Theriogenology 162 (2021) 15–21

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Effect of an altered hormonal environment by blood plasma collected after adrenocorticotropic administration on embryo development and gene expression in porcine embryos

Raquel González [a, *], Eva Pericuesta [b], Alfonso Gutiérrez-Adán [b], Ylva C.B. Sjunnesson [a]

[a] Department of Clinical Sciences, Reproduction. the Centre for Reproductive Biology in Uppsala (CRU), Faculty of Veterinary Medicine and Animal Sciences, Swedish University of Agricultural Sciences (SLU), P.O. Box 7054, SE-750 07, Uppsala, Sweden
[b] Departamento de Reproducción Animal, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Ctra de La Coruña, Km 5.9, 28040, Madrid, Spain

ARTICLE INFO

Article history:
Received 9 May 2020
Received in revised form
17 November 2020
Accepted 19 November 2020
Available online 24 November 2020

Keywords:
Pig
qRT-PCR
In vitro culture
Cortisol
ACTH

ABSTRACT

Early embryonic development may be affected by adrenal hyperactivity in stressful situations which may lead to endocrine changes in the embryo environment. A sensitive period in porcine embryo development is the 4-cell stage when the embryo genome activation occurs. A mixed *in vivo-in vitro* system was implemented to test whether an altered milieu around this stage could affect embryo development and blastocyst quality in the porcine model. After *in vitro* maturation and fertilisation, presumptive zygotes were exposed for 24 h to plasma collected after ovulation from adrenocorticotropic hormone (ACTH)-treated, non-ACTH-treated sows; and, medium without plasma, supplemented with bovine serum albumin. Subsequently, embryo development and differences in gene expression were tested among treatments. Cleavage and blastocyst rates did not differ between treatments. Blastocyst quality by morphology assessment was similar when all the resulting blastocysts were included in the analysis. However, when only expanded blastocysts (and onwards) were included in the analysis, the blastocysts from the non-ACTH plasma group showed better quality score. Blastocyst quality by morphological assessment was not mirrored by the transcription levels of various important genes for embryo development whose gene expression profile did not significantly differ among groups. It is likely that the effect of the altered environment provided by plasma from ACTH-treated sows was too short to affect embryo development. Therefore, a brief exposure to an altered endocrine environment may not have harmful consequences for the embryo once fertilisation occurs.

1. Introduction

Adrenal hyperactivity in stressful situations may lead to interferences in the hypothalamic-pituitary-gonadal axis that can affect female reproduction [1,2]. Studies on psychosocial stress both in humans and animals have described inhibitory effects on reproduction, although its impact at the gamete or embryo level is less understood [3–8].

The pig is a social species which may experience stress of management in modern farming conditions. Regrouping of sows requires around 2–7 days to establish a new hierarchy within the group [9,10]. These management practices are coincident with the periovulatory period and may interfere with the neuroendocrine system, affecting delicate processes such as ovulation, fertilisation and establishment of pregnancy. To evaluate the potential impact of management practices on sow reproduction, several *in vivo* studies aimed to evaluate the effect of an altered hormonal environment caused by adrenocorticotropic hormone (ACTH) administration at distinct moments around ovulation (reviewed by Ref. [11]). However, the possible direct effects caused by simulating psychogenic stress, through ACTH administration, on gametes or embryos were difficult to address in these *in vivo* studies. Previous *in vitro* approaches have assessed the direct effect of natural or synthetic glucocorticoids in different species on *in vitro* oocyte maturation [12–15] and on *in vitro* pre-implantation embryo development [16]. However, the scenario in which gametes or embryos are exposed under stressful situations is very difficult to simulate

* Corresponding author.
E-mail address: rrraquelgh2@hotmail.com (R. González).

https://doi.org/10.1016/j.theriogenology.2020.11.012

in vitro and it would not be mimicked by supplementing culture media with glucocorticoids. Therefore, a mixed *in vivo-in vitro* model was previously used to assess the effect of an altered endocrine environment by ACTH administration during the periovulatory period on oocyte maturation and fertilisation in the pig [17,18]. In this combined *in vivo-in vitro* model, blood plasma collected from sows which received ACTH every 4 h for ~48 h from the beginning of standing oestrus, was added *in vitro* at corresponding points of the reproductive cycle to assess the impact at specific reproductive events. It is known that the repeated administration of exogenous ACTH altered reproductive behaviour and led to modifications in reproductive hormone levels [19]. We have previously described that a brief exposure to plasma from ACTH-treated sows during *in vitro* maturation may induce alterations in mitochondrial and actin patterns in oocytes, but did not prevent embryo development [18]. Furthermore, an altered hormonal environment provided by plasma from ACTH-administrated sows seemed to negatively impact the sperm fertilising ability through alterations in the acrosome reaction and the kinetics of tyrosine phosphorylation, which ultimately led to a detrimental impact on fertilisation and embryo development [17]. Whether an altered hormonal environment derived from ACTH administration has an impact on post-fertilisation events has not yet been analysed.

The major transcriptional activation of the embryonic genome occurs during the 4-cell stage in the pig [20,21]. Additionally, in the *in vitro* system, most pig embryos are found at the 4-cell (and some have already reached the 5-8-cell) stage from 24 to 48 h of culture [22]. Therefore, the time frame between 24–48 h of *in vitro* culture corresponds to the most sensitive period for pig embryo development since they will be undergoing the transcriptional activation of their genome and will be at the stage where the developmental block occurs [23].

Gene expression has become an important tool to determine the quality of blastocysts generated after *in vitro* techniques and some genes are considered useful markers of blastocyst quality [24,25]. Disturbances on the embryonic environment may affect the proper development of the embryo, driving compensatory cellular responses that would, in turn, lead to alterations in gene expression during early embryonic development. So far, a possible effect of the altered hormonal profile caused by ACTH administration directly at the embryo level during this critical period has not been studied. Whether stressful situations affect the early development of the embryo during the critical period of 24–48 h of culture and this would eventually affect the gene expression profile in the resulting blastocysts is unknown.

Thus, the aims of the present study were to: 1) assess if cleavage and the progression up to the blastocyst stage were affected by blood plasma supplementation, collected from ACTH-treated sows after ovulation; and 2) evaluate whether transcript gene expression at the blastocyst stage was affected by altered circulating levels of reproductive and stress hormones in blood plasma collected from ACTH-treated sows after ovulation.

2. Materials and methods

Unless otherwise stated, all the reagents used were purchased from Sigma-Aldrich, Stockholm, Sweden.

2.1. Plasma samples and animals

No additional *in vivo* experiments were carried out to withdraw blood plasma samples. The plasma used in the current study was collected from sows administered ACTH (n = 2) or control females (n = 2) from a previous *in vivo* experiment [19]. Briefly, simulated stress was done by the administration of ACTH every 4 h during the periovulatory period, from the beginning of oestrus up to ~12 h after ovulation, for a total of 48 h after standing oestrus. Ovulation was monitored by transrectal ultrasonography. For the present experiment, plasma from representative sows with similar oestrous cycles was selected from each group at 12 ± 2 h post-ovulation. Blood circulation levels of cortisol and progesterone differed between groups [19] (Table 1). The boar used for semen collection was kept on straw in an individual pen at the Division of Reproduction, Department of Clinical Sciences (SLU, Uppsala). Water was provided *ad libitum* and it was fed according to Swedish standards [26]. The experimental design was approved in advance by the Ethics Committee for Experimentation with Animals, Uppsala, Sweden.

2.2. Oocyte collection and in vitro maturation

Ovaries from prepubertal gilts were transported at 32–34 °C within 3–4 h after slaughter in 0.9% (wt/vol) sodium chloride solution without antibiotics. Cumulus oocyte complexes (COCs) were collected by follicle puncture from 3 to 6 mm antral follicles with a 5 mL syringe connected to a 18 ga needle. The follicular content was transferred into 25 mL tubes containing collection medium warmed at 37–38 °C. The collection medium was Hepes-buffered TCM-199 (M – 2520) supplemented with 2.2 g/L $NaHCO_3$ (S-5761), 1 mM L-glutamine (G-8540), 0.5 mg/mL polyvinyl alcohol (PVA, P-8136), 20 IU/mL heparin (H-3149) and 50 μg/mL gentamicin (G-1264). Using collection medium without heparin, the COCs were searched, washed three times and selected. They were then rinsed once in maturation medium that consisted of: TCM-199 (M – 2154) supplemented with 1 mM L-glutamine, 1 mM sodium pyruvate (P-3662), 1 mg/mL BSA (A-3311), 100 μM 2β-mercaptoethanol (M – 7522), 50 μg/mL gentamicin, 50 ng/mL epidermal growth factor (E–4127), 5 μl/mL insulin-transferrin-selenite (ITS, I-3146), 10 IU/mL PMSG and 5 IU/mL hCG (Suigonan, Intervet Scandinavia, Skovlunde, Denmark). Groups of 35–55 COCs were placed in 500 μL maturation medium into 4-well culture dishes (Nunclon, Nalgene, Nunc International, Roskilde, Denmark) for the first 22 h (with all the supplements) and for an additional period of 22 h in maturation medium without hormones and without ITS at 38.5 °C under 5% CO_2 in humidified air.

2.3. Semen collection and sperm preparation

Fresh semen was collected on several occasions from the same boar using the gloved-hand method and immediately extended (1:1, v:v) in Beltsville thawing solution (BTS, 005974-ZG863, IMV Technologies, L'Aigle Cedex, France), [27]. Semen extender was removed by standard centrifugation. Briefly, 0.5 mL of semen was transferred to a 15 mL tube containing 4 mL of non-capacitating medium [18,28] and centrifuged at 300×*g* for 5 min at room temperature. The supernatant was discarded and the pellet was washed again under the same conditions. After the second centrifugation, the pellet was gently resuspended in *in vitro* fertilisation medium (see below), and kept at 38.5 °C under 5% CO_2 in air for 30 min before being added to fertilisation wells.

2.4. In vitro fertilisation

After maturation, oocytes were gently washed in IVF medium and rinsed twice before to transfer them to the 4-well culture dish containing a final volume of 500 μL IVF medium [17,28]. The IVF medium was composed of the following: 90 mM NaCl, 12 mM KCl, 0.5 mM NaH_2PO_4 (S-5011), 25 mM $NaHCO_3$, 0.5 mM $MgSO_4$, 2 mM sodium pyruvate, 8 mM $CaCl_2 \cdot 2H_2O$ (C-7902), 6 mM lactic acid, 6 mg/mL BSA, 1.9 mM caffeine (C-0750), 50 μg/mL gentamicin and

Table 1
Mean (±SEM) hormonal concentration in pooled plasma collected from ACTH-treated or control sows at 12 h after ovulation.

Group	Cortisol (nmol/l)	Progesterone (nmol/l)	17β-Estradiol (pmol/l)	Inhibin alpha (ng/ml)
Control (n = 2)	35.4 ± 15.7	5.33 ± 1.40	6.0 ± 3.0	0.27 ± 0.03
ACTH (n = 2)	527 ± 116	12.6 ± 0.04	5.0 ± 0.0	0.34 ± 0.02

ACTH was administered every 4 h during the periovulatory period, from the beginning of oestrus until 12 ± 2 h after ovulation. Plasma collected was added to *in vitro* embryo culture medium for the first 24 h of culture. For a representative statistical analysis, see Brandt et al. (2007).

5 μg/mL phenol red. Gametes were co-incubated for 24 h at 38.5 °C under 5% CO_2 in air. The final sperm concentration used was 5×10^5 cells/mL.

2.5. In vitro culture

After gamete co-incubation, the oocytes were transferred to 1.5 mL tubes containing 750 μL Hepes-buffered North Carolina State University medium-23 (Hepes-NCSU-23) [29]. The remaining cumulus cells and spermatozoa loosely attached were removed using a vortex for 1 min. Presumptive zygotes (and some already cleaved) were then washed three times in the same medium and rinsed once in porcine culture medium (PZM [30]) before placing them in 500 μL PZM. They were randomly split in three groups and exposed to the following treatments during the first 24 h of *in vitro* culture: a) 10% (v:v) plasma collected after ovulation from ACTH-treated sows; b) 10% (v:v) plasma from non-ACTH-treated sows or; c) plasma-free group, supplemented only with 3 mg/mL bovine serum albumin (BSA). Culture medium consisted of 108 mM NaCl, 25.07 mM $NaHCO_3$, 10 mM KCl, 0.35 mM KH_2PO_4, 0.4 mM $MgSO_4 \cdot 7H_2O$, 2 mM calcium lactate (C-8356), 2.78 mM myo-inositol (I-7508), 1 mM L-glutamine, 5 mM hypotaurine, 2% (v:v) essential amino acids (B-6766), 1% (v:v) non-essential amino acids (M − 7145), 3 mg/mL BSA, 50 μg/mL gentamicin and 5 μg/mL phenol red. After 24 h of treatment in PZM, which corresponded with 48 h post-insemination (hpi), cleavage rate was assessed in Hepes-NCSU-23 and the non-cleaved oocytes were removed. Embryos were transferred to fresh PZM, covered with 300 μL mineral oil (M − 8410) and were kept in culture up to day 7. Culture was carried out in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2. Cleavage rate was expressed as the percentage of embryos found at 48 hpi of culture over the total of presumptive zygotes in culture. A corrected cleavage rate was also calculated having taken into account the number of embryos already present at the beginning of exposure to treatment. Blastocyst rate was expressed as the percentage of blastocysts found on day 7 of culture over the total of embryos. Quality of blastocysts was classified according to the International Embryo Transfer Society embryo system [31] as grade 1 (excellent to good), 2 (fair), 3 (poor) or 4 (dead or degenerating).

2.6. RNA purification, reverse transcription and quantification of mRNA transcript abundance

For gene expression analysis, only grade 1–2 expanded and hatching blastocysts were selected on day 7 of *in vitro* culture. Pools of 10 embryos per treatment were used and the number of blastocysts from the selected stages of development was the same in all the treatments to minimise variations. Embryos were moved to PBS supplemented with 0.1% (wt/vol) polyvinylpyrrolidone (PVP) and washed five times before transferring the embryos to a RNase- and DNase-free centrifuge tube. The tubes were then plunged into LN_2 and stored at −80 °C until analysis.

PolyA RNA was extracted from pooled blastocysts using the Dynabeads mRNA Direct Extraction KIT (610.21, Dynal Biotech, Oslo, Norway) following the manufacturer's instructions. After 5 min incubation in 50 μL lysis buffer, pre-washed Dynabeads were added and samples were further incubated for 5 min. PolyA RNA attached to the Dynabeads was extracted with a magnet and suspended in 50 μL washing buffers A and B (twice, each). Thereafter, the polyA RNA was finally eluted in 30 μL of 10 mM Tris-HCl. Immediately after extraction, the reverse transcription (RT) reaction was carried out following the manufacturer's instructions (Bioline, Ecogen, Madrid, Spain) using polyT primer, random primers and moloney murine leukemia virus reverse transcriptase enzyme in a total volume of 40 μL to prime the RT reaction and to produce cDNA. Tubes were heated to 70 °C for 5 min to denature the secondary RNA structure and then the RT mix was completed with the addition of 100 units of reverse transcriptase. They were then incubated at 42 °C for 60 min to allow the reverse transcription of RNA, followed by incubation at 70 °C for 10 min to denature the RT enzyme. The quantification of all mRNA transcripts was performed by real-time quantitative (q)RT-PCR. For qPCR, three groups of cDNA per experimental treatment, each obtained from 10 embryos, were used with two repetitions for all the studied genes. Experiments were conducted to compare relative levels of each transcript with those of histone H2Az (*H2AFZ*) in each sample. The PCR was performed by adding a 2 μL aliquot of each sample to the final PCR mix (20 μL) containing specific primers to amplify *H2AFZ*, BCL2-associated X protein (*BAX*), BCL2-like protein 1 (*BCL2L1*), gap junction protein, alpha 1, 43 kDa (*GJA1*), DNA cytosine-5-methyltransferase 3 alpha (*DNMT3A*), mitochondrial polymerase gamma (*POLGA*), mitochondrial transcription factor A (*TFAM*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), insulin-like growth factor binding protein 2 (*IGFBP2*) and homeodomain transcription factor Nanog (*NANOG*). The information of the primers used is shown in Table 2.

The comparative cycle threshold (C_T) method was used to quantify expression levels [32,33]. Quantification was normalised against that of the endogenous control, *H2AFZ*. Fluorescence was acquired in each cycle to determine the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above background for each sample. Within this region of the amplification curve, a difference of one cycle is equivalent to a doubling of the amplified PCR product. According to the comparative C_T method, the ΔC_T value was determined by subtracting the *H2AFZ* C_T value for each sample from the C_T value for each gene in the sample. Calculation of $\Delta\Delta C_T$ involved the use of the highest sample ΔC_T value (*i.e.* the sample with the lowest target expression) as an arbitrary constant to subtract from all other ΔC_T sample values. Fold changes in the relative gene expression of the target gene were determined using formula $2^{-\Delta\Delta CT}$.

2.7. Statistical analysis

The data were analysed using one-way ANOVA. The *post hoc* Tukey test was used to detect differences between treatments when significant differences were found. Variables expressed as percentages were arcsine-transformed to satisfy parametric assumptions when needed. When data did not fulfil the assumptions of the model after transformation, Kruskal-Wallis ANOVA was used, followed by Mann-Whitney *U* test to detect differences between

Table 2
Information of the primers used for real time RT-PCR.

Gene	Primer sequence (5′-3′)	Fragment Size (bp)	Gene Bank Accession No.
H2AFZ	AGGACGACTAGCCATGGACGTGTG CCACCACCAGCAATTGTAGCCTTG	212	NM_016750
BAX	CTGGAGCAGGTGCCTCAGGA ATCTCGAAGGAAGTCCAGCGTC	300	XM_003355974.1
BCL2L1	GGAGCTGGTGGTTGACTTTC CTAGGTGGTCATTCAGGTAAG	518	AF216205
DNMT3A	CTGAGAAGCCCAAGGTCAAG CAGCAGATGGTGCAGTAGGA	238	CJ026384
GAPDH	ACCCAGAAGACTGTGGATGG ACGCCTGCTTCACCACCTTC	247	BC102589
GJA1	TGCCTTTCGTTGTAACACTCA AGAACACATGAGCCAGGTACA	142	AY382593.1
IGFBP2	AGACAATGGCGACGATGCTGAG TGCTCCGTGACTTTCTCCCG	150	HQ432890.1
NANOG	CCAGCGAATGAAATGTAAGAGGTG TGGTGGTAGGAATAGAAGCCCG	110	FJ882402.1
POLGA	ACTGGCTGGACATCAGCAGT GACAGTACTGCATCAGGTCC	194	AK291281.1
TFAM	GGCAGACTGGCAGGTGTA CGAGGTCTTTTTGGTTTTCCA	164	AF311909

experimental groups. Probability values of less than 0.05 were considered significant. Statistical analyses were performed using Statistica (Statsoft, Inc. 2001. STATISTICA, data analysis software system, version 6; www.statsoft.com).

3. Results

Outcomes from *in vitro* culture are shown in Table 3. There was no effect of treatment on cleavage rate ($P > 0.05$) on day 2 of culture. At the beginning of treatment (24 h post-fertilisation) some of the zygotes had already undergone cleavage. When cleavage rate was corrected, having taken into account the number of embryos that were present at the beginning of the treatment exposure per experimental group, the cleavage rate remained similar between treatments ($P > 0.05$). Blastocyst yield was not affected by plasma addition from ACTH-treated sows in comparison to controls ($P > 0.05$). The source of protein (*i.e.* plasma or BSA) did not affect the *in vitro* outcomes ($P > 0.05$).

Blastocyst classification according to the degree of development was not significantly influenced by treatment ($P > 0.05$) (data not shown). Subjective quality scores ranging from grade 1 (best quality) to 4 (worst quality) did not differ between treatments when all the resulting blastocysts were included in the statistical analysis ($P = 0.07$). However, when only expanded blastocysts (and onwards) were included in the analysis, the quality score was significantly lower (*i.e.* better quality) ($P < 0.05$) in the group supplemented with plasma from non-ACTH-treated sows (Table 4).

The relative mRNA expression of nine genes analysed to evaluate blastocyst quality remained unaffected by the altered hormonal environment provided by plasma from ACTH-treated sows or by the protein source present in the culture medium ($P > 0.05$) (Fig. 1).

4. Discussion

Reproduction is an important function that may be affected by psychosocial stress. The timing and duration of stress in relation to the reproductive female cycle might be crucial for having consequences on reproductive performance. In this study, we assessed the effect of an altered microenvironment, caused by blood plasma collected after ACTH administration, on early embryonic development. Regardless of the provided *in vitro* environment, embryo yields were similar in all groups. This was confirmed by the absence of differences under the different experimental conditions in mRNA transcription profile of various analysed genes that are important for embryo development. Our results showed that a brief exposure to plasma from ACTH-treated sows may not exert a harmful effect on pre-implantation embryonic development, but this extrapolation to the in *vivo* situation should be done cautiously and further explored.

Using an *in vivo-in vitro* system as a model to study the effect of simulated psychosocial stress (by blood plasma collected under ACTH administration) on gametes and embryo development, we aimed to implement a better model than the sole inclusion of corticoids in culture media to study the possible influences of stress on gamete/embryo quality. Nonetheless, the proposed model does not perfectly mimic the *in vivo* situation since oviductal fluid is a complex fluid and differs in some features from blood plasma

Table 3
Results of *in vitro* culture (mean ± SEM).

Treatment	Cleavage rate		Corrected Cleavage rate		Blastocyst rate	
	n	% (No. embryos /No. presumptive zygotes)	n	% (No. embryos /No. presumptive zygotes)	n	% (No. blastocysts/No. embryos)
ACTH	14	54.0 ± 2.9 (340/626)	14	49.1 ± 2.9 (281/571)	13	19.0 ± 2.3 (64/319)
Control BSA	14	54.0 ± 3.2 (313/580)	14	49.4 ± 3.1 (261/528)	13	20.1 ± 2.8 (61/296)
Control No ACTH	14	55.7 ± 2.9 (358/637)	14	50.8 ± 2.8 (300/581)	13	20.7 ± 2.0 (64/337)

Cleavage rate was calculated as the percentage of embryos found on day 2 of culture over the total of presumptive zygotes in culture. Blastocyst rate was calculated as the percentage of blastocysts found on day 7 of culture over the total of embryos. Statistical analysis was done using one way-ANOVA. No significant differences were found between treatments ($P > 0.05$). The number of replicates is indicated (n).

Table 4
Blastocyst quality (mean ± SEM).

Treatment	n	Blastocyst quality (all blastocysts)	n	Blastocyst quality (expanded blastocysts)
ACTH	61	2.16 ± 0.11	34	1.74 ± 0.09[a]
Control BSA	58	2.22 ± 0.10	28	1.77 ± 0.11[a]
Control No ACTH	61	1.97 ± 0.11	32	1.45 ± 0.06[b]

Blastocyst quality on day 7 of *in vitro* culture. Statistical analysis was done using Kruskal-Wallis ANOVA and Mann-Whitney *U* test. Different letters represent significant differences between treatments ($P < 0.05$). The number of blastocysts analysed are indicated (n).

[34–36]; although the concentration of systemic compounds such as progesterone can influence oviductal fluid composition [37]. In this regard, progesterone was elevated in ACTH-treated sows and that seemed to alter the intraluminal environment of the oviduct reservoir [38]. Plasma proteins account for most of the protein content of oviductal fluid, their concentration being 5–10% of that found in serum [35], which is approximately provided in our *in vitro* culture system with 10% blood plasma supplementation in culture medium. Earlier studies have evaluated the supplementation of culture media with glucocorticoids [12–16], but this scenario could be far from reflecting the *in vivo* situation. Conversely, it is difficult to perform studies in *in vivo* conditions. Previous reports attempted to assess the effect of ACTH administration to mimic stress on the sow reproduction at different times of the oestrus cycle (reviewed by Ref. [11]). Important findings derived from these studies showed that ACTH administration caused alterations in the hormonal profile and disturbances in sexual display [19], modifications in the oviductal intraluminal milieu [38] and fewer ova/embryos were retrieved in the ACTH-group compared to controls [39]. In the latter study, no differences were found in cleavage rates in the ACTH and control group or on the features of 4-cell stage embryos, but the developmental competence to the blastocyst stage was not investigated. Additionally, in these *in vivo* studies, to what extent the effects on embryo development may arise from the potential influence of stress on oocyte quality rather than a direct effect on the embryo could not be addressed [39]. To address these questions, we explored the effect of an altered hormonal environment, caused by ACTH administration at critical events of reproduction. We have previously described that a brief exposure to plasma from ACTH-treated sows during *in vitro* maturation may induce alterations in mitochondrial and actin patterns in oocytes, but did not preclude embryo development [18]. However, fertilisation was very sensitive and detrimentally affected by plasma from ACTH-administrated sows compared to controls [17]. In the present study, we further explored the effect of an altered hormonal environment, using plasma from ACTH-treated sows, on embryo quality, specifically during a critical period of porcine embryo development. This time frame from 24 to 48 h of culture corresponds to the third cell cycle (4-cell stage) when the major transcriptional activation of the embryo genome takes place and the developmental block occurs [20,21,23]. *In vivo*, porcine embryos can be recovered around 14–16 h (timing of first cleavage) after ovulation and embryos enter the uterus at the 4-cell stage around 46 h after ovulation [40]. This chronology of development would fit approximately with plasma collected at 12 ± 2 h post-ovulation from ACTH-treated or non-treated sows [19] provided in the present study. However, *in vitro* development of pig embryos displays some delay in comparison to their *in vivo* counterparts [22,41]. Most pig embryos are found at the 4-cell stage from 24 to 48 h of *in vitro* culture [22]. Thus, this interval was selected for plasma supplementation from ACTH-treated or control sows in the current work.

Plasma collected from ACTH-treated sows had a ~15-fold greater

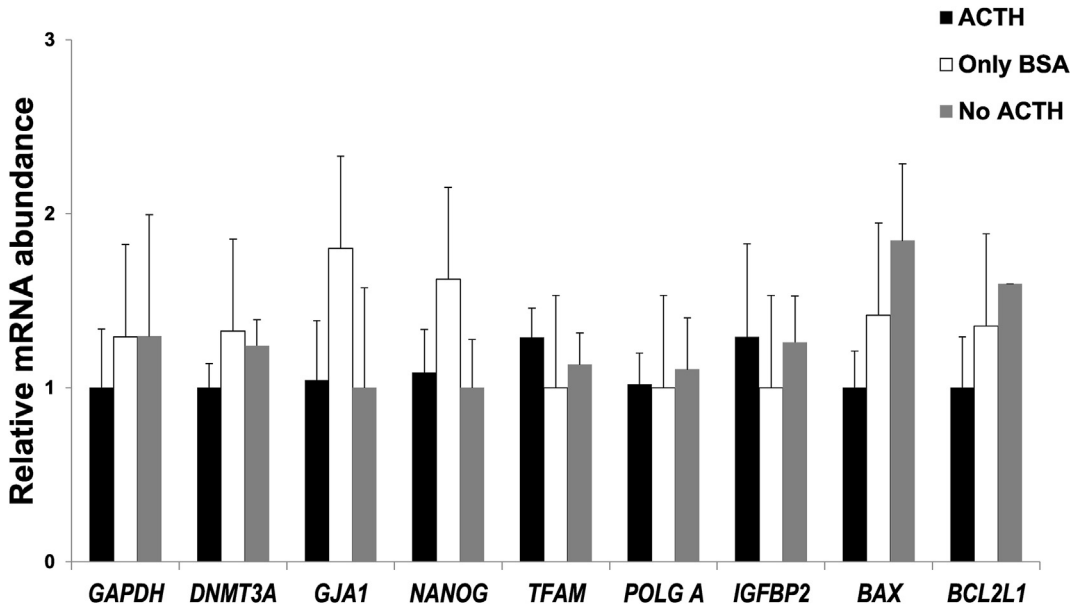

Fig. 1. Relative mRNA expression of various important genes for embryo development assessed by qRT-PCR in porcine blastocysts. Blastocysts were obtained from *in vitro* matured and fertilised oocytes. Presumptive zygotes were exposed, during the first 24 h of *in vitro* culture, to plasma collected after ovulation from ACTH-treated sows, control non-ACTH-treated sows or control without plasma (BSA). Genes analysed included: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*); DNA cytosine-5-methyltransferase 3 alpha (*DNMT3A*); gap junction protein, alpha 1, 43 kDa (*GJA1*); homeodomain transcription factor Nanog (*NANOG*); mitochondrial transcription factor A (*TFAM*); mitochondrial polymerase gamma (*POLGA*); insulin-like growth factor binding protein 2 (*IGFBP2*) and; BCL2-associated X protein (*BAX*), BCL2-like protein 1 (*BCL2L1*). Statistical analysis was done using one way-ANOVA. Data are represented as mean ± SEM. No significant differences were found between treatments ($P > 0.05$).

concentration of cortisol and a two-fold increase in progesterone levels compared to plasma from control sows. However, this altered hormonal environmental did not have any impact on cleavage rates and blastocyst yield in comparison to non-ACTH or BSA controls. Moreover, the proportion of blastocysts at different stages of development did not differ regardless of treatment. An explanation to the lack of differences in cleavage or blastocyst rates would be that in our work, the effect of an altered environment provided by blood plasma supplementation on embryo culture might have been underestimated, due to a dilution effect, in comparison to the environment within the oviduct under ACTH exposure. However, similar to the present findings, repeated ACTH administration *in vivo* did not affect cleavage rates or embryo development since the features of the 4-cell embryos were similar to those of the control sows [39].

It was previously described that the addition of fetal calf serum to the embryo culture medium did not support development of porcine 1 to 2-cell embryos [42]. This effect was not observed in the present study, but it could be attributed to differences in methodology or likely, the composition of porcine plasma collected at an equivalent time point of the oestrus cycle may differ to fetal calf serum and be able to support embryo development in the pig. One caveat to the use of plasma, including our present study, is that plasma is an undefined medium and its composition may be affected by multiple conditions. Plasma samples collected from ACTH-stimulated and non-ACTH-stimulated sows were partially characterized since only the main reproductive hormones were measured. Thus, we cannot completely rule out whether other biological factors were present in blood samples that may differ after ACTH administration and which could have impacted the observed results.

Blastocyst quality according to morphologic criteria was similar between treatments, when all the resulting blastocysts were compared. However, when only expanded (and onwards) blastocysts were included in the statistical analysis, the quality of the blastocysts obtained in the plasma from the non-ACTH group was greater than in the other two treatments. Blastocyst quality was also assessed by analysing several important genes for embryo development which are considered as markers of blastocyst quality [24,25,43]. The set of genes analysed were involved in compaction and blastocyst formation (*GJA1*); related to metabolism (*GAPDH*); growth arrest and apoptosis (*BAX*, pro-apoptotic and *BCL2L1*, anti-apoptotic); *de novo* methylation (*DNMT3A*); related to pluripotency (*NANOG*); mitochondrial function (*POLGA* and *TFAM*) and; cell proliferation and differentiation (*IGF2BP*). None of the studied genes differed in transcription levels between treatments. Therefore, the effect of simulated psychosocial stress applied only for 24 h around the 4-cell stage may not be sufficient to cause any impact on embryo development and embryo quality. The major activation of the embryonic genome occurs during the 4-cell stage in the pig, but it is unknown if it is preceded by a minor activation as demonstrated in cattle. It is likely that the effect of the altered environment provided by the exposure to plasma from ACTH-treated sows was too brief to induce changes in gene expression patterns. Further experiments using microarrays to evaluate global gene expression profiles of the resulting blastocysts might help elucidating underlying mechanisms of the potential impact of stressful situations on embryo development.

Moreover, the different source of proteins did not significantly affect the mRNA transcription abundance in any of the analysed genes in the current study. Fetal calf serum affected apoptosis-related gene expression in porcine parthenotes, enhancing the expression of the pro-apoptotic gene BAK and decreasing the expression of the anti-apoptotic gene *BCL-XL* (officially *BCL2L1*) [44]. The presence of serum during bovine embryo culture negatively affected blastocyst quality inducing differences in relative mRNA abundance in genes related to apoptosis, oxidative stress, gap junctions, differentiation and implantation [45]. Although the differences were not statistically different in our data, there were different trends in apoptosis-related genes *BAX* and *BCL2L1* regarding the source of protein. In this regard, the control group supplemented with BSA and the ACTH-plasma group tended to express lower levels of both genes compared to the control (non-ACTH) plasma group. Therefore, the plasma from the ACTH-group, with elevated levels of cortisol, may have reduced the expression of the apoptotic *BAX* gene, although the *BAX*/*BCL2L1* ratio was similar in all groups. Studies in cows evaluating the effect of dexamethasone on apoptosis in early embryos, found no effect of the synthetic glucocorticoid on cleavage, blastocysts or apoptosis rates, but had a beneficial effect on embryo developmental kinetics and cell proliferation [46]. This is in contrast with studies in mice which revealed that exposure to mothers to restraint stress, repeatedly from day 1–4 of pregnancy, caused a reduction in blastocyst cell numbers, with alterations in the inner cell mass/trophectoderm ratio compared to control dams [47]. In this study, gene expression or apoptosis rates were not evaluated in early embryos, but the number of implantation sites was reduced in females subjected to restraint stress versus non-stressed mothers. Additionally, some detrimental effects of stress were observed later into postnatal life of their offspring [47].

Gap junction protein, alpha 1, 43 kDa (*GJA1*) plays an essential role in the maintenance of compaction and subsequent blastocyst formation [48]. Reduced *GJA1* (connexin 43) expression has been related to low quality blastocysts produced in presence of serum [45,49]. *NANOG* is a transcription factor which is specific to maintain the pluripotency. The transcription levels of *NANOG* were significantly increased in *in vivo* derived embryos compared to IVF or parthenogenetic blastocysts in the pig [43]. In line with this, plasma-supplemented groups in the present study showed a downward trend both in *GJA1* and *NANOG* transcription levels which might indicate compromised embryo competence. A higher number of blastocysts would be needed to be analysed in order to confirm these tendencies.

In conclusion, an altered hormonal microenvironment, caused by blood plasma collected after adrenocorticotropic administration, applied briefly for a period of 24 h to zygotes/early cleaved porcine embryos was not sufficient to exert harmful effects on pre-implantation embryonic development. Cleavage rate, blastocyst rate and blastocyst morphology were not affected by the altered milieu provided by plasma supplementation from ACTH-sows. This was confirmed by the absence of differences in mRNA expression profile of various analysed genes important for embryo development. Our in *vitro* results indicate that early post-fertilisation events may not be as sensitive as oocyte maturation and fertilisation to a brief exposure to plasma from ACTH-treated sows [17,18].

CRediT authorship contribution statement

Raquel González: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft. **Eva Pericuesta:** Conceptualization, Methodology, Writing - review & editing. **Alfonso Gutiérrez-Adán:** Conceptualization, Methodology, Writing - review & editing. **Ylva C.B. Sjunnesson:** Conceptualization, Methodology, Investigation, Writing - review & editing.

Acknowledgements

The authors would like to thank Ickholmen abattoir for providing the ovaries and Monica Nilsson for her help collecting them. This manuscript was language reviewed by the English

Editing Service available at SLU (CBG Konsult & Information AB). This work was supported by The Swedish Research Council Formas (Project number 221-2007-374 and 221-2010-549); the Spanish Ministry of Science and Innovation (Grants AGL2009-11358 and RTI2018-093548-B-I00); and a travel grant from the Swedish Farmers' Foundation.

References

[1] Kalantaridou SN, Makrigiannakis A, Zoumakis E, Chrousos GP. Stress and the female reproductive system. J Reprod Immunol 2004;62:61–8.
[2] Moberg GP. Biological response to stress: implications for animal welfare. In: Moberg GP, Mench JA, editors. The biology of animal stress Basic principles and implications for animal welfare. New York, USA: CABI publishing; 2000. p. 1–22.
[3] Ralph CR, Lehman MN, Goodman RL, Tilbrook AJ. Impact of psychosocial stress on gonadotrophins and sexual behaviour in females: role for cortisol? Reproduction 2016;152:R1–r14.
[4] Liu YX, Cheng YN, Miao YL, Wei DL, Zhao LH, Luo MJ, et al. Psychological stress on female mice diminishes the developmental potential of oocytes: a study using the predatory stress model. PloS One 2012;7:e48083.
[5] Zhang SY, Wang JZ, Li JJ, Wei DL, Sui HS, Zhang ZH, et al. Maternal restraint stress diminishes the developmental potential of oocytes. Biol Reprod 2011;84:672–81.
[6] Zhou P, Lian HY, Cui W, Wei DL, Li Q, Liu YX, et al. Maternal-restraint stress increases oocyte aneuploidy by impairing metaphase I spindle assembly and reducing spindle assembly checkpoint proteins in mice. Biol Reprod 2012;86(83):1–14.
[7] Campagne DM. Should fertilization treatment start with reducing stress? Hum Reprod 2006;21:1651–8.
[8] Cwikel J, Gidron Y, Sheiner E. Psychological interactions with infertility among women. Eur J Obstet Gynecol Reprod Biol 2004;117:126–31.
[9] Arey DS. Time course for the formation and disruption of social organisation in group-housed sows. Appl Anim Behav Sci 1999;62:199–207.
[10] Tsuma VT, Einarsson S, Madej A, Kindahl H, Lundeheim N, Rojkittikhun T. Endocrine changes during group housing of primiparous sows in early pregnancy. Acta Vet Scand 1996;37:481–9.
[11] Einarsson S, Brandt Y, Rodriguez-Martinez H, Madej A. Conference lecture: influence of stress on estrus, gametes and early embryo development in the sow. Theriogenology 2008;70:1197–201.
[12] Andersen CY. Effect of glucocorticoids on spontaneous and follicle-stimulating hormone induced oocyte maturation in mouse oocytes during culture. J Steroid Biochem Mol Biol 2003;85:423–7.
[13] Gonzalez R, Ruiz-Leon Y, Gomendio M, Roldan ER. The effect of glucocorticoids on mouse oocyte *in vitro* maturation and subsequent fertilization and embryo development. Toxicol Vitro 2010;24:108–15.
[14] Gonzalez R, Ruiz-Leon Y, Gomendio M, Roldan ER. The effect of glucocorticoids on ERK-1/2 phosphorylation during maturation of lamb oocytes and their subsequent fertilization and cleavage ability *in vitro*. Reprod Toxicol 2010;29:198–205.
[15] Yang JG, Chen WY, Li PS. Effects of glucocorticoids on maturation of pig oocytes and their subsequent fertilizing capacity *in vitro*. Biol Reprod 1999;60:929–36.
[16] Van Merris V, Van Wemmel K, Cortvrindt R. *In vitro* effects of dexamethasone on mouse ovarian function and pre-implantation embryo development. Reprod Toxicol 2007;23:32–41.
[17] Gonzalez R, Kumaresan A, Bergqvist AS, Sjunnesson YC. Blood plasma collected after adrenocorticotropic hormone administration during the preovulatory period in the sow negatively affects *in vitro* fertilization by disturbing spermatozoa function. Theriogenology 2015;83:1128–39.
[18] Gonzalez R, Sjunnesson YC. Effect of blood plasma collected after adrenocorticotropic hormone administration during the preovulatory period in the sow on oocyte *in vitro* maturation. Theriogenology 2013;80:673–83.
[19] Brandt Y, Lundeheim N, Madej A, Rodriguez-Martinez H, Einarsson S. Effects of ACTH injections during estrus on concentrations and patterns of progesterone, estradiol, LH, and inhibin alpha and time of ovulation in the sow. Domest Anim Endocrinol 2007;32:122–37.
[20] Hyttel P. Electron microscopy of mammalian oocyte development, maturation and fertilization. In: Tosti E, Boni R, editors. Oocyte maturation and fertilization: a long history for a short event. USA: Bentham Science Publishers Ltd.; 2011. p. 1–37.
[21] Hyttel P, Laurincik J, Rosenkranz C, Rath D, Niemann H, Ochs RL, et al. Nuclear proteins and ultrastructure in preimplantation porcine embryos developed *in vivo*. Biol Reprod 2000;63:1848–56.
[22] Wang WH, Abeydeera LR, Han YM, Prather RS, Day BN. Morphologic evaluation and actin filament distribution in porcine embryos produced *in vitro* and *in vivo*. Biol Reprod 1999;60:1020–8.
[23] Telford NA, Watson AJ, Schultz GA. Transition from maternal to embryonic control in early mammalian development: a comparison of several species. Mol Reprod Dev 1990;26:90–100.
[24] Gutiérrez-Adán A, Rizos D, Fair T, Moreira PN, Pintado B, de la Fuente J, et al. Effect of speed of development on mRNA expression pattern in early bovine embryos cultured *in vivo* or *in vitro*. Mol Reprod Dev 2004;68:441–8.
[25] Miles JR, Blomberg LA, Krisher RL, Everts RE, Sonstegard TS, Van Tassell CP, et al. Comparative transcriptome analysis of *in vivo*- and *in vitro*-produced porcine blastocysts by small amplified RNA-serial analysis of gene expression (SAR-SAGE). Mol Reprod Dev 2008;75:976–88.
[26] Simonsson A. Näringsrekommendationer och fodermedelstabeller till svin [Nutrient and metabolizable energy recommendations for swine, in Swedish]. Swedish University of Agricultural Sciences, SLU Info Rapporter, Husdjur, p 751994.
[27] Pursel VG, Johnson LA. Freezing of boar spermatozoa; fertilizing capacity with concentrated semen and a new thawing procedure. J Anim Sci 1975;40:99–102.
[28] Sjunnesson YC, Morrell JM, Gonzalez R. Single layer centrifugation-selected boar spermatozoa are capable of fertilization *in vitro*. Acta Vet Scand 2013;55:20.
[29] Petters RM, Wells KD. Culture of pig embryos. J Reprod Fertil Suppl 1993;48:61–73.
[30] Yoshioka K, Suzuki C, Tanaka A, Anas IM, Iwamura S. Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. Biol Reprod 2002;66:112–9.
[31] Gordon I. Laboratory production of cattle embryos. In: Biotechnology in agriculture series No. 27 United Kingdom. second ed. CABI Publishing; 2003.
[32] Bermejo-Alvarez P, Rizos D, Rath D, Lonergan P, Gutierrez-Adan A. Epigenetic differences between male and female bovine blastocysts produced *in vitro*. Physiol Genom 2008;32:264–72.
[33] Schmittgen TD, Lee EJ, Jiang J, Sarkar A, Yang L, Elton TS, et al. Real-time PCR quantification of precursor and mature microRNA. Methods 2008;44:31–8.
[34] Hugentobler SA, Humpherson PG, Leese HJ, Sreenan JM, Morris DG. Energy substrates in bovine oviduct and uterine fluid and blood plasma during the oestrous cycle. Mol Reprod Dev 2008;75:496–503.
[35] Leese HJ. The formation and function of oviduct fluid. J Reprod Fertil 1988;82:843–56.
[36] Nichol R, Hunter RH, Gardner DK, Leese HJ, Cooke GM. Concentrations of energy substrates in oviductal fluid and blood plasma of pigs during the periovulatory period. J Reprod Fertil 1992;96:699–707.
[37] Hugentobler SA, Sreenan JM, Humpherson PG, Leese HJ, Diskin MG, Morris DG. Effects of changes in the concentration of systemic progesterone on ions, amino acids and energy substrates in cattle oviduct and uterine fluid and blood. Reprod Fertil Dev 2010;22:684–94.
[38] Brandt Y, Lang A, Rodriguez-Martinez H, Madej A, Einarsson S. Impact of ACTH during oestrus on the ultrastructure of the spermatozoa and their environment in the tubal reservoir of the postovulatory sow. Anim Reprod Sci 2006;93:231–45.
[39] Brandt Y, Madej A, Rodriguez-Martinez H, Einarsson S. Effects of exogenous ACTH during oestrus on early embryo development and oviductal transport in the sow. Reprod Domest Anim 2007;42:118–25.
[40] Hunter RH. Chronological and cytological details of fertilization and early embryonic development in the domestic pig, Sus scrofa. Anat Rec 1974;178:169–85.
[41] Funahashi H, Stumpf TT, Terlouw SL, Cantley TC, Rieke A, Day BN. Developmental ability of porcine oocytes matured and fertilized *in vitro*. Theriogenology 1994;41:1425–33.
[42] Koo DB, Kim NH, Lee HT, Chung KS. Effects of fetal calf serum, amino acids, vitamins and insulin on blastocoel formation and hatching of *in vivo* and IVM/IVF-derived porcine embryos developing *in vitro*. Theriogenology 1997;48:791–802.
[43] Magnani L, Cabot RA. *In vitro* and *in vivo* derived porcine embryos possess similar, but not identical, patterns of Oct4, Nanog, and Sox2 mRNA expression during cleavage development. Mol Reprod Dev 2008;75:1726–35.
[44] Cui XS, Jeong YJ, Lee HY, Cheon SH, Kim NH. Fetal bovine serum influences apoptosis and apoptosis-related gene expression in porcine parthenotes developing *in vitro*. Reproduction 2004;127:125–30.
[45] Rizos D, Gutierrez-Adan A, Perez-Garnelo S, De La Fuente J, Boland MP, Lonergan P. Bovine embryo culture in the presence or absence of serum: implications for blastocyst development, cryotolerance, and messenger RNA expression. Biol Reprod 2003;68:236–43.
[46] Santana PP, Carvalho CM, da Costa NN, Silva TV, Ramos PC, Cordeiro MS, et al. Effect of dexamethasone on development of in vitro-produced bovine embryos. Theriogenology 2014;82:10–6.
[47] Burkus J, Kacmarova M, Kubandova J, Kokosova N, Fabianova K, Fabian D, et al. Stress exposure during the preimplantation period affects blastocyst lineages and offspring development. J Reprod Dev 2015;61:325–31.
[48] Lee S, Gilula NB, Warner AE. Gap junctional communication and compaction during preimplantation stages of mouse development. Cell 1987;51:851–60.
[49] Wrenzycki C, Herrmann D, Carnwath JW, Niemann H. Expression of the gap junction gene connexin43 (Cx43) in preimplantation bovine embryos derived *in vitro* or *in vivo*. J Reprod Fertil 1996;108:17–24.