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Running title

Oral low-dose E2 impact female embryo development

Summary sentence

Maternal oral low-dose estrogen exposure during the preimplantation period specifically targeted female embryos by inducing a male-like gene expression profile.

Keywords

preimplantation embryo; endometrium; pig; estradiol; gene expression; endocrine disruptors

Footnotes

† Accession Number: RNA-sequencing data have been deposited in the ArrayExpress database at EMBL-EBI, accession number E-MTAB-6242 (endometrium) and E-MTAB-6263 (embryos).

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Abstract

Maternal exposure to estrogens can induce long-term adverse effects in the offspring. The epigenetic programming may start as early as the period of preimplantation development. We analyzed the effects of gestational estradiol-17β (E2) exposure on blastocysts with two distinct low doses, corresponding to the acceptable daily intake ‘ADI’ and close to the no-observed-effect level ‘NOEL’, and a high dose (0.05, 10 and 1000 µg E2/kg body weight daily, respectively). The E2 doses were orally applied to sows from insemination until sampling at day 10 of pregnancy and compared to carrier-treated controls leading to a significant increase in E2 in plasma, bile and selected somatic tissues including the endometrium in the high dose group. Conjugated and unconjugated E2 metabolites were as well elevated in the NOEL group. Although RNA-sequencing revealed a dose-dependent effect of 14, 17 and 27 differentially expressed genes (DEG) in the endometrium, single embryos were much more affected with 982 DEG in female blastocysts of the high dose group, while none were present in the corresponding male embryos. Moreover, the NOEL treatment caused 62 and 3 DEG in female and male embryos, respectively. Thus, we detected a perturbed sex-specific gene expression profile leading to a leveling of the transcriptome profiles of female and male embryos. The preimplantation period therefore demonstrates a vulnerable time window for estrogen exposure, potentially constituting the cause for lasting consequences. The molecular fingerprint of low-dose estrogen exposure on developing embryos warrants a careful revisit of effect level thresholds.

Introduction

Estrogens are important mediators for the preparation of the uterus towards implantation [1–4]. During the early embryonic cleavages maternal plasma estrogen concentrations are low [5, 6]. This is followed by an increase of estrogens around implantation (in mice at day 4) [4]. The role of mid-luteal estrogens is not as clear in primates, although human data indicates that slightly higher estrogen concentration might favor implantation [3–5]. Sows depict increasing peripheral plasma estrogen concentration after implantation [6, 7], whereas a first local rise through secretion from the elongating preimplantation embryo occurs at day 11 to 12 after fertilization [1]. Estrogens hereby function as maternal pregnancy recognition signal to inhibit luteolytic signals.

Humans are exposed to various estrogenic substances with the potential to affect the endogenous hormone systems [8, 9]. These may be natural as well as synthetic substances, so-called endocrine disrupting chemicals (EDC), which can adversely impact on developing organisms. As this has also been shown for estradiol-17β (E2) [10, 11], the latter is regarded as an EDC [9]. Especially prenatal development, starting as early as the preimplantation phase, has been demonstrated a sensitive period, when disruptive stimuli may induce long-term consequences [8, 12–14]. A preimplantation estrogen treatment has been shown to impact on the uterus, leading to an abnormal endometrial function, a perturbed intrauterine environment and a disturbed embryo-maternal communication. Various effects, ranging from subtle changes in endometrial gene expression to pregnancy losses, have been described in mice [15–18] and pigs [19–25]. The observed alterations in the endometrium involved mRNA [15, 17, 19, 20] and protein [26, 27] expression changes, as well as differences in its secretory activity [23, 24, 28] and morphology [15, 17, 23]. In addition, estrogens reaching the embryo may also exert direct effects as indicated by in vitro studies [29–31].

The timing of the estrogen exposure seems to be highly important. In pigs, a short treatment on days 9 - 10 or 7 - 10 had strong disrupting capacity [19–25], whereas treatment after day 10 of pregnancy demonstrated none or only minor alterations [21, 32, 33]. Estrogen treatment only during the preimplantation period resulted in sex-specific changes on sexual

development in murine offspring [13, 14]. This may be attributed to differences between the sexes prevailing during the preimplantation period including changes in the methylome and the metabolome [34–38]. Additionally, gestational low-dose E2 treatment in pigs has been shown to alter body composition in the male offspring [10], while bone development was affected in females [39].

The pharmacokinetic behavior of estrogens differs depending on the route of exposure and contributes to possible direct mechanisms involved in early EDC effects on tissues such as the uterus [40–43]. In the pig, the gut wall largely metabolizes orally ingested E2, while further rapid transformation occurs in the liver [43–45]. Estrogens are transported to the bile and subjected to an enterohepatic cycling [45, 46]. Thus, no [43, 45] or only low [10] concentrations of E2 are detected in the circulation following oral administration. In pigs, the predominant metabolite is estrone-glucuronide (E1G), while lower concentrations of E2-glucoronide, E1-sulfate, E1 and other minor metabolites prevail [43–45]. The main route of excretion of estrogens in the pig is the urine [47]. E1G and other glucuronides together with sulfates of E1 and E2 account for more than 90% of the metabolized E2 [43, 44].

In pigs, the circulating unconjugated estrogens are mainly bound to albumin [48, 49] and are distributed throughout the body and its tissues. Both retention and accumulation depend, at least in part, on the cellular status, such as the estrogen receptor content, which also determines potential cell-specific effects [40, 50–53]. The uterus is a major target organ where estrogens accumulate. In contrast to unconjugated estrogens, the conjugated forms possess little or no direct estrogenic activity, however, they also appear in tissues [54–56]. Although the liver is the main organ of estrogen metabolism, other tissues are able to conjugate and deconjugate estrogens likewise [54, 56, 57]. This has been particularly established for breast cancer cells converting sulfated estrogen, a major circulating conjugated form of estrogens in humans [54], into its free form, thus increasing their local amount of active estrogens [57].

In the present study, the main and most potent naturally occurring estrogen in females, namely E2, was used as potential EDC. As its pharmacokinetic behavior and mode of action

through its classical and non-classical receptors is thoroughly described [58, 59], it qualifies as model substance for estrogenic environmental low-dose exposures effects. Interestingly, the pig placenta produces considerable amounts of E2 during late gestation, and thus displays an environment likely comparable to the women [10, 60–62]. In rodents, circulating estrogens are lower during pregnancy.

We investigated the plasma elimination kinetics and tissue concentrations of E2 and its metabolites after oral intake of three distinct doses. We elucidated direct E2 effects on sows and sex-specific effects on blastocysts at day 10 of gestation by introducing a next-generation sequencing approach. To our knowledge, this is the first study, investigating in vivo effects of estrogens on the embryonic transcriptome.

Materials and Methods

Animals and sampling

Study 1: E2 elimination kinetics

Male castrated piglets (approximately 20 weeks old) were used as most sensitive model because of lowest concentrations of endogenous E2 in order to being able to even detect small elevations in plasma estrogen concentrations. This was performed as described earlier [10]. They were fed a defined amount of E2 once to determine kinetics of plasma estrogen concentrations. In brief, the animals received a single oral E2 dose, either 0.025 (n=3), 5 (n=2) or 500 µg E2/kg body weight (bw) (n=3), respectively or an ethanol carrier only (n=2). Blood samples were taken at one hours or fifteen minutes interval, centrifuged and ethylenediaminetetraacetic acid (EDTA) plasma was stored at -20 °C.

Study 2: Direct maternal E2 exposure

This is the second part of a long-term large animal trial. In the first part the sows had been exposed to E2 over the entire length of gestation [10]. Due to management reasons, the sows underwent further breeding until this second part started, where the same sows were again allocated to the same treatment group as in the first part. All details about the complete study design have been published by van der Weijden et al. [63] in the supplementary information. The second part (here named “study 2”) was conducted as follows. German

Landrace sows (n=4-6/treatment) were cycle synchronized using Altrenogest ReguMate® for twelve days, then Intergonan® (PMSG) at 750 iU was applied once the following evening, and Ovogest® (human chorion gonadotropin) at 750 iU was applied once 3.5 days later. The next day (day 0), all animals were inseminated with sperm of the same single Pietrain boar twice, in the morning and in the evening. From insemination until day 10, sows were orally exposed to different doses of E2 (1, 3, 5(10)-ESTRATRIEN-3, 17β-DIOL, Steraloids, Newport. USA), namely with 0.05, 10 and 1000 µg E2/kg bw daily, respectively, or with ethanol carrier only (control group). The E2 concentrations were selected according to reference values for humans [58] and have been reported earlier [10, 39]. The lowest dose corresponds to the 'acceptable daily intake' (ADI), the second low dose is close to the 'no-observed-effect level' (NOEL). The high dose was integrated as positive control possibly reflecting e.g. a mistaken use of oral contraceptives during early pregnancy. Half the dose was fed in the morning and the other half in the evening. One hour after ingestion of the last dose, sows were slaughtered at day 10 of pregnancy. The uterus was removed and embryos were flushed from the uterus using 10 ml phosphate-buffered saline (PBS, autoclaved, pH 7.4) per horn. These first flushings were collected, centrifuged and stored at -20 °C. Each horn was again flushed using 50 ml to ensure that all embryos were recovered. All embryos were transferred into a petri-dish containing PBS. Single embryos as well as tissue samples (from endometrium, skeletal muscle and heart) were shock frozen in liquid nitrogen and stored at -80 °C. As published earlier [64], all embryos were hatched spherical blastocysts according to the expected stage and contained an embryonic disc. There was neither significant difference in number (overall n = 230, p = 0.33) nor in size (2.2 mm ± 0.1 mm (mean ± SEM); p = 0.08) of the embryos. EDTA plasma was obtained from blood samples after centrifugation at 4 °C. Bile were collected and stored along with the plasma samples at -20 °C. Animals were only included in the RNA sequencing (RNA-Seq) analyses if embryos were at the blastocyst stage. Three sows of different treatment groups were excluded depicting only unfertilized oocytes.

The experiments were performed in accordance with the accepted standards of humane animal care and were approved by the District Government of Upper Bavaria, reference # 55.2-1-54-2531-68-09.

Hormone analyses

Plasma concentrations of E2, total estrogens (estrone (E1), E2 and estradiol-17α), testosterone, and progesterone were analyzed by enzyme immunoassay (EIA) as described recently [39]. Total estrogens were measured to estimate E1, the main unconjugated metabolite in pigs, as well as to approximate the amount of conjugated estrogen metabolites, as there was no E1 antibody available at our institute. In order to analyze conjugated steroid hormones an additional step was added to the protocol. During the steroid extraction process after phase separation, the frozen phase including the conjugated steroids was defrosted at room temperature and further processed. Two ml hydrolysis buffer (50 mM Na-acetate-buffer), containing 16 µl of the enzyme mixture β-glucuronidase/arylsulfatase (Merck KGaA, Darmstadt, Germany), was added. After an incubation step overnight at room temperature, 6 ml tert-butylmethylether/petrolether 30/70 v/v were added. The samples were agitated for 2 hours at room temperature, rested for half an hour at room temperature, and were then frozen overnight at -60 °C. The decanted supernatant was then dried in a vacuum concentrator and 500 µl assay buffer were added to the residues. Subsequently, the EIAs were performed.

In order to analyze hormone concentrations in endometrial, muscle and heart tissue, frozen tissue aliquots were grounded using a pestle and a mortar on dry ice. One hundred mg were transferred into an extraction tube, 500 µl of physiologic salt solution was added, and samples were stored at -20 °C. Next, an ether extraction was performed. Tert-butylmethylether/petrolether 30/70 v/v (6.5 ml) was added to each sample and agitated overnight. After phase separation they were frozen over the weekend at -60 °C. The decanted supernatant was then dried in a vacuum concentrator, 500 µl assay buffer were added, and the EIAs were performed. In addition, after the phase separation, the frozen part was used to obtain the conjugated steroids as described above. Minor modifications were

integrated for bile. The hydrolysis buffer was used at 500 mM and with twice the amount of enzyme mixture. After the incubation overnight at room temperature and for another two hours at 37 °C, 6.5 ml tert-butylmethylether/petrolether 30/70 v/v was added.

RNA and DNA extraction

Total RNA from endometrial samples of pregnant sows was extracted according to the manufacturer recommendations using TRIzol (Invitrogen, Karlsruhe, Germany) (n = 4 per treatment group, except for the ADI dose group (n = 3), where a repeated tissue extraction was performed). Next, total RNA and DNA from single embryos were extracted using the AllPrep RNA/DNA Micro Kit (Qiagen, Hilden, Germany) following the manufacturers protocol for cells with slight modifications. In brief, 700 µl of Buffer RLT Plus supplemented with 1% β-mercaptoethanol was added to the frozen embryo. Disruption was achieved by pipetting up and down and by a single brief vortexing. Homogenization was performed using a syringe and needle. After centrifugation of the lysate using a DNA spin column, the column was stored at 4 °C, while the flow-through was processed following the protocol for “purification of total RNA containing small RNAs from cells”. In order to improve RNA purity, the column was incubated with Buffer RPE at step D3 and D4 before centrifugation for 4 min and 2 min, respectively. RNA elution was repeated using the first eluate to increase the final concentration. The DNA was purified subsequently. Samples were immediately put on ice. RNA and DNA samples were stored at -80 °C and -20 °C, respectively. Purity and quantity were assessed spectrophotometrically using the NanoDrop 1000 (peqLab, Erlangen, Germany). Additionally, RNA quantity of embryos was determined using the Qubit (Invitrogen) with the Qubit™ RNA BR Assay. RNA integrity was measured by means of the Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany) with the RNA 6000 Nano Kit (Agilent). The mean RNA Integrity Number (RIN) of the endometrial samples and the embryos were at 9.3 ± 0.3 (± SD) and 9.7 ± 0.3 (± SD), respectively.

Sex determination of embryos

At least four embryos per sow from four sows per treatment group (control, NOEL, high dose), were used for the concurrent RNA and DNA extraction (n = 65). Unfortunately, the number of embryos of an appropriate quality for analysis was limited in the ADI group. Therefore, these needed to be excluded from the analysis. The sex of the embryos was determined by means of a quantitative real-time polymerase chain reaction (qPCR) using the DNA with primers specific for the y- chromosomal gene sex determining region Y (*SRY*) in addition to primers for the autosomal histone gene H3 histone family member 3A (*H3F3A*) (Supplemental Table S1). Primers were designed using NCBI primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and their specificity was checked using gel electrophoresis. The SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR Kit (Invitrogen) was used on the LightCycler 2.0 (Roche Diagnostics GmbH, Mannheim, Germany). One µl of DNA was added to 9 µl of the mastermix (5 µl of 2x SYBR® Green Reaction Mix (includes 0.4 mM of each dNTP and 6 mM $MgSO_4$), 2.4 µl of nuclease free water, 1 µl of 20x Bovine Serum Albumin (ultrapure, non-acetylated) (1 mg/ml), 0.2 µl of forward primer [20 µM], 0.2 µl of reverse primer [20 µM], and 0.2 µl of SuperScript® III RT/Platinum® *Taq* Mix (includes RNaseOUT™ Ribonuclease Inhibitor)). The program – reverse transcription at 50 °C for 10 min, then 95 °C for 2 min, followed by 50 amplification cycles with 5 sec at 95 °C, 10 sec at 60 °C, and 15 sec at 72 °C; the melting curve was performed from 55 to 95 °C with 0.1 °C/sec, then samples were cooled to 40 °C – was run with each sample in duplicate. A positive control DNA was included in each run. In the beginning, each primer pair was checked for its specificity by sequencing of the amplification product. Then, the melting point was used to identify the product. The following RNA-Seq analysis (n = 36) demonstrated that with exception of one embryo the gender had been correctly assigned.

RNA-Sequencing and data analyses of endometrium and embryos

The library preparation starting from 125 ng total RNA of each endometrial sample was performed with the Encore Complete RNA-Seq Multiplex System IB (NuGEN, AC Leek, The Netherlands) following the manufacturers protocol. Quality and quantity were assessed with Qubit (Invitrogen) and the Bioanalyzer 2100 (Agilent). The libraries were sequenced on a Genome Analyzer IIx system (Illumina). The cBot single Read Cluster Generation kit (Illumina) and 36 Cycle Sequencing Kit v4 (Illumina) were applied to generate single-end reads (74 bp). A multiplex of the 16 samples was analyzed on four lanes. Demultiplexing was conducted by using the barcode sequence consisting of four nucleotides at the beginning of each read.

Regarding the RNA-Seq of single embryos, 6 embryos were selected per sex and treatment group (control, NOEL, high dose; n = 36) as well as at least one male and one female embryo per sow (n = 4 per treatment group). However, as one suspected male embryo turned out to be female in the NOEL dose group, one sow had three female and no male embryo. Thus, the NOEL group consists of 5 male embryos from 3 different sows and 7 female embryos from 4 different sows, while all other groups comprise 6 embryos from 4 different sows. Library preparation with 100 ng RNA per sample was performed using the TruSeq Stranded mRNA Sample Prep Kit (Illumina, Inc, California, USA) according to the manufacturer protocol. The RNA quality and quantity were assessed with Qubit and the Bioanalyzer 2100. The pooled libraries were used for cluster generation with the TruSeq SR Cluster Kit v3-cBot-HS (Illumina). Single-end 100 bp reads were produced on an Illumina HiSeq 2000 with the TruSeq SBS Kit v3-HS (Illumina).

The RNA-Seq data were analyzed using Genomatix (Genomatix Software GmbH, Munich, Germany). Mapping was performed on the Genomatix Mining Station (Sus scrofa, Genome Library, NCBI build 4, ElDorado Version 12-2012, mapping type “fast”, alignment minimum quality 92%).

The mapped reads of the endometrium were analyzed for differential expression on the Genomatix Genome Analyzer using edgeR with default settings (p-value threshold 0.05, with

adjusted p-value, and log2 fold change of >= or <= 1). Further handling of the significantly regulated transcripts was done with Galaxy [65] installed at the Gene Center (LMU Munich, Germany, AG Blum). The cut-off for defining a gene as being transcribed in a sample was set to having at least 10 reads. At least 3 out of 4 samples of one treatment group had to have more than 9 reads for not being discarded in order to allow genes to be turned on or off by the treatment.

The mapped reads of the blastocysts were analyzed differently, as due to the higher number of samples per treatment group ($n \geq 5$). Thus, analysis of differential gene expression was performed with the BioConductor package EdgeR using the 'estimateGLMRobustDisp'[66]. A false discovery rate (FDR) of 5% was used as threshold for significance of differential gene expression. Venn diagrams were generated for genes from all four comparisons with p-values smaller than 0.0001 ($p < 0.0001$) including a fold change cut-off of 1.5 using the webtool Venny 2.1 [67]. Hierarchical clustering (HCL) was performed by the use of MeV_4_8 v10.2 [68] for the same genes used for the Venn diagrams.

RNA-sequencing data from both experiments have been deposited in the ArrayExpress database at EMBL-EBI under the accession number E-MTAB-6242 (https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-6242) for the endometrium and E-MTAB-6263 (https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-6263) for the embryos.

A functional annotation clustering was computed using the database for annotation, visualization and integrated discovery (DAVID 6.8) (https://david.ncifcrf.gov) [69] in order to visualize biological motifs. Homo sapiens was used as reference species. A gene list containing the gene symbols of differentially expressed transcripts (DEG) were clustered based on the assignment of the genes to the gene ontology (GO) FAT terms of biological process, cellular component, and molecular function. Some of the default options were adjusted, amongst others due to the relatively small number of DEG (similarity threshold = 0.6, initial and final group membership = 2, EASE score = 0.2). Only DEG where gene symbols were available and annotated in the database could be analyzed.

Technical validation of the endometrial RNA-sequencing data

A subset of genes that were differentially expressed according to the RNA-Seq analysis was additionally technically validated by a two-step reverse transcription qPCR (RT-qPCR). In many cases, more than one transcript of a single gene was shown to be regulated in the RNA-Seq analysis. If possible, primers were designed for each transcript. However, often this was not possible. Therefore, one qPCR primer pair may fit to more than one transcript determined in the RNA-Seq analysis. This is indicated in the list of all primers (Supplemental Table S1) in a separate column containing the accession number of each transcript fitting to the respective primer pair. The identical RNA samples were used. They were reverse transcribed into cDNA as described by Klein and colleagues [70]. Quantitative real-time PCR was conducted using the SsoFast™ EvaGreen® Supermix (Bio-Rad, Munich, Germany) along with 384 well plates and a final volume of 10 µl per sample. The master mix consisted of 5 µl SsoFast™ EvaGreen® Supermix, 0.2 µl of the forward primer [20 µM], 0.2 µl of the reverse primer [20 µM], 0.07 µl Visi-Blue™ (TATAA Biocenter AB, Goteborg, Sweden), and 3.53 µl RNase free water. One µl of cDNA was added into each well containing the master mix, while 1 µl of nuclease free water and 1 µl of an endometrial cDNA mixture served as negative and positive control, respectively. Quantitative real-time PCR runs were performed on the CFX384™ Real-Time PCR Detection System (Bio-Rad) with the following settings of 30 sec at 95 °C, 40 cycles with 5 sec at 95 °C, and 10 sec at 60 °C to 64 °C depending on the primers annealing temperature (Supplemental Table S1), the melting curve was performed from 65 °C to 95 °C with steps of 0.5 °C and 5 sec per increment, finally the plate was cooled to 4 °C. A qPCR product from each set of primers was sequenced to confirm product identity. Subsequently, the melting curve analysis with the specific melting point of each product was used. Data analysis using the obtained Cq values was performed as previously described [71]. For relative quantification, four reference genes were selected using NormFinder (GenEx Pro Ver 4.3.4 software multiD Analyses AB, Gothenburg, Sweden), namely *H3F3A*, ubiquitin B (*UBB*), glyceraldehyde-3-phosphate dehydrogenase

(*GAPDH*), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*) (Supplemental Table S1).

Statistics

The SigmaPlot program package release 11.0 (SPSS, Chicago, IL, USA) was used for all statistical analyses and graphical presentations except for the statistic evaluation of the RNA-Seq data. The logarithmic values of steroid hormone concentrations from plasma, bile, endometrium, skeletal muscle and heart tissue samples were analyzed using ANOVA with the Dunnett post hoc test for comparison of the three treatment groups with the control group. In order to compare the number of male and female embryos between the treatment groups a contingency table was made and subsequently a Chi^2 test was applied. The RNA-Seq data from the endometrium as well as from the embryos were statistically analyzed using the edgeR algorithm of the Genomatix Genome Analyzer. Thus, the treatment groups were compared with the control group and the male and female data sets from the same treatment group were tested for differential gene expression, respectively. For the statistical analyses of the normalized qPCR data t-tests were applied between the treatment group that had been found significantly regulated in the RNA-Seq experiment and the control group. Regarding the linear regression analysis and its graphical presentation between the fold changes of the qPCR results and the respective fold changes from the RNA-Seq experiment, if values for more than one transcript were available from the RNA-Seq data that were simultaneously amplified with one primer pair, the mean was used. Thus, for each qPCR fold change value there was one corresponding value from the RNA-Seq experiment. The data are depicted as mean ± SEM. Significant difference was assumed with $p < 0.05$.

Results

Elimination kinetics in male castrated pigs

The blood plasma concentrations before and after feeding the respective dose of E2 (0, 0.025, 5 and 500 µg/kg bw, respectively) were determined. E2 concentrations were measured and published earlier [10]. In brief, the two low doses did not lead to any notable increase in plasma E2 concentrations with average values of 5.6 ± 1.3 pg/ml (mean ± SEM)

and 5.5 ± 1.9 pg/ml, respectively. The control animals depicted an average concentration of 5.3 ± 2.5 pg/ml. The high dose showed a peak after 15 minutes with an average of 77.3 ± 23.9 pg/ml. Plasma E2 concentrations did not decline to basic levels but remained elevated from 6 to 12 hours at 23.5 ± 4.0 pg/ml. Yet unpublished data showed that even after 21 to 24 hours, there was still an average plasma concentration of 22.2 ± 7.5 pg/ml.

The animals receiving the high dose showed an increase in plasma concentration of total estrogens with a first maximum at 15 minutes with 115.3 ± 26.6 pg/ml, and a second maximum at 2 hours and 45 minutes with 115.8 ± 70.4 pg/ml (Fig.1a). The concentration remained elevated over 24 hours with a plateau phase at about 46 pg/ml. The other treatment groups did not show an increase after feeding and revealed average concentrations of 21 ± 5 pg/ml (control), 10 ± 1 pg/ml (ADI) and 11 ± 2 pg/ml (NOEL), respectively.

The pattern for conjugated E2 is shown in Figure 1b. The high dose group reached a first maximum of 9'523.9 ± 5'318.7 pg/ml at half an hour, a second maximum at 2 hours and 45 minutes with 10'232.3 ± 7'213.9 pg/ml, and decreased then to a plateau phase at about 4'200 pg/ml. In NOEL gilts, there was also an increase reaching a maximum at 45 minutes with 335.1 ± 207.1 pg/ml, then it declined to almost basal levels at 3 hours. The ADI group depicted 64 ± 28 pg/ml, and the control group had mean concentrations of 40 ± 12 pg/ml.

The conjugated total estrogens showed a similar pattern (Fig.1c). The high dose group depicted a first maximum of 65'128.7 ± 27'963.6 pg/ml at 30 minutes and a second maximum at 2 hours and 45 minutes with 91'262.3 ± 64'961.1 pg/ml. Again, the plateau phase lasted for at least 24 hours at about 33'000 pg/ml. Neither the ADI nor the control animals depicted an increase. Average concentrations were 49.3 ± 8.7 pg/ml and 51.6 ± 9.0 pg/ml, respectively. The NOEL animals showed a maximum of 2'509.9 ± 1'456.3 pg/ml after 45 minutes. In addition, there was a plateau phase at about 500 pg/ml.

Steroid hormones at day 10 of pregnancy

One hour after feeding one half of the daily dose, all analyzed free and conjugated estrogens showed significantly elevated estrogen concentrations in the high dose group (Table 1). Selected ones also had significantly elevated total estrogens in the NOEL group.

The plasma concentrations of E2 were 3-fold higher in the high dose group compared to the controls ($p = 0.003$). The respective fold change differences of significantly altered estrogen concentrations compared to the control animals are shown in Table 2. The amounts of total estrogens were also significantly altered ($p < 0.001$) with 3-fold and 17-fold higher concentrations in the NOEL dose group and the high dose group, respectively.

Concurrently, high concentrations of E2 ($p < 0.001$) and total estrogens ($p < 0.001$) were determined in the bile after feeding the high dose (Table 1). The concentration was about 2'500-fold and about 3'100-fold higher for E2 and total estrogens, respectively (Table 2). Similar to plasma, total estrogens were also significantly higher in the bile from the NOEL dose group with 49-fold higher concentrations compared to the control animals (Table 2). Thus, large quantities of unconjugated estrogens appeared in the bile, either as E2 or after conversion as E1.

In the endometrium, heart and skeletal muscle, E2 and total estrogens were significantly higher in the high dose group, with increases of about 15-fold and about 150-fold, respectively (Table 2). Additionally, in the NOEL dose group, total estrogens were significantly 6- and 5- fold higher in the endometrium and the heart, respectively.

In the bile, where the concentrations of conjugated estrogens exceeded the unconjugated forms, the relative increase (Table 2) was much more pronounced for the unconjugated forms. The conjugated forms had only about 800-fold and about 400-fold higher conjugated E2 and conjugated total estrogens, respectively, in the high dose group compared to the controls. In contrast, in the plasma, the increase of conjugated E2 and conjugated total estrogens with 361-fold and about 2300-fold, respectively, was much more pronounced compared to the unconjugated forms (Table 2).

Elevated concentrations of conjugated estrogens were detected in the tissue samples (Table 1). The increase in the high dose group compared to the control group was in a similar range in all three tissues with about 10-fold and about 100-fold for conjugated E2 and conjugated total estrogens, respectively, thus showing a slightly lower increase compared to the unconjugated estrogens (Table 2).

Overall, there were marked increases in the high dose group regarding all analytes and considerable changes occurred in the NOEL dose group while no effects were found in the animals fed the ADI dose.

Progesterone and testosterone were similarly analyzed. There were no significant differences of progesterone ($p > 0.5$) in plasma, bile, and tissues (endometrium, skeletal muscle, heart). The average concentrations were 14.1 ± 1.7 ng/ml in the plasma, 33'474.3 ± 13'042.4 ng/ml in the bile, 28.1 ± 1.8 ng/g in the endometrium, 46.5 ± 3.0 ng/g, in the skeletal muscle, and 94.3 ± 9.4 ng/g in the heart. Testosterone showed an overall significant difference in the plasma samples (41.1 ± 7.1 pg/g (control), 65.8 ± 7.1 pg/g (ADI), 36.6 ± 6.4 pg/g (NOEL), and 29.8 ± 4.7 pg/g (high dose); $p = 0.03$), and significantly higher values in the high dose group compared to the control animals in skeletal muscle tissue (82.5 ± 10.3 pg/g (control), 88.6 ± 10.0 pg/g (ADI), 104.8 ± 21.1 pg/g (NOEL), and 181.5 ± 29.1 pg/g (high); $p = 0.02$). Testosterone was neither different in the bile (1805.8 ± 464.8 pg/ml, $p = 0.6$) nor in the heart (281.0 ± 86.7 pg/g, $p = 0.5$).

Embryo sexing

The number of embryos per sex and treatment group is shown in the contingency table (Table 3). The Chi^2 test depicted that the proportion of male and female embryos was not statistically significantly associated with treatment dose ($p = 0.892$).

Effects on gene expression in endometrium

Differentially expressed genes ($p < 0.05$) were determined in the endometrium of all treatment groups resulting in 14, 17 and 27 DEG in the ADI, NOEL and the high dose group compared to the control, respectively. Thus, the highest dose revealed the highest number of regulated genes. Most of the genes were upregulated, and only few overlapping genes

between the different E2 treatment groups were found (Fig. 2). The respective genes are listed in Supplemental Table S2.

For the functional annotation analysis of all endometrial DEG (of the ADI, NOEL and high dose group comparisons in sum), there were 42 gene symbols from which 40 were found in the DAVID database. Sixteen clusters were formed. The 10 most enriched clusters are depicted in Table 4. In general, extracellular structure organization, response to stimulus, cell activation, multiple aspects of apoptosis, catalytic activities, regulation of transport, secretion, signaling and cell communication, developmental processes, metabolic processes, particularly including phosphorus metabolic processes, and regulation of immune system process are the most represented functional categories.

Validation of endometrial RNA-sequencing data

Overall, 21 genes including 23 transcripts, found to be differentially expressed in the RNA-Seq experiment, were technically validated by RT-qPCR using the identical samples. The results are shown in Table 5. The two datasets fit well, as the linear regression analysis revealed an overall significant correlation ($p < 0.001$; $R^2 = 0.505$, adj. $R^2 = 0.486$). In general, most genes differentially expressed in the high dose group could be validated. In the low-dose exposure groups, there were often similar fold changes between the two experimental approaches; although, in many cases the RT-qPCR data did not reach significance. This might also be due to the use of a p-value threshold of 0.05 in the RNA-Seq data analysis.

Effects on gene expression in day 10 embryos

By applying an FDR of 5%, 982 and 62 DEG were detected in the female blastocysts of the high dose and the NOEL group compared to the controls, respectively. This included 373 down- and 609 upregulated genes in the high dose group and 31 down- and 31 upregulated genes in the NOEL group. In the male blastocysts none and 3 DEG were found in the high dose and the NOEL group compared to the controls, respectively. There were two downregulated and one upregulated transcripts. Thus, there was a more pronounced effect in the female embryos compared to males, demonstrating sex-specific effects of the E2 treatment. All regulated genes are named in Supplemental Table S3, while detailed transcript

lists were deposited in the ArrayExpress database at EMBL-EBI under the accession number E-MTAB-6263 (https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-6263).

Concerning general sex-specific differences an analysis with an FDR of 5% between male and female controls was performed. There were 35 DEG higher expressed in the male embryos, including 8 Y-and 7 X-chromosomal genes. In addition, 50 DEG were higher expressed in the female embryos, containing 33 X-chromosomal genes. All respective transcripts are shown in Supplemental Table S4.

In order to perform a comparison of the results from the different analyses between treatments and controls that is not biased by the algorithm calculating the correction for multiple testing, a cut-off for the nominal p-value of $p < 0.0001$ together with a fold change cut-off of 1.5 was used. These results are shown in Figure 3 (Supplemental Table S5). In the female embryos, there were 32 genes in the NOEL group, 18 with lower and 14 with higher expression after E2 treatment, while in the high dose group there were 73 genes, 18 with lower and 55 with higher expression after E2 treatment. These overall 99 different DEG included only one X-chromosomal gene (Supplemental Table S6), integrator complex subunit 6 like (*INTS6L*, previously known as DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 26B (*DDX26B*)), which was 2.7-fold higher expressed in the high dose group compared to the control group. This indicates no specific treatment effect on the expression of X-chromosomal genes in the female embryos. Only few genes were detected for the male embryos with a total of 9 and 5 genes in the NOEL and the high dose group, respectively (Fig. 3).

From the commonly regulated genes between treatment groups, there were 4 DEG regulated in the female NOEL and high dose group, namely ral guanine nucleotide dissociation stimulator (*RALGDS)* and *LOC100624113* upregulated as well as vitamin D receptor *(VDR)* and selenoprotein I (*SELENOI* ;previously known as ethanolaminephosphotransferase 1 (*EPT1))* downregulated. Furthermore, one differentially expressed gene (folate hydrolase 1 (*FOLH1*)) was upregulated in the female treatment groups as well as in the male NOEL group.

Hierarchical clustering of the same genes as used for the Venn diagram ($p < 0.0001$, fold change cut-off 1.5, Supplemental Table S5 and S6), grouped according to their sex and the applied dose is shown in Figure 4a, while in Figure 4b clustering was performed for samples as well as for genes. Both HCL indicate female embryos becoming more similar to male embryos due to E2 treatment, particularly evident in the high dose group of female embryos. The functional annotation clustering was performed for the female embryos, as males did not depict sufficient numbers of DEG. Again, the same genes as for the Venn diagram were used. Eighty-five DEG from the comparisons of treated female embryos (of the NOEL and high dose group comparisons in sum) with female controls could be used having an official gene symbol. Seventy-seven of these DEG were detected in the DAVID database leading to 22 clusters of which the 10 most enriched are shown in Table 6. These 10 clusters encompass biological processes involved in cell cycle, organic acid metabolism, catabolic processes particularly including organic substances, regulation of catalytic activity, multicellular organism development including embryonic organ development, signal transduction, as well as the positive regulation of biosynthetic processes and gene expression, cellular components of to the endoplasmic reticulum, the cytoplasmic region and synapses, as well as molecular functions regarding nucleotide binding and hydrolase activity.

Discussion

The preimplantation phase is a sensitive time window where gestational administration of estrogens may affect dams and embryos [15, 16, 18–20, 23–25] possibly impacting on the offspring later in life [13, 14]. EDC such as certain low-dose estrogens are taken up orally through food [9, 58, 72, 73]. Therefore, we modeled the effects of a continuous oral low-dose E2 administration on the transcriptome of embryos. We delineated potential mechanisms of E2 tissue distribution and metabolism and focused on the endometrium as a major target of estrogens, which may impact on embryo development.

Fuerst and colleagues [10] have shown a fast increase in circulating plasma E2 after feeding a single high dose to male castrated pigs. The maximum concentration was measured after 15 minutes. This concentration declined rapidly and reached a plateau phase that still

persisted with slightly elevated concentrations at 12 hours. As by the definition of low-dose and thus as intended by the study design, plasma E2 did not increases in the two low-dose groups. In contrast, in the present study analyzing the same animals, we observed a fast increase in plasma concentrations of conjugated estrogens after feeding not only the high dose of E2 but additionally in the NOEL group. Other studies introducing E2 into the stomach of prepubertal gilts have demonstrated that E2 is rapidly converted into conjugated metabolites [43–45]. Analyses of blood from portal veins have shown that E2 had been mainly converted into E1G already by the gut wall [44, 45]. In addition to further processing in the liver, this may explain the fast and strong increase of the conjugated estrogens in the plasma found in the present study. Similar to data from Ruoff and colleagues [45], concentrations of free estrogens in peripheral plasma after exposure to the two low doses remained at basal levels. The second maximum after 2 and 3 hours in the high dose group probably resulted from both potentially remaining estrogens in the stomach [43] as well as from enterohepatic cycling of estrogens [45, 46]. The latter may also explain the plateau phases until 24 hours after the application. Concordantly, plateau phases in the pig have been shown to prevail for more than 12 hours [43, 74, 75]. A dose response with two peaks and a plateau phase after oral treatment is as well-known from humans [42].

Although sows and castrated male piglets might differ in their metabolism of E2, important aspects can be drawn from the study on male piglets for the study in sows. At first, it shows that a very fast peak of E2 in plasma was observed, indicating that the amounts measured after one hour in the sows were most probably below the maximum levels achieved after E2 intake. Similar to the results in castrated boars after a single E2 dose, all plasma estrogen concentrations were elevated in the high dose group in continuously exposed sows at day 10 of pregnancy one hour following the last oral exposition. Conjugated estrogens were as well elevated in the NOEL dose group. Second, E2 was continuously present at a plateau phase, still remaining elevated after 12 hours. This leads to the assumption that when feeding the E2 dose to the sows every 12 hours an accumulation may occur. As unconjugated total estrogens were elevated in the sows in the NOEL dose group, an accumulation due to the

continuous exposure is indicated. Third, as even in this sensitive male model no low-dose effect on the plasma E2 concentration was observed – similar to the results in the sows – it is likely that the effects observed in the sows and embryos are due to E2 metabolites, possibly also the conjugated ones.

In the bile of the continually exposed sows, the relative increase with increasing doses of E2 was much more pronounced for the unconjugated compared to the conjugated estrogens. We assume that the ability of the liver to conjugate estrogens might have been saturated, so that a higher amount of remaining unconjugated estrogens was transported to the bile. The opposite was found in the plasma with a much stronger relative increase of conjugated estrogens compared to the unconjugated forms. Thus, quite some of the glucuronide and sulfate conjugates reached the plasma before being excreted by the kidney. Bottoms and colleagues have shown that by using an even higher dose as herein, the main route of excretion still remained urine with E1G as main metabolite [43].

The tissue concentrations of estrogens were determined as estrogens can hereby exert direct estrogenic effects on cellular functions. While plasma concentrations are rapidly cleared by the liver, tissues are capable of retaining the steroids for a longer time [40, 51]. Concordantly, in the sows at one hour after the application, the relative increase in unconjugated estrogen concentrations observed in the NOEL and high dose was less pronounced in plasma compared to the increase found in the different tissues. The clearance can be tissue dependent [40, 50, 51, 76]. One main factor is the presence of estrogen receptors, as increasing amounts enable the tissue to better retain estrogens [40, 50–53]. The uterus as a major target organ for estrogens possesses particularly high amounts of estrogen receptors [40, 77]. Yet, little differences between the tissues analyzed herein were detected. One reason may be that the last E2 feeding had only been applied one hour before slaughter. The effect is assumed to increase with time, more pronounced from 2 hours on, as indicated by other studies [51, 53].

Estrogens are important for uterine receptivity [1–3, 5, 24]. Disruption of pregnancy through estrogen exposure at days 9 and 10 has been associated with endometrial mRNA

expression changes [19, 20, 24]. Using microarray analyses, Ross and colleagues [19] have found 9, 71 and 21 genes differentially expressed at day 10, 13 and 15, respectively. Thus, only relatively few alterations were detected. One reason may be the analysis of the complete endometrial tissue, as particular alterations only occur in certain cell types such as the epithelium [19, 24, 78]. Still, the E2-cypionate (E2C) treatment induced one down- and 8 upregulated genes at day 10 [19]. This is comparable to the present study with overall 6 down- and 45 upregulated genes. Additionally, we detected a dose dependent increase in regulated genes and even low-dose effects were demonstrated. Some identical genes were similarly upregulated in the high dose group in the present study as compared to the study by Ross and colleagues [19]. Namely, retinol binding protein 4 (*RBP4*) was higher expressed at day 10, while vanin 2 (*VNN2*), sulfotransferase family 2A member 1 (*SULT2A1*), and solute carrier family 39 member 2 (*SLC39A2*) have been increased at day 13 but not at day 10 [19]. The genes affected by our E2 treatment were grouped into functional clusters, depicting some biological processes known to be involved in uterine preparation such as positive regulation of secretion and transport, extracellular region, and leukocyte activation [24, 79]. Some of the involved functional categories are similar to results from other studies such as processes related to cell death, transport, leukocyte activation, cell activation and developmental processes [19, 20]. However, the comparison is difficult as the other studies had most alterations at gestational days 13 and 15 and led to embryonic losses. Thus, although we similarly found gene expression changes in the endometrium, mainly other genes were affected. In addition, the continuous E2 exposure did not lead to embryonic losses [10]. Interestingly, our preimplantation E2 exposure did not affect the endometrial miRNA expression profile [64].

The increasing concentrations of E2 and/or its metabolites reaching the uterus as depicted in this manuscript and elsewhere [64], respectively, are one possible explanation for the expression differences found in the NOEL and the high dose group compared to the control animals. Despite a 160-fold increase in unconjugated total estrogens in the endometrium, there was no difference in the number of offspring at birth after treatment over the entire

pregnancy [10]. Other studies with estrogenic treatments only at days 9 - 10 or 7 – 10 of gestation demonstrated endometrial mRNA expression changes [19, 20, 24] and embryonic losses [22, 23, 25]. Thus, pigs, with estrogens as their maternal recognition signal, seem highly sensitive concerning treatments starting slightly before implantation, presumably due to a desynchronizing of the uterine and the embryonic development [24]. This phenomenon seemingly does not occur with continuous exposure even at a relative high dose as used in the present study and earlier [10].

To our knowledge, our results are the first to report sex-specific mRNA expression differences in blastocysts after in vivo estrogen exposure. There was a pronounced treatment effect on female but not male embryos. These sex-specific effects may be related to the fact that differences between the sex prevail during the preimplantation embryo development such as in their methylome, transcriptome, proteome and metabolome [35–38]. In line, adaptations to environmental changes such as diet and nutrients have been shown to be sex-specific [35, 80].

Sex-specific analyses using microarrays revealed that in bovine in vitro produced blastocyst at day 7, one third of the genes showed sex-specific expression (FDR $P < 0.05$) [38]. This is not reflected in our findings of 85 DEG between male and female control embryos. However, Bermejo-Alvarez et al. [38] also reported that by using a fold change larger than 2, only 53 transcripts were higher expressed in females and 2 in males. Next to general differences (in vitro – in vivo; bovine – porcine; day 7 – day 10); in the present study, interestingly, the comparison between the control groups revealed a similar total number of transcripts with 41 higher expressed in males and 19 higher expressed in females when setting a cut-off fold change of 2 for the differential expressed transcripts. Heras et al. [80] also selected bovine blastocysts at day 7 and analyzed in vivo as well as in vitro (serum and serum-free) produced embryos after RNA-Seq with EdgeR (FDR corrected p-value < 0.05). Comparing male and female embryos of the same treatment group without a cut-off fold change or with a fold change of at least 2, they observed 225 and 119 (in vivo), 54 and 54 (in vitro with serum) and 54 and 48 (in vitro serum-free) DEG, respectively. Thus, they did not observe a large

number of genes differentially regulated between the sexes, which is similar to the present study.

Strikingly, with increasing E2 dose, the gene expression profile of female embryos became more similar to the males. There are reports of sex-specific differences in the speed of embryo development [35]. Thus, there may be the possibility that the estrogen treatment led to alterations in the normal timing of the development of the female embryos making them appear more similar to the males at this point in time. Otherwise, they may have adapted a phenotype more similar to the male embryos. Unfortunately, there are only few in vivo studies regarding the sex-specific velocity of early embryo development [35]. Most studies used in vitro produced embryos depicting more often a faster development of male embryos, but this also seems to depend on the culture conditions. For example, in the pig, the energy substrate has been demonstrated to be important in this respect [81].

The disruptive potential of estrogens including E2 has been shown in vitro [29–31]. In vivo, short-term application of estrogens directly before implantation has demonstrated direct effects on the endometrial gene expression profile [19, 20, 24] as well as disruption of the gestational process later on, including embryonic losses [21–25]. In the present study, we also observed endometrial gene expression changes. However, as shown by continuously administering E2 over the entire gestation, to the same sows as used in the study at hand in a previous pregnancy, neither alterations in body weight nor litter size nor sex distribution were found at birth [10]. Thus, our continuous E2 treatment starting with insemination was less disruptive as treatments only directly before implantation [21–25]. Still, lasting consequences were observed in the offspring, namely a bone density phenotype, a shift in body composition as well as gene expression differences mainly in the prostate [10, 39, 82]. Likewise, a study in mice demonstrated that continuous estrogen exposure only during the preimplantation phase led to sex-specific alterations in the offspring [13, 14]. Both sexes were affected, including a masculinization of the female offspring [14]. Although, we neither observed changes in genes involved in the process of modifying DNA methylation such as DNA methyltransferases nor obtained a GO term involving epigenetics in the DAVID

analyses, a separate analysis of DNA methylation changes in the embryos and offspring showed that epigenetic marks have been affected in both [63]. Three genes were analyzed from which two were significantly affected in the embryos and offspring. These are the cell cycle regulator Cyclin Dependent Kinase Inhibitor 2D (*CDKN2D*) and the tumor suppressor gene Phosphoserine Aminotransferase 1 (*PSAT1*). A subtle but significant hypomethylation was observed in the embryos, while in the liver of the one-year-old female offspring a similar small effect, but in this case a hypermethylation was determined. Although detailed underlying mechanisms remain to be explored, this indicates the possibility of lasting changes due to the preimplantation E2 exposure.

Overall, we evidence that oral maternal E2 exposure targeted the endometrium and particularly the developing blastocysts by leveling their physiologically inherent sex-specific gene expression profile. This perturbation was either induced through direct effects of E2 metabolites or through alterations in the endometrial secretion impacting on the embryo. It may imply both a functional perturbation of the embryo and/or a shift of its developmental velocity. Notably, the molecular fingerprint at a low dose currently considered as NOEL is thereby of considerable importance. The disturbed embryonic development may likely entail sex-specific adult phenotypes increasingly described in offspring of EDC exposed mothers. Therefore, a careful revisit of effect level thresholds seems warranted.

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Author contributions

SEU, VLF designed the research. VLF, RWF, MR, SK performed the research. VLF, SB, SK, HB analyzed the data. VLF, SEU wrote the manuscript. All authors discussed the results and commented on the manuscript.

Conflict of Interest

The authors have declared that no conflict of interest exists.

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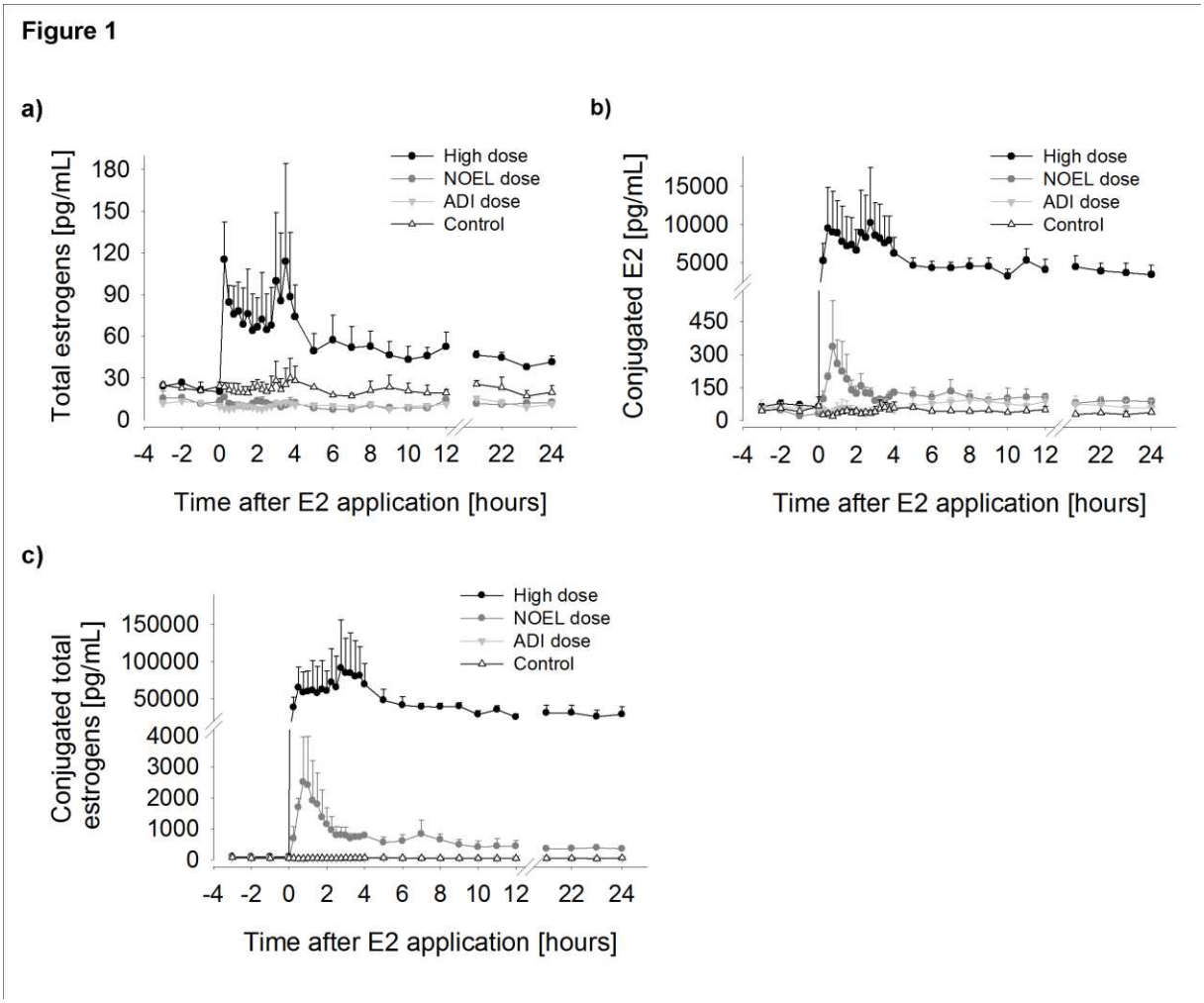

Figure 1.

Plasma kinetics of distinct oral doses of E2 in male castrated pigs. There were four treatment groups (0, 0.025, 5 and 500 µg E2/kg bw, respectively); the two low E2 doses represent half of the daily dose of the ADI (acceptable daily intake) and close to the NOEL (no-observed-effect level) as announced for humans; similarly, half of the daily dose of the high dose group as applied in the study 2 was fed. Plasma total estrogen (a), conjugated E2 (b), and conjugated total estrogen (c) concentrations are depicted as mean ± SEM (n = 2-3 / treatment group).

Figure 2

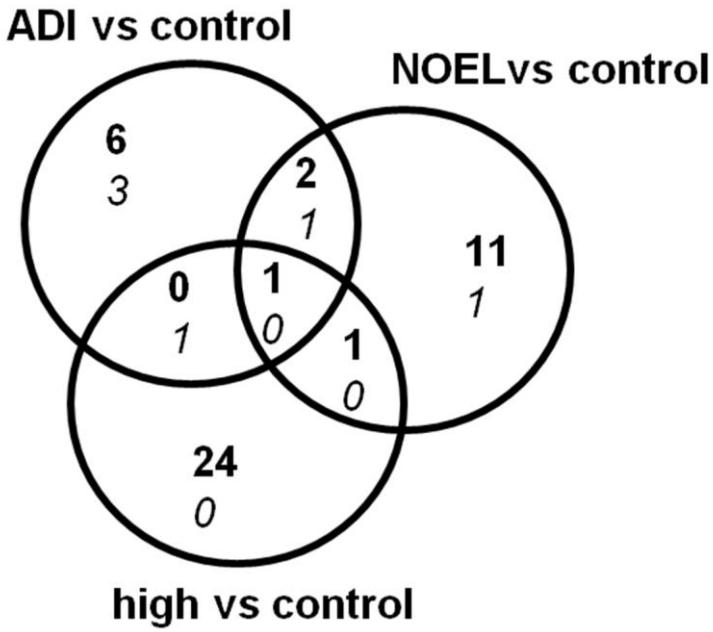

Figure 2.

Venn diagram of the differentially expressed genes (DEG) in the endometrium. The number of DEG from the RNA-Seq experiment of sows treated with distinct doses of E2 until day 10 of pregnancy (n = 4 per treatment group). Bold letters indicate higher expression, italic letters indicate lower expression after E2 treatment compared to the control. ADI - acceptable daily intake, NOEL - no-observed-effect level.

Figure 3

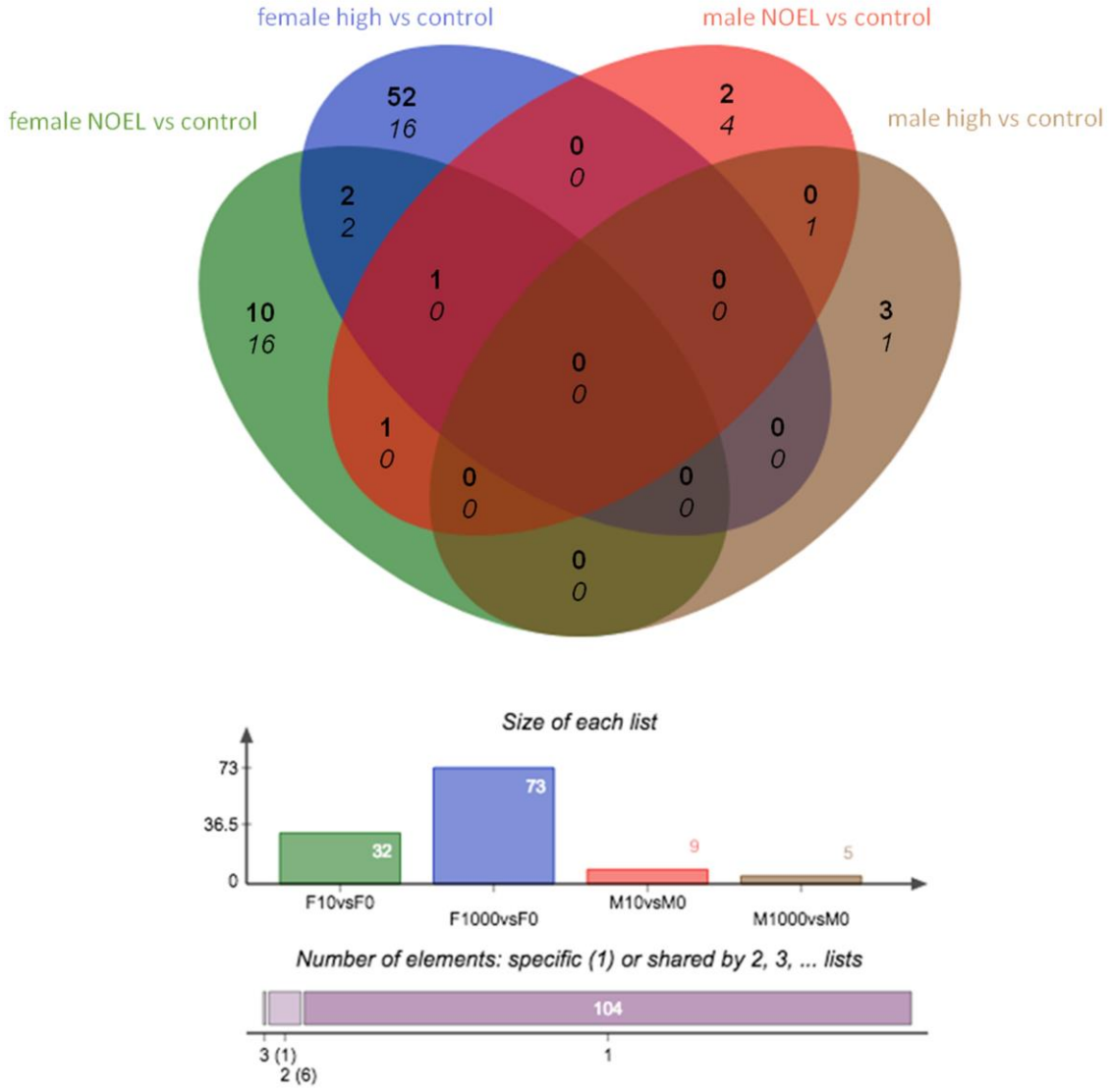

Figure 3.

Venn diagram of RNA-Seq results in embryos. The number of genes with $p < 0.0001$ and a cut-off fold change of 1.5 from the RNA-Seq experiment of embryos (n = 5-7 /treatment group) are shown. The sows were treated with carrier only, a dose close to the no-observed-effect level (NOEL) or a high dose of E2 (0, 10 and 1000 µg E2/kg bw/d, respectively) until day 10 of pregnancy. Bold letters indicate higher expression, italic letters indicate lower expression after E2 treatment compared to the control.

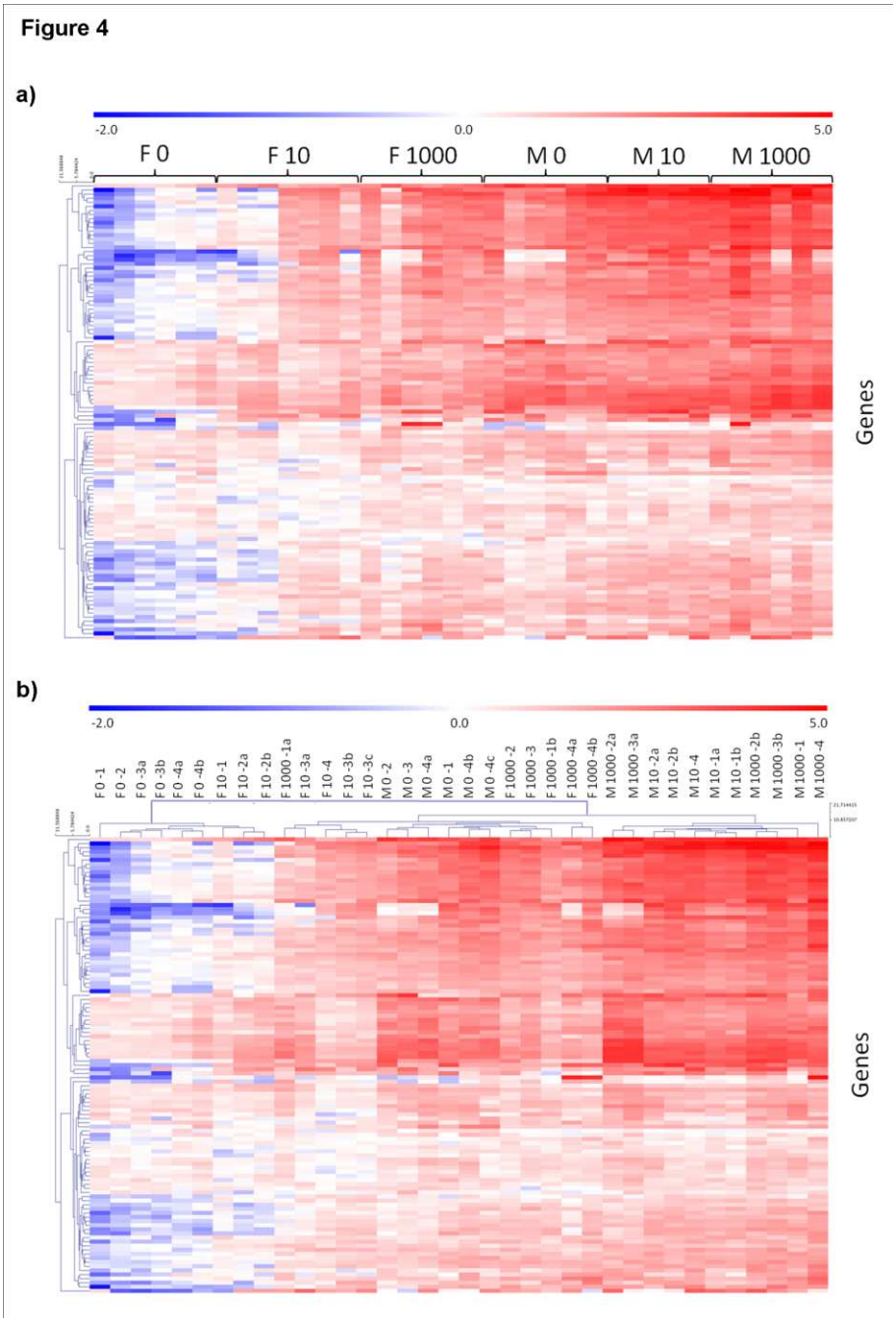

Figure 4.

Hierarchical clustering of RNA-Seq results in embryos. Genes with $p < 0.0001$ and a cut-off fold change of 1.5 from the RNA-Seq experiment of embryos (n = 5-7 /treatment group) are shown. The sows were treated with distinct doses of E2 until day 10 of pregnancy. Clustering of the genes only (a) and clustering of genes and samples (b) are depicted. F - female, M - male; treatment doses [µg/kg bw/d] are indicated by the letters 0, 10 and 1000; within each treatment group and sex differing mother sows are name with 1 to 4, while siblings additionally contain letters a to c.

Supplementary Data Legends

Supplemental Table S1. List of all primers used for qPCR validation of endometrial transcripts.

Supplemental Table S2. Differentially expressed genes in the endometrial samples (RNA-Seq).

Supplemental Table S3. Differentially expressed genes (gene symbols) in the embryos (RNA-Seq).

Supplemental Table S4. Differentially expressed transcripts of the comparison between male and female control embryos.

Supplemental Table S5. Differentially expressed genes (gene symbols) of the embryonic Venn Diagram (Fig. 3).

Supplemental Table S6. Differentially expressed genes in the embryonic samples ($p < 0.0001$, fold change cut-off 1.5).