from the same stock solutions, three were sent for concentration validation using mass spectrometry. This analysis revealed that the exposure concentrations were lower than intended, being approximately 75% (range 73–80%) of the anticipated. The nominal concentrations, validated exposure concentrations, and calculated exposure in the media (when the stock solution was diluted by 40 ×) are presented in Table 1.

3.2. Developmental competence (experiment 1)

Following exposure to PFHxS during IVM, developmental competence of the oocytes was evaluated until 8 dpf. There was a decreased likelihood of the COC to reach cleaved stage 44hpf if it had been exposed to 10, 40, or 100 μg mL^{-1} PFHxS (Fig. 3). This could also be seen as decreased chance of reaching beyond the two-cell stage in the same exposure groups (Fig. 3, exact proportions and results from logistic regression in Table 2). The initial delay in development was also observed at later stages when the likelihood of reaching blastocyst stage was decreased upon exposure to ≥ 40 μg mL^{-1} PFHxS during IVM (Fig. 3, Table 2).

3.3. Embryo phenotype upon exposure of PFHxS during IVM

Embryo quality presented as the proportions of top-quality embryos are presented in Fig. 4B, details can be found in Table S3 of the SI. When using cell-count as a proxy for advanced development, we could observe decreased developmental rate upon exposure to PFHxS (experiment 1). When we investigated the fate of all COCs cultured, and how many cell divisions they had reached, we observed a decrease in total cell count upon exposure to concentrations ≥ 40 μg mL^{-1} PFHxS. This concentration also decreased the likelihood of reaching blastocyst stage (Section 3.2). In blastocysts, there was a decreased cell-count upon exposure to even lower concentrations (≥20 μg mL^{-1} PFHxS). In the highest exposure group (100 μg mL^{-1} PFHxS), the number of developing blastocysts was too small to draw reliable conclusions of parameters assessed by confocal imaging (n = 2). In a subset of blastocysts, lipid distribution was evaluated by confocal imaging. Exposure to 1–10 μg mL^{-1} PFHxS during IVM induced alterations in the lipid distribution in the blastocysts, observed as increased average total volume lipids ($p = .01$ and $p = .02$ respectively, Table 3). There was also a tendency towards a lower amount of lipid/cell ($p = .05$) in the 1 μg mL^{-1} PFHxS group, which was not seen at other concentrations. In lower (< 1 μg mL^{-1} PFHxS) and higher (>10 μg mL^{-1} PFHxS) concentrations, there were no observed changes in lipid distribution in the blastocysts.

To address the effects at the blastocyst stage of a concentration that was not expected to induce embryo lethality or developmental delay, a second experiment was conducted with a larger set of COCs upon PFHxS treatment (0.1 μg mL^{-1} PFHxS, n = 674) or control (n = 642). At this concentration, PFHxS increased the likelihood of blastocysts to reach higher developmental stages compared to the control. That is, a higher proportion of embryos was observed at hatching/hatched stage in the treated group (24%) compared to the control (17%) and a lower proportion of early blastocysts/blastocyst in the treated group (20%) compared to the control (27%) ($p = .04$) (Fig. 4 A). At the same exposure concentration (0.1 μg mL^{-1} PFHxS), there was no observed risk of changed quality grade upon exposure ($p = .21$).

3.4. Gene-expression changes upon oocyte exposure to PFHxS (experiment 2)

To investigate molecular changes upon low-dose exposure to PFHxS, 8 dpf embryos from the control and 0.1 μg mL^{-1} PFHxS treatment, matched for blastocyst stage and grade, were pooled in groups of ten (in four replicates = 80 embryos in total) (Table S2). The pools of embryos were analysed using Agilent transcriptomic microarray to determine changes in gene expression. Fourteen differentially expressed genes (DEGs) (fold change > 1.5, $p < .05$) were identified between the control and the PFHxS treated group of which four were upregulated and ten downregulated. A volcano plot of gene expression following PFHxS exposure during IVM and a heatmap of the DEGs are presented in Fig. 5. Following Benjamini Hochberg correction for FDR, no transcript reached p-values $< .05$.

3.4.1. Functional analysis by IPA

To identify common biological functions of affected genes, Ingenuity Pathway analysis (IPA) was employed. Using the DEGs defined as $p < .05$ and fold change > 1.5 did not generate enriched biological functions. To obtain a general overview of pathways and biological functions affected, we also considered smaller changes in expression

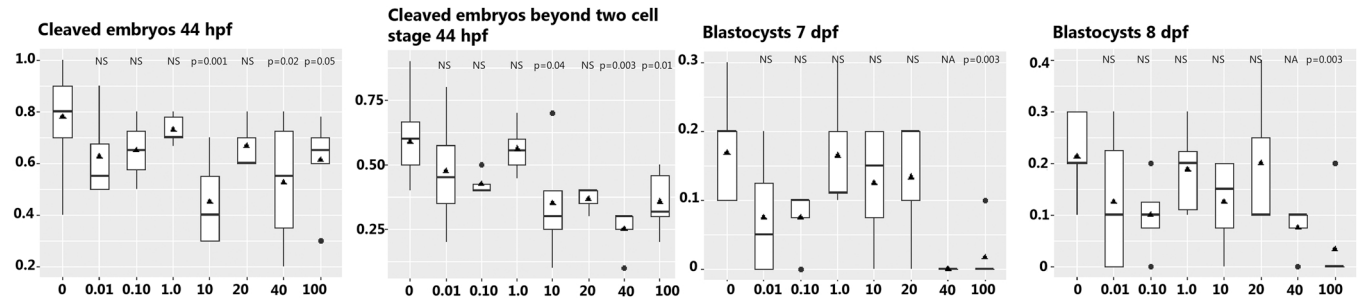

Fig. 3. Developmental competence following PFHxS exposure. COCs were exposed to PFHxS in concentrations from 0.01 to 100 μg mL^{-1} during IVM. Boxplots show the proportion of cleaved embryos and embryos cleaved beyond two-cell stage at 44 h post fertilisation (hpf). Proportion of embryos developed at 7 and 8 days post fertilisation (dpf) calculated from cultured COCs. The line represent median, the triangle is the mean, the box interquartile range (IQR) and whiskers are 1.5 × IQR. Outliers are depicted with dots and represent values outside 1.5 × IQR. P-values >[T 0.05re interpreted not significant (ns) and significant p-values are printed in the figure.

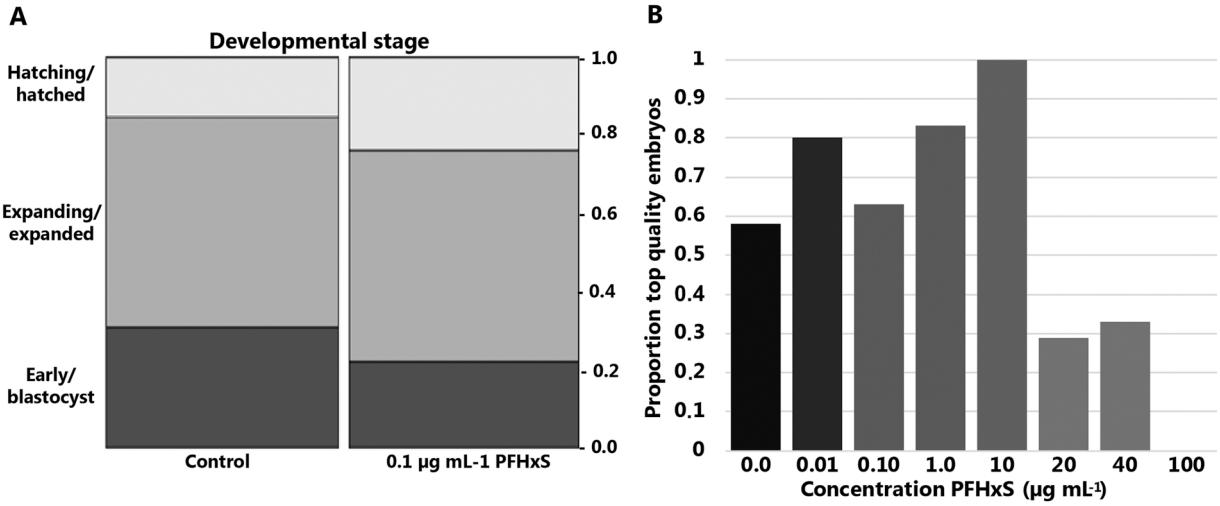

Fig. 4. Blastocyst stage/grade upon PFHxS exposure during IVM. A: Plot showing the proportion (y-axis) of blastocysts in each developmental stage (early/blastocyst, expanding/expanded and hatching/hatched) in the control and groups exposed to 0.1 μg mL^{-1} PFHxS during IVM. The likelihood of reaching higher developmental stages after exposure to 0.1 μg mL^{-1} PFHxS was significant ($p = .04$). B: Proportion of blastocysts of high quality (grade 1–1.5) at 8 dpf upon exposure to PFHxS from 0.0 to 100 μg mL^{-1} during IVM. There were too few observations for statistical comparison between groups.

Table 3
Blastocyst lipid distribution and blastomere count upon exposure to PFHxS during IVM.

PFHxS, μg mL^{-1}	Total cell count[a]			Blastocyst cell count[b]		Lipid/cell		Lipid droplet size		Total volume lipids	
	cells (SEM)	p	n	cells (SEM)	p	μm^3 (SEM)	p	μm^3 (SEM)	p	× 1000 μm^3 (SEM)	p
.0	28 (6.5)	NA	7	123 (11)	NA	909 (165)	NA	84 (5.6)	NA	89.2 (13.1)	NA
0.01	23 (5.7)	.72	5	107 (13)	.35	875 (223)	.49	111 (19)	.25	95.1 (30)	.69
0.10	18 (5.5)	.15	4	113 (18)	.58	959 (230)	.51	106 (11.6)	.38	92.0 (8.3)	.66
1	32 (7.6)	.44	5	120 (16)	.86	822 (166)	.05	127 (29)	.06	126.3 (32)*	.01
10	25 (9.3)	.36	4	145 (15)	.25	1278 (370)	.43	125 (19)	.10	193 (63)*	.02
20	27 (6.5)	.95	7	81 (8.1)*	.01	810 (238)	.80	81 (5.9)	.89	58 (13.5)	.65
40	11 (2.7)*	.03	3	61 (5.5)*	.005	1809 (1113)	.13	82 (6.2)	.93	107.7 (59)	.46
100	12 (3.6)*	.02	2	–	–	–	–	–	–	–	–

[a] The fate of all COCs subjected to culture was calculated, i.e., the cell number was counted at 8 dpf in all stages between oocyte (one cell) and blastocyst (see further "2.9 Statistical analysis"), [b]Average cell count in blastocysts developed 8 dpf. Asterisks denote significant values of $p < .05$

including all significant transcripts ($p < .05$, 312 transcripts). Using this dataset, IPA identified biological functions associated with PFHxS exposure related to increased synthesis and production of reactive oxygen species (ROS) as well as cancer-related pathways. The main biological functions altered upon exposure to PFHxS are shown in Table 4.

Pathways upstream of the changed biological functions were related to oxidative stress, impact on metabolism, and hormonal regulation. Oxidative stress pathways included, e.g., through *serine/threonine kinase (ATM)* signalling ($p = 6.32$ e-05, contributing genes e.g., *NR4A1, CLU*), *tumour protein 53 (TP53)*, *nuclear factor kappa B subunit 1 (NFKB1)* and *transforming growth factor (TGF) beta* ($p = 7.69$e-04, contributing gene, e.g., *CCR7*). Impact on metabolism was predicted due to altered genes related to PPARγ inhibition (rosiglitazone inhibition, $p = 2.73$e-03, *PPARγ* inhibition p = .02, *PPARγ coactivator 1 alpha (PPARGC1A)* $p = .001$), contributing genes e.g., *ACOX1, ADIPOR1, NR4A1, CCR7, TGFBR1*), while impact on hormonal regulation was predicted due to genes linked to *beta-oestradiol* signalling inhibition ($p = 3.8$e-06, contributing genes e.g., *NR4A1, PLAU*) (see Table S4 for full list of

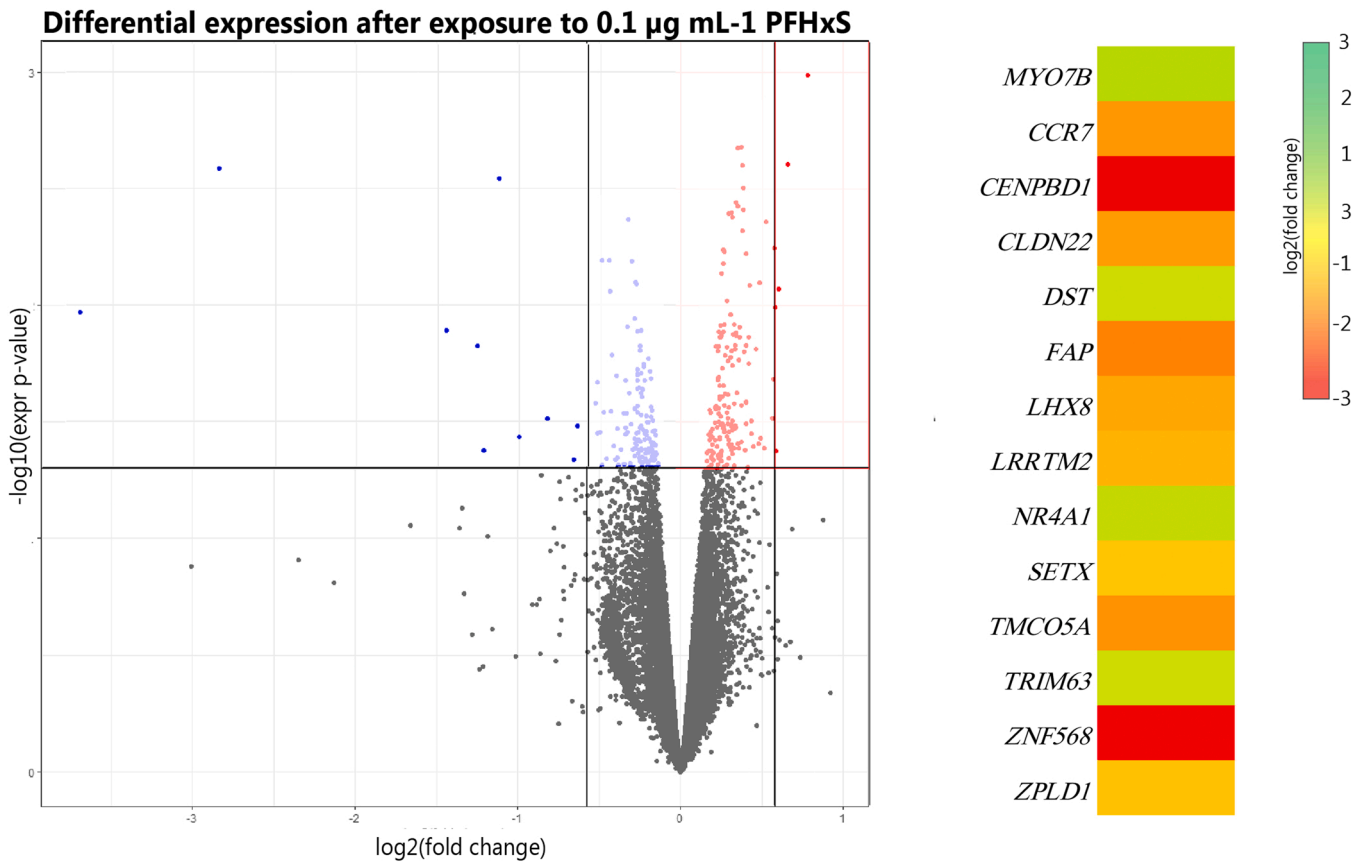

Fig. 5. Differential expression in blastocysts upon exposure of PFHxS during IVM. Volcano plot showing differently expressed genes. Black lines illustrating $p <$ [TS8 0.05axis) and fold change > 1.5 (x-axis). Differently expressed genes (DEGs, $p < .05$, fold change > 1.5), colour indicate direction of expression (log2 (fold change)).

Table 4
Main biological functions altered in blastocysts after PFHxS exposure during IVM.

Diseases or Functions Annotation	*p*-value	Predicted Activation State	Activation z-score
Development of malignant tumour	2.69E-08	Increased	2.113
Lymphohematopoietic neoplasia	2.05E-05	Increased	2.041
Lymphohematopoietic cancer	2.07E-05	Increased	2.086
Synthesis of reactive oxygen species	2.69E-04	Increased	2.147
Production of reactive oxygen species	8.25E-04	Increased	2.403
Differentiation of helper T lymphocytes	1.00E-03	Increased	2.154
Quantity of nitric oxide	1.54E-03	Increased	2.2

activated/inhibited upstream regulators).

3.5. DNA methylation changes upon PFHxS exposure (Experiment 2)

To address if PFHxS treatment affects DNA methylation and whether a possible link to transcriptomic changes could be seen, the same pools of embryos used for the transcriptomic microarray were analysed for DNA methylation pattern differences using Agilent EDMA microarray. Variation in DNA methylation patterns at the interrogated regions in blastocysts exposed to PFHxS during IVM compared to controls is presented in Fig. 6. 668 differentially methylated regions (DMRs), ($p < .05$, fold change > 1.5) were identified. These DMRs were significantly enriched in CpG-Islands ($p = .002$, Fig. S1-S2, SI) and 104 overlapped with the significantly ($p = .05$) changed genes. Yet, applying Benjamini Hochberg adjustment of *p*-values did not generate any DMRs.

3.5.1. Functional analysis by IPA

The number of DMRs defined as fold change > 1.5 and $p < .05$ was not sufficient to perform a biological functions analysis by IPA. In order to obtain an overview of pathways affected based on data from the DNA-methylation platform, all significant regions were included in the analysis ($n = 4639$, $p < .05$). IPA identified enrichment of molecular and cellular functions related to cellular assembly as well as cellular organisation, function, and cell death based on the genes altered by PFHxS treatment. As in the transcriptomic data, enriched pathways were related to *TP53* ($p = 2.04e-29$) and *TGFB1* (8.18e-17). Furthermore, altered *beta-oestradiol* signalling ($p = 7.97e-11$) was identified as a possible upstream regulator of the predicted changed biological functions (top upstream regulators in Table S5, SI). In pathways common to both arrays, 28 genes showed both transcriptomic and DNA methylation changes upon PFHxS exposure (Table S6, SI).

4. Discussion

4.1. Oocyte developmental competence following PFHxS exposure during maturation

Decreased developmental competence of the oocyte (developmental toxicity) was observed in response to exposure to ≥ 40 µg mL^{-1} PFHxS during IVM. This concentration was validated to 29.2 µg mL^{-1} (73 µM). At this concentration, a decreased proportion of embryos went through

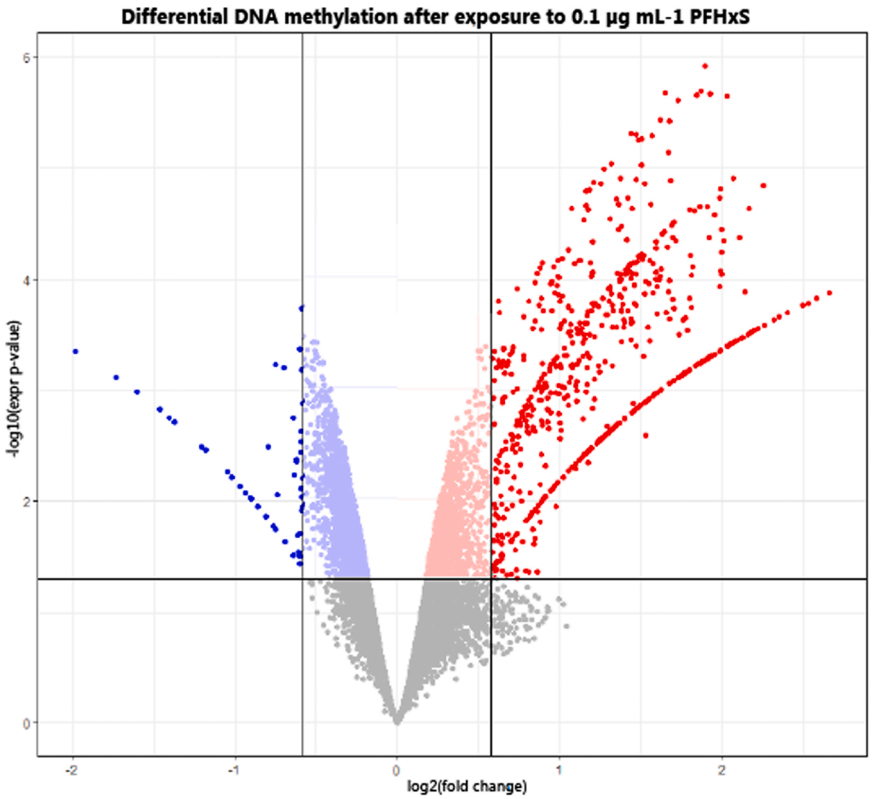

Fig. 6. Variation in DNA methylation in blastocysts after exposure to 0.1 µg mL^{-1} PFHxS during IVM. Volcano plot showing differently methylated regions. Black lines illustrating $p < .05$ (y-axis) and fold change > 1.5 (x-axis).

the first cleavages at 44 hpf and decreased proportion of blastocysts developed until 8 dpf. A reduced average cell count in blastocysts was seen at lower concentrations, from 20 µg mL^{-1} PFHxS (validated to 16 µg mL^{-1} or 40 µM). It could be noted that the actual proportion of high-quality embryos was lower in concentrations above 10 µg mL^{-1} PFHxS (validated to 8 µg mL^{-1} or 20 µM). However, there were not enough observations for statistical comparisons. One previous study on porcine oocytes investigated endpoints on the oocyte directly after IVM (44 h) [25]. Upon exposure, there was decreased oocyte viability ≥ 300 µM and maturation was affected at concentrations ≥ 50 µM. In zebrafish larvae, the LC50 has been assessed to 340 µM after exposure at 3–120 hpf [50]. Avian developmental toxicity has been assessed through *in ovo* exposure and revealed how concentrations of 38 µg g^{-1} (90 µM) decreased embryonic mass [51]. Taken together, the developmental competence of bovine oocytes (capacity to cleave and form a blastocyst) seems to be a relatively sensitive in vitro endpoint.

Compared to other PFAS investigated, bovine oocytes seem to be less sensitive to PFHxS in this system. We have previously shown that 10 µg mL^{-1} PFNA [33], as well as human relevant concentrations of PFOS (0.053 µg mL^{-1}), during IVM cause decreased embryo cleavage 44 hpf [34]. However, only PFNA caused diminished blastocyst development at these concentrations. These results are in line with studies on other experimental animals, where PFOS developmental toxicity exceeded the PFHxS toxicity [52–54]. The higher toxicity of PFOS compared to PFHxS is also expected since longer chains of PFAS in general cause higher toxicity compared to shorter chains [55].

4.2. *Variation in lipid distribution in blastocysts 8 dpf upon exposure to PFHxS during oocyte maturation*

We could see an increased average total amount of lipid in the blastocysts at 8 dpf after PFHxS exposure of 1–10 µg mL^{-1} during oocyte maturation. This, however, was not observed at higher or lower concentrations, suggesting a non-monotonic response to lipid alterations upon exposure. Previous studies have shown altered lipid configuration as increased lipid accumulation after PFOS exposure in several species [28,56–58]. In the bovine model, we have shown altered lipid configuration in blastocysts upon PFAS exposure (PFNA, PFOS) of the maturing oocyte [33,34]. Related to lipid homeostasis, one of the hallmark toxicity endpoints for PFHxS in animal experiments is increased liver weight and liver steatosis [54,59,60], which is *de facto* related to lipid metabolism. After developmental exposure to PFHxS in combination with a mixture of endocrine disruptors not causing toxicity alone, exposure has been associated with increased lipid accumulation in rats [61] and increased serum cholesterol in mice [54]. In humans, exposure to high concentrations of PFHxS and PFOS has been associated with higher serum lipids [62].

4.3. *Activated and inhibited gene pathways and possible consequences for the outcome of embryos*

Analysis of biological functions by IPA predicted increased synthesis and production of ROS in 8 dpf embryos. As we did not observe any signs of toxicity at the morphological level at this PFHxS concentration, and increased ROS production might be a sign of an adaptive response. Based on the predicted upstream pathways, this could involve, e.g., *ATM* activation, which is a well-described stress response pathway in cell cultures and embryos and is activated after previous stressors such as oxidative stress damage [63]. *ATM,* in turn, has been shown to activate *TP53* [64], which promotes apoptosis [65]. IPA further predicted *TGFb* activation based on altered genes upon PFHxS exposure. When applied during culture of bovine blastocysts, TGFb1 has been shown to improve blastocyst rate [66], which supports the notion of an adaptive response. Activation of *TP53* in combination with activated *TGFb1* pathways has previously been observed as a response to ethanol exposure in porcine embryos [45], suggestively a mechanism of the embryo to cope with the

toxic insult during development. Notably, *TP53* and *TGF beta* signalling was also predicted to lie upstream of genes linked to DMRs upon PFHxS exposure. Of the common genes showing both transcriptional and DNA-methylation changes in these pathways, about half (8/17 in TP53, 3/5 in TGF beta) showed inverse relationships between gene expression and DNA methylation. This is in line with the general suppressive function on gene expression by methylation of the DNA. However, DNA methylation might also be linked to increased transcription [67], which could explain why some genes were changed in the same direction between the arrays.

DNA methylation is reprogrammed and methylation is depleted during early embryo development, and the bovine genome is only starting to become *de novo* methylated 8 dpf [68]. It is quite remarkable that the narrow window of exposure (22 h IVM) can cause changes persistent in the blastocyst one week after exposure, both as altered gene expression and as modified DNA methylation.

Based on our transcriptomic analyses, PFHxS affected pathways downstream of PPARγ and ER signalling. While we have not shown their involvement in PFHxS's effects on oocytes, this finding is corroborated by a previous study showing that PFHxS-induced liver toxicity involves *PPARγ* and *ER* [27]. While PFHxS' toxicity has mainly been attributed to its ability to interact with *PPARα*, this study suggested that *PPARα*-independent effects involved *PPARγ* and *ER* [27]. Moreover, during differentiation of adipocytes in vitro, the gene-expression profile upon exposure appeared similar to that observed upon PPARγ –agonist treatments [69]. PPARγ has been implicated in the regulation of fatty acid oxidation in murine oocytes following early embryo development [70]. Thus, PPARγ might link transcriptomic changes induced by PFHxS to the observed alterations in lipid distribution upon exposure observed at concentrations of 1–10 μg mL^{-1} PFHxS.

Inhibition of oestrogen –activated pathways was another event predicted to occur upstream of the gene alterations induced by PFHxS exposure during oocyte maturation. This result was corroborated by the DNA methylation data predicting altered beta oestradiol signalling to lie upstream of the differentially methylated genes. PFHxS has been predicted to interact with ER in silico and modulate its activity in vitro [71,72], yet ER's role in mediating PFHxS's in vivo has so far not been confirmed [23,24]. Notably, oestrogen is known to increase developmental competence in combination with FSH/LH supplementation during IVM [73]. Thus, the oestrogenic activity of PFHxS might have led to the increased developmental rate seen in the subset of blastocysts in the second experiment. Altered beta-oestradiol regulated pathways were also predicted to lie upstream of genes linked to DMRs, where 7 out of 17 probes showed an inverse relationship between DNA methylation and gene expression changes. This may suggest that changes seen in the gene expression profile might be due to epigenetic changes and potentially persist during development. However, additional studies will be necessary to demonstrate the role of ERs in mediating this effect of PFHxS.

4.4. Human health implications

The exposure of PFHxS in women undergoing ovum pick-up for ART procedures ranged on the average from 0.3 to 1.75 ng mL^{-1} PFHxS (0.00075–0.004 μM) with a min to max range of 0.1–9.07 ng mL^{-1} [6,22,74]. The results presented here show several gene expression and DNA methylation changes without morphological effects on the embryos after exposure to 0.1 μg mL^{-1} PFHxS (validated to 0.08 μg mL^{-1} or 2 μM) after a short window of exposure during IVM. The lowest dose at which we could observe negative effect on blastocyst phenotype was at 1 μg mL^{-1} (altered lipid configuration) and 20 μg mL^{-1} (decreased amount of cells in blastocyst). The concentration of 20 μg mL^{-1} is about 10,000 × the approximate concentration in human sera [6,22,74–77], which can be used as a proxy for follicular fluid concentration, as the ratio is close to one [78]. However, 20 μg mL^{-1} is less than 100 × the average concentration in the heavily exposed community in Sweden [79] and less than 10 × the average concentration in factory workers in China [80]. In these highly exposed populations, some individuals have exposure exceeding 1 μg mL^{-1}, which is the concentration where we could see altered lipid distribution [79,80]. In a population without marked exposure to PFHxS, there has been association with altered serum lipids indicating possible effects on lipid metabolism even in the general human population [62]. The narrow window of exposure tested in this experiment (IVM) must be considered. This might lead to concern that exposures in vivo could exceed these findings, considering the longer exposure time. Furthermore, humans are exposed to a wide range of chemicals with the potential to disrupt the hormonal system as well as the delicate process of oocyte maturation [10]. Synergistic effects are not only plausible but even probable. For example, increased toxic effects have been seen in rats after exposure to a mixture of endocrine disrupting chemicals in combination with PFHxS, where PFHxS alone did not cause toxicity [53].

5. Conclusion

In this study, we have shown how PFHxS exposure of bovine oocytes in vitro during the narrow window of the final maturation affects subsequent developmental competence of the oocyte. Further, small yet significant changes in lipid configuration were observed in blastocysts, as well as molecular alterations, both on the level of gene expression and DNA-methylation. Together, these findings provide evidence that PFHxS is one of the many PFASs that can affect final oocyte maturation, leading to compromised embryo quality and development. This research contributes to the assessment of PFHxS impact on ovarian function and subsequent female fertility.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.reprotox.2022.02.004.

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