Combined synchronization and superovulation treatments negatively impact embryo viability possibly by the downregulation of WNT/β-catenin and Notch signaling genes in the porcine endometrium

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Abstract

The combination of estrus synchronization and superovulation treatments introduces molecular modifications whose effects are yet to be disclosed. Here, reproductive parameters and gene expression changes in ovaries and endometrium were explored on day 6 after artificial insemination (AI), when synthetic progesterin altrenogest (ALT) was combined with gonadotropins. Sows were administered ALT for 7 d beginning on the day of weaning and superovulated with equine chorionic gonadotropin (eCG) 24 h later and human chorionic gonadotropins (hCG) at the onset of estrus (SS-7 group; n = 6). The controls were either superovulated sows with eCG 24 h postweaning and hCG at the onset of estrus (SC group; n = 6) or sows with postweaning spontaneous estrus (NC group; n = 6). Ovary examination and embryo and tissue collection were performed in all sows via laparotomy on day 6 post-AI. RNA-Seq was conducted to analyze differentially expressed genes (DEGs) between groups. Statistical analysis of the reproductive parameters was conducted with ANOVA and Tukey post hoc tests. DEGs were analyzed with an ANOVA (fold changes ≥2 or ≤2, P value <0.05). Hormonal treatments almost doubled (P < 0.03) the number of corpora lutea (39.8 ± 10.2 and 38.3 ± 11.1 in SS-7 and SC sows, respectively) compared with that in the NC group (23.1 ± 3.8). In contrast, embryo viability significantly decreased (P < 0.003) in response to SS-7 treatment (75.1 ± 15.2%) compared to SC and NC groups (93.8 ± 7.6% and 91.8 ± 6.9%, respectively). RNA-Seq analyses revealed 675 and 1,583 DEGs in the SS-7 group compared to both SC and NC groups in endometrial and ovarian samples, respectively. Interestingly, many genes with key roles in the Wnt/β-catenin and Notch signaling pathways were differentially expressed in SS-7 sows relative to SC and NC groups (e.g., Ctnnb1, Myc, Gli3, Scyl2, Ccny, Daam1, Ppm1n, Rbpj, and Usp8). A key finding in this study was the downregulation of β-catenin (Ctnnb1) gene expression in the SS-7 endometrium, suggesting that this treatment influences embryo–uterine dialogue by triggering a cascade of events leading to embryo maldevelopment. These data explain the proliferative defects in SS-7 embryos and suggest a novel mechanism of a porcine embryo–maternal crosstalk.

Lay Summary

Methods for porcine superovulation (increasing the number of ovulated oocytes per cycle) and estrus synchronization (grouping estrus sows on the same day) are available for assisted reproductive technologies, using hormonal treatments. The main goal of the present study was to understand how hormones used for these purposes influence gene expression patterns in the female reproductive tract (ovaries and endometrium). We observed that hormonal treatments (synchronization combined with superovulation) have the potential to alter ovarian and endometrial gene expression patterns, triggering improper follicle development and oocyte growth, and leading to abnormal embryonic development before implantation. Genes involved in two key metabolic pathways for embryo development (Wnt/β-catenin and Notch signaling pathways) were dysregulated in reproductive tissues.

Key words: altrenogest, embryo death, pathways, sequencing, synchronization

Abbreviations: AI, artificial insemination; ALT, altrenogest; ART, assisted reproductive technologies; cDNA, complementary DNA; DAVID, Database for annotation, visualization and integrated discovery; DEG, differentially expressed gene; DICE, Database of immune cell expression; eCG, equine chorionic gonadotropin; ET, embryo transfer; FC, fold change; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropins; KEGG, Kyoto Encyclopedia of Genes and Genomes; LH, luteinizing hormone; mRNA, messenger RNA; NC, natural control; PCR, polymerase chain reaction; RNA, ribonucleic acid; SC, superovulation control; SRA, Sequence Read Archive; SS-7, synchronization and superovulation; STAR, spliced transcripts alignment to a reference
Introduction

Effective methods for superovulation (mainly in weaned sows; Angel et al., 2014; Martinez et al., 2016) and estrus synchronization in cyclic pigs (mainly gilts) (Martinet-Botté et al., 1985; Wang et al., 2018) are available. Superovulation treatment with gonadotropins is widely used for assisted reproductive technologies (ART), including embryo transfer (ET) because it stimulates follicle growth and development and promotes oocyte maturation and ovulation rates (Cooke and Lenton, 1994; Insler and Lunefeld, 1996). Superovulation treatment with equine (eCG) and human (hCG) chorigonic gonadotropins supplied 24 h postweaning and at the beginning of estrus, respectively, significantly increases the ovulation rate and the percentage of viable embryos compared with non-superovulated sows (Angel et al., 2014; Gonzalez-Ramiro et al., 2021). Moreover, superovulated embryos present similar in vivo development capacity after ET as control embryos (Angel et al., 2014). The optimum technique for simultaneously synchronizing estrus and superovulating a group of sows in current ET programs is weaning combined with superovulation treatments (Knox, 2015). However, these programs often require large cohorts, implying that multiple weaning must be combined to obtain enough sows. In this regard, various studies have shown that a short estrus synchronization treatment postweaning with the progesterin altrenogest (ALT) has the potential to modify weaning to estrus interval (Kraeling and Webel, 2013) and positively affect embryonic and fetal survival rates (Patterson et al., 2008) without affecting the ovulation rate (van Leeuwen et al., 2010). Short-term ALT treatment suppresses follicular development by negative feedback on follicle-stimulating hormone (FSH) and luteinizing hormone (LH) release (Lopes et al., 2017), preventing estrus from spontaneously occurring (De Rensis et al., 2017) outside a window of 5 to 7 d post-ALT. However, despite this apparent effectiveness, if combined with a superovulation treatment, it dramatically reduces pregnancy rates and embryo production efficiency compared with those achieved in superovulated or hormonal non-treated sows (Gonzalez-Ramiro et al., 2021).

It is known that hormones play a significant role in regulating reproductive physiology via the regulation of gene expression. mRNAs encoding hormone receptors are usually modulated by their specific hormones via autoregulatory feedback (Béchet, 1986; Ing, 2005). Understanding how hormones influence mRNA stability and expression when combined synchronization and superovulation treatments are used might generate new methods to control ART and reproductive physiology in animals. This study aimed to determine how gene expression changes in ovaries and endometrium on day 6 after artificial inseminations (AIs) in response to a short-term protocol for synchronization of estrus combined with conventional superovulation in weaned sows.

Materials and Methods

Ethics

The study was approved by the Ethics Committee for experiments with animals at the University of Murcia (Code: 486/2018) and followed the Directive 2010/63/EU for animal experimentation.

Experimental design

We evaluated the gene expression patterns in endometria and ovaries after short-term ALT (Regumate, Merck Sharp & Dohme Animal Health, S.L., Salamanca, Spain) treatment postweaning followed by superovulation compared to the following two control sets: a conventional superovulation procedure and spontaneous estrus. All sows used in the experiment presented a body condition score (0 to 5 scale) and a reproductive index (fertility and prolificacy history) greater than 2.6, 90%, and 10.5 piglets, respectively. Sows were treated with ALT for 7 d beginning on the day of weaning and superovulated with eCG (Foligon, Intervet, Boxmeer, The Netherlands) 24 h after the last ALT administration and hCG at the onset of estrus (Synchronization and Superovulation, SS-7 group; n = 6). Superovulated sows treated with eCG 24 h after weaning and hCG (Veterin Corion, Divasa, Farmavic S.A., Barcelona, Spain) at the beginning of estrus (Superovulation Control, SC group; n = 6) and sows with postweaning spontaneous estrus (Natural Control, NC; n = 6) constituted the two control groups.

Sows in estrus were inseminated with sperm doses from the same boar and laparotomized on day 6 (day 0 = onset of estrus) for ovary examination and embryo collection and evaluation. Viable embryos, unfertilized oocytes, and degenerated embryos were noted, and the recovery (structures retrieved or the number of corpora lutea on the ovaries) and fertilization (viable embryos or number of structures retrieved) rates were also recorded. Samples from the uterine horn and ovary were collected from each sow, directly snap-frozen in liquid nitrogen, and kept at −80 °C until further use for transcriptome analysis. A total of 30 samples were analyzed corresponding to 5 samples from ovaries and 5 samples from endometrial tissue from each of the three groups studied.

Animals

Multiparous Landrace × Large-White sows (3 to 7 parities) with a lactation period of 21 to 24 d were randomly selected on the day of weaning. All sows had a previous fertility rate and prolificacy greater than 85% and 10.5 piglets born, respectively, and a body condition score ranging from 2.75 to 3.25 on a five-point scale on the day of weaning, without differences between groups. Animals were individually allocated under controlled environmental conditions in a commercial farm (Agropor SL, Murcia, Spain). The doses for AIs were obtained from fertile Duroc boars belonging to an AI station.

Hormonal treatments, estrus detection, and AI

Oral administration of ALT at a dose of 20 mg per sow per day was used for estrus synchronization. Superovulation was induced by intramuscular administration of 1,000 IU eCG and 750 IU hCG. The detection of estrus was conducted daily by qualified personnel beginning on the last day of ALT administration or the day of weaning by applying back pressure after nose–nose contact with a boar. Estrus was considered when the sow presented a standing reflex of more than 1 min.

The sows were inseminated at 0 and 24 h of the beginning of estrus with 3 × 105 spermatozoa in 90 mL extender (Beltsville thawing solution; Pursel and Johnson, 1975). The percentage of motile and morphologically normal spermatozoa in the AI doses was greater than 75% at insemination.
Embryo and tissue collection

Laparotomies were performed on day 6 as previously described (Martínez et al., 2017). Briefly, after intramuscular sedation with azaperone (Stresnil, Landegger Strasse, Austria), sows were anesthetized by intravenous administration of sodium thiopental (B. Braun VetCare SA, Barcelona, Spain) and then isoflurane (IsoFlo, Madrid, Spain). During surgery, the number of corpora lutea in each ovary was counted to assess the ovulatory response to the hormonal treatments. Embryos were collected from the uterus with Tyrode’s medium supplemented with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and polyvinyl alcohol (Funahashi et al., 2000; Martínez et al., 2014) and morphologically evaluated for viability. Tissue samples were recovered from the mesometrial side of the left uterine horn and the entire left ovary, washed with THP medium, snap-frozen, and stored at −80 °C until RNA extraction.

Total RNA extraction

Total RNA isolation was performed as previously reported (Martínez et al., 2020). Briefly, endometrial or ovary samples were disrupted in 1 mL of TRIzol reagent (Thermo Scientific, Waltham, MA, USA) with a Tissuelyser II (Qiagen, Hilden, Germany) and centrifuged. The supernatant was incubated with bromochloropropane, centrifuged again, and re-incubated with isopropanol and RNA precipitation solution. The precipitate was mixed with 1 mL of 75% ethanol and centrifuged, and the resulting pellet was air-dried and dissolved in 30 µL of RNase-free water. The RNA quantity and integrity were measured with a Bioanalyzer 2100 (Agilent, Santa Clara, USA). All RNA samples had satisfactory quality, with a RNA integrity number >8.

RNA sequencing

RNA-Seq technique was used to identify differentially expressed genes (DEGs) among groups. Libaries were generated as previously reported (Martínez et al., 2020) with the TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer’s protocol. Briefly, total RNA was diluted in nuclease-free water, and after purification and fragmentation, mRNA was converted into a cDNA library through an end repair process, the addition of a single “A” base to the 3’ end and the ligation of Illumina adaptors. The products were purified and PCR-enriched to produce the final double-stranded cDNA library. The NextSeq 500/550 High Output kit v2.5 (150 cycles) was used for library sequencing (sequencing setup of 2 × 75 bp paired-end). The outcomes of the sequencing process are depicted in Supplementary File S1. The raw datasets were deposited in the Sequence Read Archive under accession number PRJNA862977 (http://www.ncbi.nlm.nih.gov/bioproject/862977).

Analysis of sequencing data

Clean reads were aligned to the pig genome (SusScrofa 10.2) by using Spliced Transcripts Alignment to a Reference (STAR; Dobin et al., 2013) method. Normalization of the data was performed by the Robust Multichip Average method (Bolstad et al., 2003). Raw intensities were background adjusted, log 2 transformed, and normalized using quantile normalization to assign a single intensity value to each probe. The data analysis was performed with Partek Genomics Suite & Partek Pathways software (Partek Incorporated, St. Louis, USA). A one-way ANOVA was used to detect DEGs among groups with the cutoff P value (p-value) and fold change (FC) values set to 0.05 and 2, respectively. Gene Ontology and pathway enrichment analyses of DEGs were performed using DAVID (database for annotation, visualization and integrated discovery), KEGG (Kyoto Encyclopedia of Genes and Genomes), and DICE (database of Immune Cell Expression) databases. Enrichment analysis was performed using g:Profiler (version e106_eg53_p16_65fdcd97) with the g:SCS multiple testing correction method applying significance threshold of 0.05 (Raudvere et al., 2019). Network connections among genes were performed using STRING software (v11.0), available online at https://string-db.org/ (Szklarczyk et al., 2019).

RNA-Seq validation

RNA-Seq data were validated by real-time quantitative PCR (RT-qPCR) of six selected DEGs. RNA samples used for RT-qPCR assay were the same samples used for RNA-Seq analysis. Primers were commercially synthesized and tested (Bio Rad, Hercules, CA, USA). Total RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). qPCR was performed in 10-µL reactions with 5 µL of PowerUp SYBR Green Master Mix (Applied Biosystems, CA, USA), 50 nM for each set of primers, 1 µL of cDNA, and water. All reactions were carried out using the Real-Time PCR Detection System (QuantStudio 5, Thermo Fisher, Waltham, MA, USA). The thermal cycling profile was 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. Each sample for each gene was run in duplicate. The gene relative expression levels were quantified using the 2−ΔΔct method and two reference genes (GAPDH and ACTB).

Statistics of reproductive data

The reproductive parameter data were compared with an ANOVA and Tukey’s test for the post hoc analysis by using GraphPad PRISM version 8.0 (GraphPad Software, Inc., San Diego, CA). A P value less than 0.05 was considered significant. The data are expressed as the mean ± SD.

Results

Estrus synchronization combined with superovulation treatments negatively impacted embryo survival on day 6 after AIs

The results of the reproductive parameters evaluated in response to the different treatments are represented in Figure 1. Although the mean number of corpora lutea per ovary was higher in both SS-7 and SC groups than in the NC group (39.8 ± 10.2, 38.3 ± 11.1, and 23.2 ± 3.8, respectively; P < 0.05), a decrease (P < 0.05) in the fertilization rates (75.1 ± 15.2%, 93.8 ± 7.6%, and 91.8 ± 6.9%, respectively) and an increase in the numbers of oocytes and degenerated embryos (9.2 ± 4.3, 2.0 ± 2.4, and 1.5 ± 1.4, respectively) were observed in the SS-7 group compared with those in both the SC and NC groups.

Overview of gene expression in reproductive tissues

In the present study, we observed 675 and 1,583 DEGs in the SS-7 group compared to both the SC and NC groups in endometrial and ovarian samples, respectively. From
this general set of genes, 530 were downregulated (with 54 having an FC < 10), and 145 were upregulated (with 29 having an FC > 10) in the endometrium. A total number of 42 downregulated transcripts (9 with an FC < 10) and 1,541 upregulated transcripts (124 with an FC > 10) were observed in the ovary when comparing the SS-7 group to both SC and NC groups.

Altered transcripts found in the SS-7 group were then plotted in a Venn diagram to visualize co-expressed genes, for example, shared between endometrial and ovarian samples (Figure 2). Only seven transcripts were commonly upregulated in both tissues. Results of DEGs identified in all comparisons have been incorporated in Supplementary File S2.
Biological interpretation of the transcriptomic response to the combined synchronization and superovulation treatment in the ovary and endometrium

All DEGs were classified into the following three main GO categories: biological processes, cellular components, and molecular functions (Figure 3). From the general set of DEGs in SS-7 sows compared to SC and NC sows, a high proportion of genes were enriched in functional groups related to cell proliferation and communication, metabolic process, developmental process, cell-to-cell signaling, energy production, etc. (Figure 3). Specifically, 43 and 31 genes (ovary and endometrium, respectively) were assigned to functional categories associated with oocyte progression and in utero-embryo development based on the information retrieved from KEGG and DAVID databases. A graphical representation of this functional classification of overexpressed genes in SS-7 sows compared to those in the SC and NC sows is shown in Figure 4, where 20 and 21 (ovary and endometrium, respectively) functional terms with the lowest P value are shown.

The combined synchronization and superovulation treatment enhanced folliculogenesis but negatively impacted embryo viability probably through the dysregulation of Wnt/β-catenin and Notch signaling genes in the endometrium

The combination of synchronization and superovulation treatments induced the activation of several genes with potential roles in ovarian folliculogenesis and oocyte progression (e.g., Insr-FC: 3.3; pv<0.02, Est1-FC: 21.1; pv<0.04).

In addition, we found a strong enrichment of genes associated with the Wnt/β-catenin and Notch signaling pathways in the endometrium of SS-7 sows compared to SC and NC groups, concomitant with detrimental endometrial responses for embryo development. Among the transcripts involved in Wnt/β-catenin signaling, we found a downregulation of Ctnnb1 (FC: −2.2; pv<0.02), Ccn1 (FC: −2.3; pv<0.01), Rbpj (FC: −3.1; P < 0.01), Mib1 (FC: −2.6; pv<0.04), among others.
A model for the possible mechanisms of action of hormonal treatments on reproductive parameters through gene modifications in reproductive tissues is presented in Figure 6. The model speculates that the combination of synchronization and superovulation treatments stimulates folliculogenesis through the activation of insulin and estrogen receptors, potentially triggering the premature ovulation of many immature oocytes as well as contributing to embryo loss potentially via the downregulation of several Wnt/β-catenin and Notch signaling genes, having key roles in blastocyst activation and development.

Validation of RNA-Seq data
We selected six genes to verify the RNA-Seq results by RT-qPCR. These genes presented similar patterns of expression under both methods (Figure 7), proving that RNA-Seq results were reliable.

Discussion
In the present study, hormonal treatments triggered an increase in the mean number of corpora lutea per ovary compared to natural weaning, resulting in a substantially higher number of embryos recovered on day 6 after AI. As widely reported, exogenous gonadotrophin administration results in an enhancement of oocyte nuclear maturation and accelerates follicle development and oocyte release (Kaneko et al., 2006). The findings observed in this study regarding reproductive parameters were consistent with the results obtained after analyzing gene expression patterns in the ovaries of superovulated sows.

Several genes with key roles in follicular development were differentially expressed in the ovary in response to superovulation (SC compared to NC) and synchronization combined with superovulation (SS-7 compared to both SC and
Figure 4. Functional enrichment analysis of selected differentially expressed transcripts in ovarian (A) and endometrial (B) tissue between SS-7 group (sows treated with altrenogest for 7 d beginning on the day of weaning and superovulated with equine chorionic gonadotropin 24 h after the last altrenogest administration and human chorionic gonadotropin at the onset of estrus) and SC (superovulated sows with equine chorionic gonadotropin 24 h after weaning and human chorionic gonadotropin at the beginning of estrus) and NC (sows with postweaning natural estrus) groups. Enrichment analysis was performed using g:Profiler (version e106_eg53_p16_b5fcd97) with the g:SCS multiple testing correction method applying significance threshold of 0.05 (Raudvere et al., 2019), where functional categories were represented in the x-axis as GO BP: Go Term-Biological Processes; KEGG: Pathways from KEGG database; REAC: Reactome pathway database.
NC groups). Examples of this fact include the upregulation of insulin receptor (Insr) and estrogen receptor 1 (Esr1) gene expression in the SS-7 group compared to SC and NC groups. In the ovary, insulin plays a key role in the development of follicles, steroidogenesis and ovulation (Das and Arur, 2017). These actions are mediated through insulin receptors (Insr), which are widely distributed throughout all ovarian tissues, including stromal tissue and granulosa and theca cells (Dupont and Scaramuzzo, 2016). Insulin, in synergy with FSH, supports the proliferation and differentiation of granulosa cells (Baumgarten et al., 2015). The upregulated expression of Insr observed in the ovary could be a sign of an increased influx of insulin contributing to follicular development. Similarly, the estrogen receptor-α encoding gene (Esr1) enhances antral ovarian follicle growth by increasing cell proliferation and progression of the antral cavity (Rovani et al., 2014). Defects in the expression of Esr1 have been associated with primary ovarian insufficiency (Kim et al., 2011; Chen et al., 2018), a disorder that occurs by the premature depletion of follicles or premature folliculogenesis arrest (M’Rabet et al., 2012).

However, it is important to mention that, in a recent study conducted by our group (Gonzalez-Ramiro et al., 2021), the combination of synchronization and superovulation treatments led to a high rate of immature oocytes among the structures recovered on day 6 after AI (more than 60% of nonviable embryos recovered on day 6 after AI in that study were germinal vesicle-stage oocytes). In the present report, the superovulation protocol (SC) caused dysregulation of growth arrest-specific gene two (Gas2) in the ovary when compared to natural weaned sows (NC). Surprisingly, Gas2 turned to be upregulated in the SS-7 group compared to both SC and NC groups. Gas2 encodes a cytoplasmic protein reported to be a critical modulator of ovarian follicle assembly and development by interacting with the Notch signaling pathway in mice (Brancolini et al., 1995). The overexpression of this gene provokes apoptotic reorganizations of the murine actin cytoskeleton (York et al., 2016). Moreover, insulin growth

Figure 5. Representative networks of selected differentially expressed transcripts in ovarian (A) and endometrial (B) tissue between SS-7 group (sows treated with altrenogest for 7 d beginning on the day of weaning and superovulated with equine chorionic gonadotropin 24 h after the last altrenogest administration and human chorionic gonadotropin at the onset of estrus) and SC (superovulated sows with equine chorionic gonadotropin 24 h after weaning and human chorionic gonadotropin at the beginning of estrus) and NC (sows with postweaning natural estrus) groups.
factor 1 (Igf1), a powerful stimulator of cell proliferation and differentiation that modulates steroidogenesis and apoptosis during folliculogenesis (Lucy, 2011), was upregulated in SC compared to NC but exhibited a decreased expression in SS-7 compared to both SC and NC groups. These results clearly indicate that despite the obvious positive effect in follicular development, which increased the number of corpora lutea in SC and SS-7 ovaries, the combined hormonal treatments (ALT and gonadotropins; SS-7) triggered inadequate oocyte-follicular communication, which is essential for correct oocyte maturation, leading to dysregulated ovarian gene expression and premature ovulation of immature oocytes.

Furthermore, successful embryo implantation and progression of pregnancy require both implantation-competent blastocysts and a receptive uterine environment (Blitek et al., 2013). The present study revealed detrimental effects on the embryonic parameters of sows treated with ALT in combination with gonadotropins (SS-7) when compared to only gonadotropins (SC) and natural estrus weaning (NC). To the best of our knowledge, the molecular responses of reproductive tissues to hormonal treatments after synchronization and superovulation protocols remain largely unknown. Here, we report interesting findings regarding gene expression patterns that could be directly associated with detrimental embryo viability rates. RNA sequencing revealed a significant downregulation of numerous genes with important roles in embryonic growth and progression.

Among a range of identified embryo development-related genes, dysregulation of many Wnt-β-catenin signaling...
genes in the endometrial tissue of the SS-7 group (e.g., clyd, dlg5, med12l, myc, gli3, kank1, scyl2, tbl1xr1, ctmb1, ccny, ppp1rn, rbpi, tnks2, and usp8) drew our attention. Wnt-β-catenin-related genes encode the transcription of proteins that regulate cell–cell interactions in many species (Huelsken and Birchmeier, 2001; Cadigan, 2008). When Wnt proteins bind their cellular receptors, classic canonical Wnt (or Wnt-β-catenin) signaling is activated (Pinson et al., 2000; Tamai et al., 2000; Mao et al., 2001). Wnt-β-catenin signaling participates in cellular proliferation and differentiation and is crucial for the development of embryos to the blastocyst stage and the acquisition of embryonic competency for implantation (Chen et al., 2009). Significant evidence from earlier research suggests that some Wnt-β-catenin ligands might be implicated in the transition from the morula to the blastocyst stage and for blastocyst activation (Mohamed et al., 2004). Nevertheless, some studies have suggested that embryo-derived Wnt5a are not needed for embryo developmental progress (Tribulo et al., 2017). Biechele et al., 2013 reported that the inhibition of Wnt-β-catenin signaling did not alter the percentage of cleaved embryos achieving the blastocyst stage in mice (Biechele et al., 2013). However, there may be differences among species in relation to the role of embryo-derived Wnt proteins in the development of a competent blastocyst. Also, it is likely that Wnts derived from the mother also participate in embryo progression (Schohl and Fagotto, 2003). As previously reported, Wnt-β-catenin silencing remarkably inhibits blastocyst competency for implantation (Xie et al., 2008). De Vries et al. (2004) observed that mice oocytes with conditional deletion of β-catenin were able to develop into blastocysts, but females yield a reduced number of pups when crossbred with wild-type males in comparison to those of wild-type to wild-type mating. A previous study using a transgenic mouse to monitor stimulation of the Wnt/β-catenin pathway showed that uterine Wnt/β-catenin signaling is transitorily and precisely provoked at the potential embryo attachment site immediately before implantation (Mohamed et al., 2005). Moreover, maternal/zygotic ctmb1 (β-catenin 1) knockout mouse embryos showed morphological abnormalities during the pre- and early postimplantation periods (Messerschmidt et al., 2016).

As shown in this study, b-catenin (ctmb1) gene expression was downregulated in the endometrium of SS-7 sows compared to SC and NC groups, where embryo development was clearly repressed. This study suspected that synchronization-superovulation hormonal treatments could potentially inhibit cell-to-cell interactions by altering the ctmb1 steady-state concentration. Furthermore, we observed that the cyclin Y (Ccny) gene was repressed in the endometrium of SS-7 sows. Ccny is known to enhance Wnt/β-catenin signaling by improving the competence of the Wnt receptor Lrp6 (lipoprotein receptor-related protein 6; a transmembrane receptor that initiates Wnt/β-catenin signaling), which is crucial for mouse embryonic development. The knockout of Ccnyrs decreases Lrp6 phosphorylation, which hampers β-catenin activity and abolishes cell expansion in vitro, leading to embryo death (Zeng et al., 2016). In addition, recent studies demonstrated that Wnt/β-catenin and Notch signaling pathways, which are the main controlling pathways related to cell fate decisions of embryos (Arteravanis-Tsakonas et al., 1999) are transitorily activated in myometrial cells during pregnancy, which might indicate a role of these pathways in embryo spacing along the uterine horns. The signals produced by pre-attachment blastocysts could activate these pathways at the myometrial level, regulating and coordinating myometrial contractions and embryo spacing.

Interestingly, some genes involved in both Wnt/β-catenin and Notch signaling pathways were repressed in endometrial SS-7 samples. One of these genes was Rbpj, the nuclear transducer of Notch signaling and a positive regulator of the Wnt signaling pathway. Rbpj gene is fundamental for normal embryonic development since it interacts with estrogen receptor α in the uterus, which is indispensable for the early orientation of the embryo with the axes of the uterus (Robinson and Fisher, 2014). The uterine-specific deletion of Rbpj caused an atypical embryo–uterine orientation and led to substantial embryo loss (Zhang et al., 2014). Moreover, such specific gene function appears essential for mouse post-implantation development (Oka et al., 1995) as null mutant mice presented growth retardation and embryonic mortality at 8.5 and 10.5 d of pregnancy, respectively, and several abnormalities, such as a reduction in size and abnormal placental development. Our findings support the hypothesis that synchronization in combination with superovulation treatment induces molecular modifications in the endometrium failing to timely regulate uterine muscular contraction accounted for normal embryo spacing and orientation and probably precludes the possibility of consequent embryo loss. Additionally, the Mind-bomb 1 (Mib1) gene interacts with and regulates Notch ligands, indicating its wide role in Notch signaling activation (Barsi et al., 2005). In mice, Mib1 knockout embryos showed complete Notch signaling reduction, cell apoptosis, reduced somitogenesis, and vascular remodeling and died earlier than embryonic day 11.5 (Koo et al., 2005). These findings are consistent with our results where Mib1 gene expression was downregulated in the endometrium of SS-7 sows. These and other genes with specific roles in the modulation of Wnt/β-catenin and Notch signaling pathways were repressed in the SS-7 group, suggesting an influence of the synchronization and superovulation treatments on endometrial Wnt/β-catenin and Notch signaling that may trigger different events leading to embryo failure to develop further.

Conclusions
Taken together, the findings observed in the present study substantiate the hypothesis that hormonal treatments (synchronization combined with superovulation) have the potential to alter ovarian and endometrial gene expression patterns, triggering improper follicle development, and oocyte growth. Moreover, these treatments lead to impaired embryonic development before implantation probably due to abnormal embryonic-uterine communication. These data contribute to explaining the low survival rates observed in SS-7 embryos and constitute novel evidence suggesting that Wnt/β-catenin and Notch signaling pathways could be associated to blastocyst survival and competency toward implantation. However, the mechanisms by which synchronization-superovulation hormonal treatments alter gene expression and the implication of these pathways need to be further investigated.

Supplementary Data
Supplementary data are available at Journal of Animal Science online.
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Author Contributions

Conceptualization, I.P., E.A.M., H.R.-M., and C.A.M.; methodology, H.-G.R., I.P., J.M.C., A.G-P., M.A.G., C.C., E.A.M., and C.A.M.; software, H.G.-R. and C.A.M.; validation, E.A.M. and H.R.-M.; writing—original draft preparation, C.A.M.; writing—review and editing, I.P., E.A.M., and H.R.-M.; supervision, E.A.M. and H.R.-M.; project administration, E.A.M.; funding acquisition, C.C., E.A.M., H.R.-M., and C.A.M. All authors contributed to manuscript revision and read and approved the submitted version.

Data and model availability statement

The raw datasets generated during and/or analyzed during the current study are available at Sequence Read Archive (SRA) with BioProject accession number: PRJNA862977 (http://www.ncbi.nlm.nih.gov/bioproject/862977).

Ethics approval

Experimental procedures were carried out according to the 2010/63/EU EEC Directive for animal experiments and were revised and approved by the Ethical Committee for Experimentation with Animals of the University of Murcia, Spain (research code: 486/2018; 27 September 2018).

Conflict of Interest Statement

The authors declare no conflict of interest.

Literature Cited

Angel, M. A., M. A. Gil, C. Cuello, J. Sanchez-Osorio, J. Gomis, I. Parrilla, J. Vila, I. Colina, M. Diaz, J. Reixach, et al. 2014. The effects of superovulation of donor sows on ovarian response and embryo development after nonsurgical deep-uterine embryo transfer. Theriogenology 81:832–839. doi:10.1016/j.theriogenology.2013.12.017
Artavanis-Tsakonas, S., M. D. Rand, and R. J. Lake. 1999. Notch signaling: cell fate control and signal integration in development. Science 284:770–776. doi:10.1126/science.284.5415.770
Barsi, J. C., R. Rajendra, J. I. Wu, and K. Artzt. 2005. Mind bomb1 is a ubiquitin ligase essential for mouse embryonic development and Notch signaling. Medec. Dev. 122:1106–1117. doi:10.1016/j.mod.2005.06.005
Baumgarten, S. C., S. M. Convissar, A. M. Zamah, M. A. Fiero, N. J. Winston, B. Scoccia, and C. Stocco. 2015. FSH regulates IGF-2 expression in human granulosa cells in an AKT-dependent manner. J. Clin. Endocrinol. Metab. 100:E1046–E1055. doi:10.1210/jc.2015-1504
Béchet, D. 1986. Control of gene expression by steroid hormones. Reprod. Nutr. Dev. 26:1025–1035. doi:10.1051/106019860701
Biechele, S., K. Cockburn, F. Lanner, B. J. Cox, and J. Rossant. 2013. Porcine-dependent Wnt signaling is not required prior to mouse gastrulation. Development 140:2961–2971. doi:10.1242/dev.094458
Blitek, A., M. M. Kaczmarek, A. Wacławik, A. J. Zielick, H. Rodriguez-Martinez, N. M. Soede, and W. L. Flowers. 2013. Embryo-maternal relationships during the peri-implantation period in non-human primate and old world monkey species. Netherlands (UK): Nottingham University Press. doi:10.1530/biosciprocs.19.0005
Bolstad, B. M., R. A. Irizarry, M. Astrand, and T. P. Speed. 2003. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19:185–193. doi:10.1093/bioinformatics/19.2.185
Brancolini, C., M. Benedetti, and C. Schneider. 1995. Microfilament reorganization during apoptosis: the role of Gas2, a possible substrate for ICE-like proteases. EMBO J. 14:5179–5190. doi:10.1002/mol.200202.x
Cadigan, K. M. 2008. Wnt-beta-catenin signaling. Curr. Biol. 18:R943–R947. doi:10.1016/j.cub.2008.08.017
Chen, Q., Y. Zhang, J. Lu, Q. Wang, S. Wang, Y. Cao, H. Wang, and E. Duan. 2009. Embryo-uterine cross-talk during implantation: the role of Wnt signaling. Mol. Hum. Reprod. 15:215–221. doi:10.1093/molehr/gap009
Chen, Y., H. Tang, L. Wang, J. He, Y. Guo, Y. Liu, X. Liu, and H. Lin. 2018. Fertility enhancement but premature ovarian failure in esr1-deficient female zebrafish. Front. Endocrinol. (Lausanne) 9:567. doi:10.3389/fendo.2018.00567
Cooke, I. D., and E. A. Lentox. 1994. Folliculogenesis – the natural way. Aust. N Z J. Obstet. Gynaecol. 34:268–271. doi:10.1111/j.1479-828x.1994.tb01071.x
Das, D., and S. Arur. 2017. Conserved insulin signaling in the regulation of oocyte growth, development, and maturation. Mol. Reprod. Dev. 84:444–459. doi:10.1002/mrd.22806
De Rensis, F., A. J. Zielick, and R. N. Kirkwood. 2017. Seasonal infertility in gilts and sows: aetiology, clinical implications and treatments. Theriogenology 96:111–117. doi:10.1016/j.theriogenology.2017.04.004
De Vries, W. N., A. V. Esvikov, B. E. Haac, K. S. Fancher, A. E. Holbrook, R. Kemler, D. Solter, and B. B. Knowles. 2004. Maternal beta-catenin and cadherin in mouse development. Development 131:4435–4445. doi:10.1242/dev.013136
Dobin, A., C. A. Davis, F. Schlesinger, J. Drenkow, C. Zaleski, S. Jha, P. Batut, M. Chaisson, and T. R. Gingeras. 2013. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29:15–21. doi:10.1093/bioinformatics/bts635
Dupont, J., and R. J. Scaramuzzino. 2016. Insulin signalling and glucose transport in the ovary and ovarian function during the ovarian cycle. Biochem. J. 473:1483–1501. doi:10.1042/BCJ20160124
Funahashi, H., H. Ekwall, and H. Rodriguez-Martinez. 2000. Zona reaction in porcine oocytes fertilized in vivo and in vitro as seen with scanning electron microscopy. Biol. Reprod. 63:1437–1442. doi:10.1095/biolreprod63.5.1437
Gonzalez-Ramiro, H., C. Cuello, J. M. Cambra, A. Gonzalez-Plaza, J. M. Vazquez, J. L. Vazquez, H. Rodriguez-Martinez, M. A. Gil, A. Lucas-Sanchez, I. Parrilla, et al. 2021. A short-term altrenogest treatment post-weaning followed by superovulation reduces pregnancy rates and embryo production efficiency in multiparous sows. Front. Vet. Sci. 8:771573. doi:10.3389/fvets.2021.771573
Huelsken, J., and W. Birchmeier. 2001. New aspects of Wnt signaling pathways in higher vertebrates. Curr. Opin Genet. Dev. 11:547–553. doi:10.1016/S0959-437X(01)00221-8
Ing, N. H. 2005. Steroid hormones regulate gene expression posttranscriptionally by altering the stabilities of messenger RNAs. Biol. Reprod. 72:1290–1296. doi:10.1095/biolreprod.105.040014
Insler, V., and B. Lunenfeld. 1996. Ovulation induction in the era of recombinant gonadotropins and GnRH analogues. Isr. J. Med. Sci. 32:79–89.
van Leeuwen, J. J. S., I. J. Williams, B. Kemp, and N. M. Soede. 2010. Post-weaning altronegrost treatment in primiparous sows; the effect of duration and dosage on follicular development and consequences for early pregnancy. *Anim. Reprod. Sci.* 119:258–264. doi:10.1016/j.anireprosci.2010.02.008

Lopes, T. P., A. Bolarín, E. A. Martínez, and J. Roca. 2017. Altrenogest involves pathways independent of nuclear CTNNB1. *Reproduction* 153:405–419. doi:10.1530/REP-16-0610

Ravoni, M. T., B. G. Gaperin, G. F. Ilha, R. Ferreira, R. C. Bohrer, R. Duggavathi, V. Bordignon, and P. B. D. Gonçalves. 2014. Expression and molecular consequences of inhibition of estrogen receptors in granulosa cells of bovine follicles. *J. Ovarian Res.* 7:96. doi:10.1186/s13048-014-0096-0

Schohl, A., and F. Fogatto. 2003. A role for maternal beta-catenin in early mesoderm induction in Xenopus. *EMBO J.* 22:3303–3313. doi:10.1093/emboj/cdg328

Pinson, K. I., T. Khanna, S. Monkley, J. Brennan, S. Monckley, B. A. Merry, and W. C. Skarnes. 2000. An LDL-receptor-related protein mediates Wnt signalling in mice. *Nature* 407:535–538. doi:10.1038/35035124

Pursel, V. G., and L. A. Johnson. 1975. Freezing of boar spermatozoa: fertilizing capacity with concentrated semen and a new thawing procedure. *J. Anim. Sci.* 40:99–102. doi:10.2527/jas1975.40199x

Raudvere, U., L. Kolberg, I. Kuzmin, T. Arak, P. Adler, H. Peterson, and J. Vilo. 2019. gProfiler: a web server for functional enrichment analysis and conversions of gene lists (2019 update). *Nucleic Acids Res.* 47:W191–W198. doi:10.1093/nar/gkz369

Robinson, J. F., and S. J. Fisher. 2014. Rbhp links uterine transformation and embryo orientation. *Cell Res.* 24:1031–1032. doi:10.1038/ct.2014.110

Rovani, G. L., G. F. Ilha, R. Ferreira, R. C. Bohrer, R. Duggavathi, V. Bordignon, and P. B. D. Gonçalves. 2014. Expression and molecular consequences of inhibition of estrogen receptors in granulosa cells of bovine follicles. *J. Ovarian Res.* 7:96. doi:10.1186/s13048-014-0096-0

van Leeuwen, J. J. S., I. J. Williams, B. Kemp, and N. M. Soede. 2010. Post-weaning altronegrost treatment in primiparous sows; the effect of duration and dosage on follicular development and consequences for early pregnancy. *Anim. Reprod. Sci.* 119:258–264. doi:10.1016/j.anireprosci.2010.02.008

Lopes, T. P., A. Bolarín, E. A. Martínez, and J. Roca. 2017. Altrenogest involves pathways independent of nuclear CTNNB1. *Reproduction* 153:405–419. doi:10.1530/REP-16-0610

Ravoni, M. T., B. G. Gaperin, G. F. Ilha, R. Ferreira, R. C. Bohrer, R. Duggavathi, V. Bordignon, and P. B. D. Gonçalves. 2014. Expression and molecular consequences of inhibition of estrogen receptors in granulosa cells of bovine follicles. *J. Ovarian Res.* 7:96. doi:10.1186/s13048-014-0096-0

Schohl, A., and F. Fogatto. 2003. A role for maternal beta-catenin in early mesoderm induction in Xenopus. *EMBO J.* 22:3303–3313. doi:10.1093/emboj/cdg328

Szkarczyk, D., A. L. Gable, D. Lyon, A. Junge, S. Wyder, J. Huerta-Cepas, M. Simonovic, N. T. Doncheva, J. H. Morris, P. Bork, et al. 2019. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res.* 47:D607–D613. doi:10.1093/nar/gky1131

Tamai, K., M. Semenov, Y. Kato, R. Spokony, C. Liu, Y. Katsymura, F. Hess, J. P. Saint-Jeannet, and X. He. 2000. LDL-receptor-related proteins in Wnt signal transduction. *Nature* 407:530–535. doi:10.1038/35035111

Teague, C. L., J. R. Rossjohn, M. J. Durand, H. Zhang, and C. A. Martinez. 2005. Production of bovine embryos using a chemically defined medium. *Biol. Reprod.* 72:417–424. doi:10.1095/biolreprod.103.025692

Mohamed, O. A., D. Dufort, and H. J. Clarke. 2004. Expression and estradiol regulation of Wnt genes in the mouse blastocyst identify a candidate pathway for embryo-maternal signaling at implantation. *Biol. Reprod.* 71:417–424. doi:10.1095/biolreprod.103.025692

Moazed, A. A., M. Jonnaert, C. Labelle-Dumais, K. Kuroda, H. J. Clarke, and D. Dufort. 2005. Uterine Wing/beta-catenin signaling is required for implantation. *Proc. Natl. Acad. Sci. U.S.A.* 102:8579–8584. doi:10.1073/pnas.0506121102

Oka, C., T. Nakano, A. Wakeham, J. L. de la Pompa, C. Mori, T. Sakai, S. Okazaki, M. Kawaiuchi, K. Shiota, T. W. Mak, et al. 1995. Disruption of the mouse RBP-J kappa gene results in early embryonic death. *Development* 121:3291–3301. doi:10.1242/dev.121.3291

Patterson, J., A. Wellen, M. Hahn, A. Pasternak, J. Lowe, S. DeHaas, D. Kraus, N. Williams, and G. Foxcroft. 2008. Responses to delayed estrus after weaning in sows using oral progesterone treatment. *J. Anim. Sci.* 86:1996–2004. doi:10.2527/jas.2007-0440

Pinson, K. I., J. Brennan, S. Monkley, B. A. Merry, and W. C. Skarnes. 2000. An LDL-receptor-related protein mediates Wnt signalling in mice. *Nature* 407:535–538. doi:10.1038/35035124

Pursel, V. G., and L. A. Johnson. 1975. Freezing of boar spermatozoa: fertilizing capacity with concentrated semen and a new thawing procedure. *J. Anim. Sci.* 40:99–102. doi:10.2527/jas1975.40199x