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Effect of early addition of bone morphogenetic protein 5 (BMP5) to embryo culture medium on *in vitro* development and expression of developmentally important genes in bovine preimplantation embryos

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ABSTRACT

Previous studies have reported that bone morphogenetic protein 5 (BMP5) is differentially expressed in the isthmus of bovine oviducts and it is present in the oviductal fluid. However, the specific action of this factor is unknown. To evaluate whether BMP5 exerts some effect during early bovine embryo development, gene expression of BMP5, BMP receptors, and the effect of exogenous BMP5 on *in vitro* development and expression of developmentally important genes were assessed. In experiment 1, pools of embryos at two-cell, four-cell, eight-cell, and blastocyst stages, derived from *in vitro* fertilization, were collected for analysis of *BMP5* and BMP receptors (*BMPR1A*, *BMPR1B*, and *BMPR2*) messenger RNA (mRNA) expression. On the basis of previous results, in experiment 2, presumptive zygotes were cultured for the first 48 hours after insemination in CR1aa medium assaying three different treatments: (1) control (CR1aa); (2) vehicle control (CR1aa + 0.04 mM HCl), and (3) BMP5 treatment (CR1aa + 100 ng/mL of BMP5). The cleavage rate was evaluated 48 hours after insemination (Day 2), and then, embryos were transferred to CR1aa + 10% fetal bovine serum. The blastocyst rate was determined on Day 7. In experiment 3, pools of embryos at two-cell, four-cell, eight-cell, and blastocyst stages, derived from control and BMP5-treated groups, were collected for analysis of *ID2* (BMP target gene), *OCT4*, *NANOG*, and *SOX2* (pluripotency genes) mRNA expression. BMP5 transcripts were not detectable in any of the embryonic stages examined, whereas the relative mRNA abundance of the three BMP receptors analyzed was greater in early embryo development stages before maternal–embryonic transition, raising the possibility of a direct effect of exogenous BMPs on the embryo during the first developmental period. Although early addition of 100 ng/mL of BMP5 to the embryo culture medium had no effect on the cleavage rate, a significantly higher proportion of cleaved embryos developed to the blastocyst stage in the BMP5 group. Moreover, reverse transcription quantitative real-time polymerase chain reaction analysis showed a significant increase in the relative abundance of *SOX2* in two-cell stage embryos, *ID2* and *OCT4* in eight-cell stage embryos, and *NANOG* and *OCT4* in blastocysts derived from BMP5-treated embryos. In conclusion,

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our results report that early addition of BMP5 to the embryo culture medium had a positive effect on the blastocyst rate and affected the relative expression of BMP target and pluripotency genes, suggesting that BMP5 could play an important role in the preimplantation development of bovine embryos.

1. Introduction

Embryo–maternal communication during the preimplantation period plays a crucial role in the establishment and maintenance of pregnancy in mammals. During early embryogenesis, this complex dialog starts in the window of time when the embryo transits through the oviduct. Therefore, the oviductal molecules appear to be one of the several factors that mediate the paracrine signaling between the embryo and the mother in the course of initial development [1,2]. In this sense, it has been reported that different growth factors and cytokines are present in oviductal secretions, whereas their corresponding receptors are expressed in the preimplantation embryo, suggesting a crosstalk between the developing early embryo and the oviduct through different regulatory pathways [3,4].

In the present work, our attention is focused on bone morphogenetic proteins (BMPs), a group of growth factors that belongs to the transforming growth factor beta (TGF-β) superfamily [5]. These factors are active as disulfide-linked dimmers that exert their effects by binding to tetrameric complexes of type I (BMPR1A, BMPR1B, or ACVR1A, also known as ALK3, ALK6, and ALK2, respectively) and type II (BMPR2, ACVR2A, or ACVR2B) serine/threonine kinase receptors leading to signal propagation through SMAD-dependent pathways [6,7]. Bone morphogenetic proteins regulate a diverse range of development processes during embryonic and adult life and they play an important role in mammalian reproduction [8]. There is extensive evidence supporting their role as autocrine and paracrine regulators of ovarian follicular development, differentiation in the female reproductive tract, implantation of the blastocyst in the uterus, and morphogenesis and organogenesis during embryo development [8–11]. However, little is known about their action during the early stages of preimplantation development when the embryo is in transit through the oviduct.

Studies in preimplantation mouse embryos revealed that multiple components of the BMP signaling pathway, including BMP ligands, receptors, and SMADs, are expressed in a developmentally regulated fashion during the preimplantation development [12–14]. Moreover, recent studies have reported that BMP signaling is required for cell cleavage and for the correct development of extra-embryonic lineages in mouse embryos [15,16]. Although little is known in other species, particularly in farm animals, there are strong lines of evidence to suggest that BMP signaling could also play an important role during the preimplantation development of bovine embryos. Transcriptome analysis in bovine preimplantation embryos produced *in vivo* revealed that different BMP signaling components, including BMP ligands (*BMP4* and *BMP15*) and receptors (*BMPR1B*), are expressed as early as the one-cell stage and until the blastocyst stage showing significant changes in their transcript levels during preimplantation development [17]. Also, it has been shown that the addition of BMP15 during IVM significantly improves the percentage of bovine embryos reaching the blastocyst stage [18]. Furthermore, addition of BMP4 or the inhibitor Noggin to the embryo culture medium affects embryo development suggesting that a correct balance of BMP signaling is needed for proper preimplantation development of bovine embryos [19]. BMPs are also involved in the regulation of trophoblast differentiation and in maintaining pluripotency in the inner cell mass of bovine blastocysts [20].

In addition to all this evidence, previous studies carried out at our laboratory revealed that different BMP genes (*BMP2*, *3*, *4*, *5*, *7*, *10*, and *15*) are expressed in the epithelial cells of the bovine oviduct during the estrous cycle, suggesting that these factors can be synthesized by the maternal tract [21]. Interestingly, one of these factors identified as BMP5 shows differential expression in isthmus epithelial cells and is present in the oviductal fluid during the estrous cycle, showing high transcriptional levels during the periovulatory phase [21]. However, at present, there is no information about the specific action of this factor in the oviduct and there is no experimental data reporting its role during bovine preimplantation development.

Within this context, the purpose of this study was to evaluate whether BMP5 exerts some effect during early bovine embryo development. Therefore, the gene expression of BMP5 and BMP receptors in *in vitro*–produced bovine embryos was initially examined by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR). After observing differential gene expression of BMP receptors during early embryo development stages (before embryonic genome activation), it was decided to evaluate the effect of the early addition of BMP5 to the embryo culture medium until the first 48 hours post insemination (hpi) on cleavage, developmental rates, and messenger RNA (mRNA) abundance of genes related to the BMP signaling pathway and pluripotency in bovine embryos obtained by IVF.

2. Materials and methods

2.1. Chemicals

All reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated. Human recombinant BMP5 (615-BMC-020/CF; R&D Systems, Inc., Minneapolis, MN, USA) was reconstituted in 4 mM HCl to give a stock concentration of 100 μg/mL. After reconstitution, the stock solution was aliquoted and stored at −70 °C. The stock solution was diluted before the experiment, and a volume of 5 μL was added to 50 μL

microdrops of bovine embryo culture medium (CR1aa) to achieve a final concentration of 100 ng BMP5/mL medium and 0.04 mM HCl.

2.2. Oocyte recovery and in vitro maturation

Ovaries were collected from beef cows (*Bos taurus*) at local abattoirs, transported to the laboratory at 25 °C to 30 °C, and washed three times with warm PBS solution, pH 7.4. Cumulus–oocyte complexes (COCs) were recovered by aspiration of ovarian follicles (2–8 mm in diameter) with an 18-G needle and a 5 mL syringe containing ~1 mL of HEPES-buffered Tyrode's albumin lactate pyruvate medium (H-TALP) plus 10% (v:v) fetal bovine serum (FBS). Only oocytes with a compact nonatretic cumulus of at least three layers and a homogeneous ooplasm were selected for IVM. Groups of 50 COCs were transferred to four-well plates (NUNC, Roskilde, Denmark) containing 500 μL of maturation medium and cultured for 22 hours at 38.5 °C under an atmosphere of 5% CO_2 in air with maximum humidity. The maturation medium was composed of bicarbonate-buffered TCM-199 (Gibco product 11150-059; Grand Island, NY, USA) supplemented with 10% (v:v) FBS (Internegocios, Buenos Aires, Argentina), 0.05 IU rFSH/mL (Puregon; Organon, Dublin, Ireland), 0.1 mM of sodium pyruvate (P4562), 20 μM cysteamine (M9768), and 1% antibiotic–antimycotic solution (Gibco product 15240).

2.3. In vitro fertilization

For IVF, frozen semen doses (0.5 mL straws) from a single bull, previously proven suitable for IVF at our laboratory, were thawed in a 35 °C water bath for 40 seconds. Then, spermatozoa were washed twice by centrifugation at 1000 rpm for 5 minutes in 5 mL of Brackett and Oliphant (BO) medium supplemented with 5 mM caffeine (C4144) and 20 μg/mL of heparin (H3149; washing BO medium). After the final wash, the motility and concentration of spermatozoa were determined and the pellet was resuspended in 50% (v:v) washing BO medium and 50% (v:v) BO medium supplemented with 20 mg/mL of fatty acid–free BSA (A6003). Sperm concentration was adjusted to 15×10^6 cells/mL, and 100 μL droplets were placed under mineral oil (Fisher Scientific, Fair Lawn, NJ, USA) in 60 mm culture dishes. After maturation, COCs were washed three times with H-TALP and about 10 matured oocytes per drop were coincubated with sperm for 5 hours at 38.5 °C under 5% CO_2 in humidified air.

2.4. In vitro culture

After the fertilization period, presumptive zygotes were removed from the insemination droplets and washed with H-TALP. Cumulus cells and attached sperm were removed by repeated pipetting, and then, inseminated oocytes were washed three times with H-TALP before culturing. Groups of 20 presumptive zygotes were cultured in 50 μL of serum-free CR1aa medium (CR1 stock contained BME essential amino acids [20 μL/mL], MEM nonessential amino acids [10 μL/mL], 1 mM L-glutamine, 1% antibiotic–antimycotic solution [Gibco, 15240], and fatty acid–free BSA [3 mg/mL]) for 48 hpi (Day 2 pi; Day 0 = day of insemination) under mineral oil at 38.5 °C, in a humidified 5% CO_2 atmosphere. Subsequently, embryos were transferred and cultured in 50 μL of fresh CR1aa supplemented with 10% (v:v) FBS until Day 7 pi.

2.5. Experimental design

2.5.1. Experiment 1: Gene expression analysis of BMP5 and BMP receptors in preimplantation embryos

In the first experiment, mRNA expressions of *BMP5* and the *BMPR1A*, *BMPR1B*, and *BMPR2* receptors were evaluated by RT-qPCR in three pools of 10 embryos harvested at the two-cell (29 hpi), four-cell, and eight-cell (48 hpi) stages and in three pools of four embryos at the blastocyst (168 hpi) stage, obtained by IVF and cultured in CR1aa medium as described previously. The blastocyst pools were reduced to four embryos to ensure the homogeneity of size between the selected embryos. For comparison purpose, the expression of *BMP7* was analyzed in the same pools of embryos. Additionally, complementary DNA (cDNA) samples of bovine oviduct epithelial cells from ampulla and isthmus regions obtained at the preovulatory stage (for detailed procedures see [21]) were included as positive controls of *BMP5* and *BMP7* expression.

2.5.2. Experiment 2: Effect of early addition of BMP5 on preimplantation embryo development

In the second experiment, to examine the effect of BMP5 during the first hours of culture on early development of *in vitro*–produced bovine embryos, a total of 723 presumptive zygotes were cultured in CR1aa medium without serum in the presence and absence of BMP5 assaying three different experimental groups: (1) control (CR1aa without supplement; n = 286); (2) vehicle control (CR1aa supplemented with 0.04 mM HCl; n = 158), and (3) BMP5 (CR1aa supplemented with 100 ng/mL of recombinant human BMP5; n = 279). The cleavage rate was assessed 48 hpi (Day 2), and embryos were transferred to CR1aa supplemented with 10% FBS and cultured until Day 7 pi. The blastocyst rate was determined on Day 7 pi. The experiment was carried out five times under the same assay conditions.

2.5.3. Experiment 3: Effect of BMP5 on mRNA abundance of genes related to the BMP signaling pathway and pluripotency during early embryogenesis

To determine if the early addition of BMP5 to the embryo culture medium has any effect at molecular level, three pools of 10 embryos at the two-cell (29 hpi), four-cell, and eight-cell (48 hpi) stages and three pools of four embryos at the blastocyst (168 hpi) stage from control and BMP5-treated groups were collected to evaluate the expression of *ID2* (a target gene of BMP signaling pathway) and *OCT4*, *NANOG*, and *SOX2* (pluripotency-associated genes) using RT-qPCR according to the procedures described in the following.

2.6. RNA isolation and reverse transcription

Embryos collected for expression analysis were washed in H-TALP and immediately placed in sterile RNase and DNase-free tubes (0.6 mL) containing 100 μL of lysis solution (NucleoSpin RNA XS Kit; Macherey–Nagel, Düren, Germany). Tubes were kept at −80 °C until RNA extraction.

Total RNA was extracted from each pool of embryos according to the manufacturer's protocol, by adding 20 ng of poly-A carrier RNA to the sample lysate. Contaminating genomic DNA of each sample was removed from the total RNA preparations by digestion with recombinant RNase-free DNase, added directly to the extraction column during the purification process. RNA was then eluted from the purification column with 10 μL of RNase-free H_2O and immediately used for cDNA synthesis. Reverse transcription was performed using Moloney Murine Leukemia Virus reverse transcriptase (Promega, Madison, WI, USA), the oligo (dT)17 primer, and random primers. The reaction mixture (25 μL) consisted of 8 μL of total embryonic RNA (heat denatured), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 0.5 mM of each dNTP, 25 pmol of oligo (dT)17, 25 pmol of random primers, 200 units of reverse transcriptase, and RNase-free water. Reactions were performed by incubating the mixture in a thermal cycler at 42 °C for 90 minutes followed by a reverse-transcriptase inactivation at 94 °C for 5 minutes. The remaining 2 μL of RNA from each sample was incubated without reverse transcriptase under the same conditions as described previously (but scaling the reaction to a lower volume) to create a negative or "no RT" qPCR control to discard genomic contamination. Resulting cDNA (or RNA in the case of the "no RT" controls) were stored at −20 °C, and in the moment of use, they were diluted appropriately for qPCR amplification.

2.7. Analysis of gene expression by quantitative real-time polymerase chain reaction

Messenger RNA levels of the selected genes were determined by RT-qPCR using specific primers designed with Primer-BLAST, an online tool of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers (see Table 1) were previously validated for adequate primer efficiency, and specificity of their PCR products was confirmed by agarose gel electrophoresis on a 2.5% agarose gel and sequencing. All target genes showed acceptable efficiency (97%–100%) and correlation coefficients (close to 1.0). Polymerase chain reactions (with a final volume of 20 μL, containing 5 μL of cDNA template [diluted 1:5], 0.25 mM of forward and reverse primers, and 10 μL of Fast EvaGreen qPCR Master Mix [Biotium, Hayward, CA, USA]) were performed in a CFX96 Real-Time PCR detection System (Bio-Rad, Hercules, CA, USA). The PCR program consisted of an initial step of 2 minutes at 95 °C, followed by 48 cycles of 15 seconds at 95 °C and 30 seconds at 58 °C for annealing and extension. At the end of each PCR run, melt curve analysis was performed for all genes to ensure single product amplification and exclude the possible interference of dimers. This was verified by the presence of a single melting temperature peak. The melting protocol was a step cycle starting at 60 °C and increasing to 95 °C with a transition rate of +0.5 °C/s increments. For negative controls and to exclude the possibility of genomic DNA contamination, no-template and no–reverse transcription controls were included. In addition, for each development stage, three biological repetitions were performed, and the mean cycle threshold value (Ct) for each repetition was obtained from a technical duplicate. Relative expression levels were quantified by the ΔΔCt method using CFX Manager Software version 3.0 (Bio-Rad Laboratories). Reverse transcription qPCR data were normalized to the geometric mean of two housekeeping genes, *GAPDH* and *SDHA*, previously used for bovine preimplantation embryos [22]. In our determinations, *GAPDH* and *SDHA* produced

Table 1
Details of specific primers used in RT-qPCR assays.

Gene	Primer sequences (5′–3′)[a]	Amplicon length (bp)	GenBank accesion number
BMP5	F: GATGTGGGTTGGCTTGTCTT R: CCTGATGAGAGCCGGATTTA	271	NM_001305016.1
BMP7	F: GGCAGGACTGGATCATCG R: GAGCACAGAGATGGCATTGA	191	NM_001206015.1
BMPR1A	F: TTGGGAAATGGCTCGTCGTT R: AGACACAATTGGCCGCAAAC	142	NM_001076800.1
BMPR1B	F: TTTGGGAGGTCGCTAGGAGA R: GCCGCAGCTTCTTGATACAC	132	NM_001105328.1
BMPR2	F: TCTTTCAGCCACCAATGTCCT R: GTTCAGTGGAAATGACCCAGG	158	NM_001304285.1
ID2	F: CGACATCAGCATCCTGTCCTT R: AGAGCCTGTGGATTTGTTGT	145	NM_001034231.2
OCT4	F: AGAAGGGCAAACGATCAAGC R: AGGGAATGGGACCGAAGAGT	173	NM_174580.2
NANOG	F: GTGCTCAATGACAGATTTCAG R: CCGTTGTTTTTCTGCCATTT	150	NM_001025344.1
SOX2	F: ACTTCACATGTCCCAGCACTACCA R: TTCTTTGAAAATGTCTCCCCCGCC	129	NM_001105463.2
GAPDH	F: AGATGGTGAAGGTCGGAGTG R: GAAGGTCAATGAAGGGGTCA	117	NM_001034034.2
SDHA	F: GCAGAACCTGATGCTTTGTG R: CGTAGGAGAGCGTGTGCTT	185	NM_174178.2
ACTB	F: GATCATTGCTCCTCCCGA R: ACTCCTGCTTGCTGATCC	101	NM_173979.3

[a] All the primers were designed with the Primer-BLAST online tool of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

uniform expression levels varying less than 0.5 Ct between control and treated cDNA samples. The target stability function of the CFX96 software determined that the combined M value for *GAPDH* and *SDHA* was M = 0.98 (Coefficient of variation = 0.34). All the study was carried out following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments recommendations [23].

In the case of the positive controls for *BMP5* and *BMP7* expression analysis included in experiment 1, 5 μL of cDNA samples (diluted 1:10) of bovine oviduct epithelial cells from ampulla and isthmus regions was used for RT-qPCR analysis following the protocol described previously. Data obtained were normalized to the expression levels of *ACTB* as housekeeping gene. Samples from three biological replicate of ampulla and isthmus regions at the preovulatory stage were assayed in duplicate for each gene of interest and the reference gene. Relative expression levels were quantified by the ΔΔCt method using CFX Manager Software version 3.0 (Bio-Rad) as described previously.

2.8. Statistical analysis

Data were analyzed using SigmaStat 3.5 and SigmaPlot 10.0 statistical software (Systat Software, Richmond, CA, USA). Cleavage and blastocyst rates between treatments were analyzed using Fisher's exact test. One-way ANOVA, followed by multiple pairwise comparisons using Tukey's test when applicable, was used to detect differences in relative mRNA abundance levels between different embryo stages and treatments. Probability values less than 0.05 were considered statistically significant.

3. Results

3.1. Gene expression analysis of BMP5 and BMP receptors in preimplantation embryos

As a first approach to determine the expression of BMP5 in bovine preimplantation embryos and to evaluate the culture time in which bovine embryos could be possible targets of exogenous BMP ligands, mRNA expression levels of *BMP5* and three BMP signaling receptors (*BMPR1A*, *BMPR1B*, and *BMPR2*) were examined before and after embryonic genome activation in *in vitro*–fertilized embryos using RT-qPCR.

As shown in Figure 1A, *BMP5* transcripts were not detected with RT-qPCR in any of the embryonic stages examined, even when the reactions were performed with cDNA derived from more than 10 embryos. As a positive control, samples of bovine oviduct epithelial cells from isthmus and ampulla regions were also analyzed. In agreement with our previous results [21], RT-qPCR analysis showed that *BMP5* is differentially expressed in isthmus epithelial cells (Fig. 1B; $P < 0.001$). For comparison purposes, expression of *BMP7* mRNA was also analyzed in the same samples considering that *BMP5* and *BMP7* belong to the same BMP subfamily (BMP5/6/7). In contrast to *BMP5*, *BMP7* transcripts were detected throughout preimplantation development (Fig. 1A). The relative abundance of *BMP7* mRNA was increased in the two-cell stage but showed a significant decrease in the blastocyst stage ($P < 0.05$; Fig. 1A). In addition, expression of *BMP7* was detected in cDNA control samples derived from both anatomical regions of the bovine oviduct (Fig. 1B).

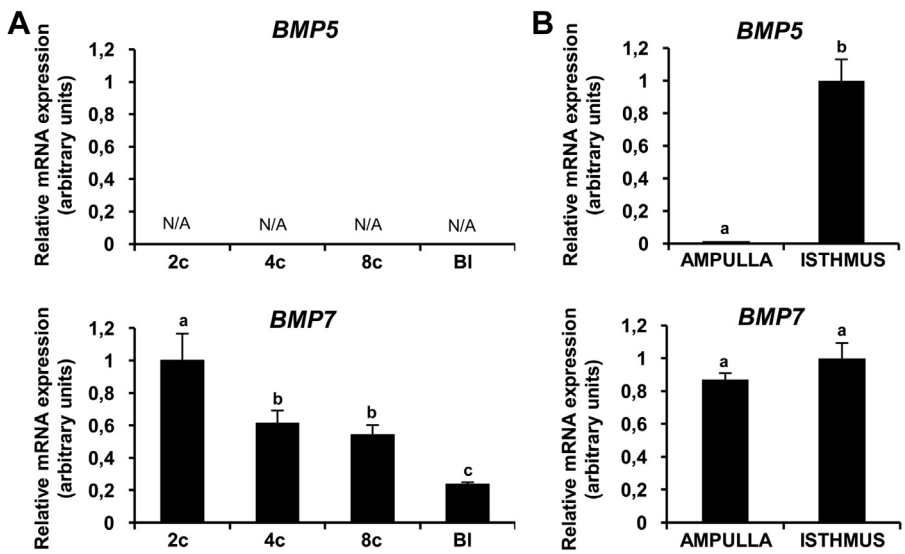

Fig. 1. Reverse transcription quantitative real-time polymerase chain reaction analysis of *BMP5* and *BMP7* messenger RNA (mRNA) expression levels in bovine preimplantation embryos and oviductal epithelial cells from ampulla and isthmus regions. (A) Bars represent *BMP5* and *BMP7* mRNA levels during early development stages normalized to the geometric mean of endogenous *GAPDH* and *SDHA* genes. (B) Bars represent *BMP5* and *BMP7* mRNA levels in oviductal epithelial cells from ampulla and isthmus regions normalized to *ACTB* mRNA levels. Results are expressed as mean ± standard error of the mean. Significant differences ($P < 0.05$) are indicated with different letters. BMP, bone morphogenetic protein; N/A, no amplification; 2c, two-cell stage; 4c, four-cell stage; 8c, eight-cell stage; Bl, blastocyst stage.

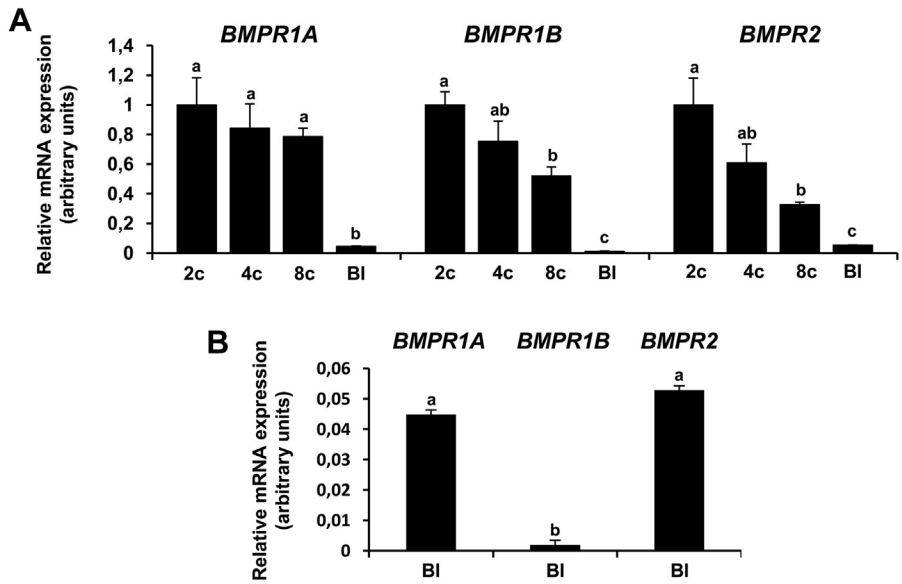

Fig. 2. Reverse transcription quantitative real-time polymerase chain reaction analysis of BMP receptors messenger RNA (mRNA) expression levels in early bovine embryo development stages. (A) Bars represent *BMPR1A*, *BMPR1B*, and *BMPR2* mRNA levels during early development stages normalized to the geometric mean of *GAPDH* and *SDHA* housekeeping genes. (B) Bars represent *BMPR1A*, *BMPR1B*, and *BMPR2* mRNA levels in Day-7 blastocysts normalized to the geometric mean of *GAPDH* and *SDHA* housekeeping genes. Results are expressed as mean ± standard error of the mean. Significant differences ($P < 0.05$) are indicated with different letters. BMP, bone morphogenetic protein; 2c, two-cell stage; 4c, four-cell stage; 8c, eight-cell stage; Bl, blastocyst stage.

Regarding BMP receptors, *BMPR1A*, *BMPR1B*, and *BMPR2* transcripts were detected in all embryonic stages analyzed, showing significant changes in the relative abundance of their mRNA during the early stages of preimplantation development (Fig. 2A). The relative mRNA abundance of the three receptors was greater in early developmental stages (two-cell, four-cell, and eight-cell stages), showing a significant decrease in the blastocyst stage ($P < 0.05$). Moreover, *BMPR2* and *BMPR1A* mRNA showed a significantly higher level of expression than *BMPR1B* mRNA in the blastocyst stage (Fig. 2B; $P < 0.001$).

3.2. Effect of early addition of BMP5 on preimplantation embryo development

On the basis of previous results that showed higher mRNA abundance for BMP receptors during early embryo stages, the effect of early addition of BMP5 to the embryo culture medium on *in vitro* development was assayed. The exogenous addition of 100 ng/mL of recombinant human BMP5 during the first 48 hpi did not affect the cleavage percentage compared to control and vehicle groups, with cleavage rates ranging from 74.6% to 76.9% (Table 2; $P > 0.05$). Similarly, the proportion of embryos that reach the first, second, and third cell cycles at 48 hpi did not show any significant difference between BMP5-supplemented and control groups (Table 2; $P > 0.05$). However, addition of BMP5 to the culture medium during the first 48 hours of *in vitro* embryo culture significantly improved the proportion of cleaved embryos that developed to the blastocyst stage on Day 7 compared to the control and vehicle groups (Table 2; $P < 0.05$).

3.3. Effect of BMP5 on mRNA abundance of genes related to the BMP signaling pathway and pluripotency during early embryogenesis

To determine whether addition of BMP5 to the embryo culture medium influences the expression levels of developmentally important genes, mRNA abundance of *ID2* (a

Table 2
Effect of early addition of BMP5 to the embryo culture medium on development of *in vitro*–fertilized bovine embryos.

Treatment	Oocytes (N)	Cleaved, n (%[c])	2c, n (%[d])	4c, n (%[d])	8c, n (%[d])	Day-7 blastocysts, n (%[e])
Control	286	220 (76.9)	27 (12.3)	83 (37.7)	110 (50.0)	62 (28.2)[a]
Vehicle	158	112 (70.9)	12 (10.7)	44 (39.3)	56 (50.0)	30 (26.8)[a]
BMP5	279	208 (74.6)	20 (9.6)	89 (42.8)	99 (47.6)	81 (38.9)[b]

The table shows the total number of oocytes used and embryos developed in a total of five different IVF experiments. Zygotes were cultured in CR1aa medium assaying three different treatments: (1) control (CR1aa); (2) vehicle control (CR1aa + 0.04 mM HCl); and (3) BMP5 treatment (CR1aa + 100 ng/mL of BMP5). At 48 hours post insemination (pi), embryos were transferred to CR1aa supplemented with 10% fetal bovine serum and cultured until Day 7 pi.
[a,b]Different letters between treatments indicate significant differences ($P < 0.05$).
[c] Percentage of presumptive zygotes that undergo cleavage evaluated at Day 2 pi.
[d] Percentage of cleaved embryos that developed to the two-cell (2c), four-cell (4c), and eight-cell (8c) stages at Day 2 pi.
[e] Percentage of cleaved embryos that developed to the blastocyst stage evaluated at Day 7 pi.

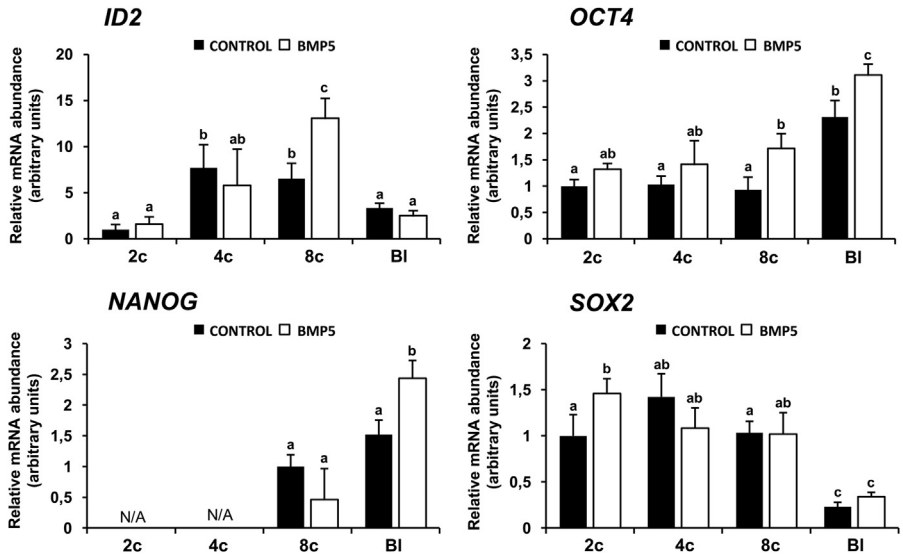

Fig. 3. Relative messenger RNA (mRNA) abundance of *ID2*, *OCT4*, *NANOG*, and *SOX2* in *in vitro*–derived bovine embryos cultured in the presence or absence of BMP5. Bars represent the relative abundance of *ID2*, *OCT4*, *NANOG*, and *SOX2* transcripts in two-cell (2c), four-cell (4c), eight-cell (8c), and blastocyst (Bl) stage embryos normalized to the geometric mean of *GAPDH* and *SDHA* housekeeping genes. The control group is represented by black columns and BMP5-treated embryos are represented by white columns. Results are expressed as mean ± standard error of the mean. Different superscripts indicate significant differences ($P < 0.05$) between treatments. Data are obtained from three replicates of independent groups of embryos: group of 10 embryos for 2c, 4c, and 8c stages and group of four embryos for the Bl stage. BMP, bone morphogenetic protein; N/A, no amplification.

target gene of BMP signaling) and *OCT4*, *NANOG*, and *SOX2* (pluripotency-associated genes) was examined in controls and BMP5-treated embryos during different stages of development.

The relative abundance of *ID2* gene transcripts detected by RT-qPCR in embryos cultured in the presence or absence of BMP5 is shown in Figure 3. *ID2* mRNA was detected in all embryonic developmental stages analyzed. Transcript levels increased after the two-cell stage, showing higher relative abundance during the four-cell and eight-cell stages (before embryonic genome activation) and a significant decrease in the blastocyst stage (Fig. 3). This mRNA profile was observed in embryos derived both from cultures with and without BMP5. However, a significant increase in the relative abundance of *ID2* mRNA was observed in eight-cell embryos supplemented with BMP5 compared with controls without BMP5 (Fig. 3; $P < 0.05$). No significant differences were found between BMP5-treated and control groups for the other embryonic stages examined.

In the case of pluripotency genes, abundance of *OCT4* mRNA at eight-cell and blastocyst stages was greater in embryos cultured in medium with BMP5 than in control embryos (Fig. 3; $P < 0.05$). Likewise, addition of BMP5 to the culture medium significantly increased *NANOG* mRNA levels in the examined blastocysts compared with embryos derived from the control group (Fig. 3; $P < 0.05$). When analyzing mRNA expression for *SOX2*, a significant increase of its mRNA abundance could be observed in BMP5-treated embryos at the two-cell stage (Fig. 3; $P < 0.05$). However, no significant differences in relative mRNA expression for *SOX2* was found in the other embryonic stages analyzed (four cells, eight cells, and blastocyst) compared to the control group (Fig. 3).

4. Discussion

To evaluate the role of BMP5 during early bovine embryo development, the present study investigated the gene expression of BMP5 and BMP receptors in *in vitro*–fertilized embryos and the effect of exogenous BMP5 supplementation on preimplantation embryo development.

Messenger RNA transcripts of BMP5 were not detectable at two-cell, four-cell, eight-cell, and blastocyst stages, suggesting no significant level of gene expression of BMP5 and also that this factor would not be produced by the embryo, at least in the embryonic stages analyzed. This finding reinforces the hypothesis of its maternal origin and remarks the importance of BMP5 as an oviductal molecule that could exert a paracrine action on early bovine embryo. To determine if other members of the family would yield the same result, expression of *BMP7* was also examined. In contrast to *BMP5*, *BMP7* mRNA was detected in all development stages analyzed showing high levels of abundance during the two-cell stage and low levels in the blastocyst stage. These results suggest that during early embryogenesis, the transcript abundance of BMP ligands in bovine embryos is differentially regulated. This observation agrees with the results of Kues et al. [17] in bovine embryos produced *in vivo* which also reported a stage-specific expression of *BMP4* and *BMP15* transcripts during embryonic development.

On the other hand, to evaluate the moment of early embryogenesis in which the bovine embryo could be target of BMPs, gene expression of three specific receptors of the BMP signaling pathway was analyzed in *in vitro*–produced embryos. High abundance of *BMPR1A*, *BMPR1B*, and *BMPR2* transcripts were observed between two- and eight-cell

stages, before maternal–embryonic transition. In contrast, a significant decrease in *BMPR1A*, *BMPR1B*, and *BMPR2* mRNA levels was evidenced in the blastocyst stage. These results are indicative of a precise temporal regulation of transcript abundance for type I and type II BMP receptors during bovine embryo development. The high abundance of these transcripts in early stages suggests that BMPs present in the oviductal microenvironment or synthesized by the embryo itself could interact with their receptors and exert a paracrine or autocrine action on the bovine embryo during its transit through the oviduct.

Considering the temporal gene expression pattern of specific BMP receptors in bovine embryos, it was decided to incorporate BMP5 to the culture medium during the first 48 hours of the *in vitro* embryo culture and determine its effect on the cleavage and developmental rates. The concentration of recombinant BMP5 used in the present study (100 ng/mL) was selected according to the concentration of others BMPs and TGF-β growth factors used in other studies that showed effect on *in vitro* embryo development [19,24]. Our results show that addition of 100 ng/mL of BMP5 had no effect on the cleavage and developmental rates evaluated 48 hpi. A similar proportion of embryos reaching the first, second, and third cell cycles 2 days pi were found both in the presence and absence of BMP5. These observations are consistent with previous studies showing that the addition of BMP2 and BMP4 to *in vitro* bovine maturation medium and embryo culture medium did not alter the first cellular divisions and percentages of cleaved embryos [19,25].

Although early addition of BMP5 to the embryo culture medium had no effect on the cleavage rate, a significantly higher proportion of cleaved embryos in the BMP5 group developed to the blastocyst stage. This result suggests that the culture of the embryos with BMP5 just for the first 48 hours is enough to improve the development to the blastocyst stage. Previous studies with other members of the BMP family showed different effects in relation to blastocyst development. Fatehi et al. [25] found that exogenously added BMP2 and BMP4 to maturation medium did not affect oocyte maturation and subsequent blastocyst formation. In addition, La Rosa et al. [19] reported that addition of BMP4 and the inhibitor Noggin to embryo culture medium reduced blastocyst developmental rates of *in vitro*–fertilized bovine embryos. However, Hussein et al. [18] reported that BMP15 added during IVM of bovine oocytes significantly improved the blastocyst yield. The medium composition, culture conditions, concentration of the recombinant factor, and the moment it is added to the culture, as well as the presence or absence of serum in the culture medium, are undoubtedly variables that have to be considered before evaluating and comparing these results [18,26–28]. In this sense, it is important to emphasize the decision in the present study to supplement BMP5 only at the beginning of the embryo culture and until 2 days pi because higher levels of BMP receptor transcripts were detected during the first stages of development, before maternal–embryonic transition. In addition, the incorporation of the protein during the early stages of development somehow simulates the physiological effect the factor could exert *in vivo*, given that the bovine embryo only stays 3 to 4 days in the oviduct before it enters the uterus.

It is known that besides their effect on ultrastructural, metabolic, and cryotolerance characteristics of embryos, *in vitro* culture conditions can also have an impact on the expression of genes that are essential for development [29–31]. Considering this, it was decided to evaluate whether early addition of BMP5 to the embryo culture medium would affect the relative abundance of developmentally important genes in embryonic stages before and after embryonic genome activation. The first gene assayed was *ID2*, a target gene of BMP signaling. *ID2* is a transcription factor that participates in the regulation of pluripotency and differentiation in embryonic stem cells, and together with other transcriptional factors, it is required for maintenance and differentiation of trophoblast cells [32–34]. We found that *ID2* is expressed throughout the early development stages analyzed, but it showed a differential profile because the relative abundance of its transcript increased in the four- and eight-cell stages and significantly decreased in the blastocyst stage. In addition, it was observed that embryos cultured in the presence of BMP5 showed a significant increase in the abundance of *ID2* mRNA in eight-cell stage embryos during the main phase of activation of the embryonic genome. In agreement with our results, transcriptome analysis by microarray in *in vivo*–produced bovine embryos reported that *ID2* together with other genes (*ZO3*, *CLDN4*, *TP53*) increases its transcriptional levels between four- and eight-cell stages during the activation of the bovine embryonic genome [17]. The fact that the transcriptional levels of BMP receptors and the *ID2* gene are higher during the early stages and significantly lower in the blastocyst stage allows us to infer that the BMP signaling pathway could play a fundamental role in the early stages of development, during the transit of the embryo through the oviduct. This would explain the positive effect of early addition of BMP5 and the negative effects observed when BMP signaling is overactivated in more advanced stages. For example, it has been reported that degenerated bovine embryos that do not reach the blastocyst stage show a significant increase in the levels of expression of BMP4, BMPR1A, and ID3, suggesting that a late imbalance in the expression of BMP signaling components can lead to abnormal embryonic development [35]. These results are consistent with other studies showing that overactivation of the BMP4 pathway induces a high rate of apoptosis during early embryonic development [36] and that the addition of BMP4 to the embryo culture medium until Day 7 decreases blastocyst production of IVF embryos [19].

Given that TGF-β family members, including BMPs, play an important role in the regulation of embryonic stem cell pluripotency and differentiation [37], it was also examined whether BMP5 affects gene expression levels of *OCT4*, *NANOG*, and *SOX2*, three important markers, and transcription factor regulators of pluripotency during early embryo development. The expression profiles obtained for the three pluripotency genes are in accordance with previous reports on IVF bovine embryos [38,39]. Particularly, *NANOG* mRNA was not detectable by RT-qPCR neither in the two-cell stage nor in the four-cell stage

embryos derived from control and BMP5-treated groups, and transcript levels are detectable from the eight-cell stage onward. This is consistent with previous reports showing that the expression of this gene starts during the major onset of embryonic genome activation [38,39]. Taking into account that four- and eight-cell embryos were collected at the same time (48 hpi) and that it has been reported a relationship between embryo developmental speed and the temporal gene expression pattern [40], we cannot rule out the possibility that the non-expression of *NANOG* in four-cell embryos collected 48 hpi could also be associated with a slower development of these embryos. On the other hand, our results showed that the early addition of BMP5 to the culture medium produced a significant increase in the relative abundance of *SOX2* mRNA in two-cell-stage embryos, *OCT4* mRNA in eight-cell-stage embryos, and *NANOG* and *OCT4* mRNA in blastocysts. These results suggest that BMP5 could affect pluripotency gene expression in early stages before maternal–embryonic transition and also in more advanced stages after embryo genome activation. However, the mechanism by which BMP5 could exert this effect is not known and needs to be explored.

Studies using mouse embryonic stem cells have reported that the BMP signaling pathway has a close relationship with OCT4, NANOG, and SOX2. Once the SMAD proteins involved in the BMP signaling pathway (SMAD1, 5, 8, and 4) enter the nucleus, they have the ability to bind DNA directly and interact with specific transcription factors to regulate expression of different target genes [41]. Between the many cofactors that can interact with SMAD complexes are included the transcriptional factors OCT4, NANOG, and SOX2 [37,42]. Moreover, SMAD1 with Stat3 (transcriptional factor activated by the LIF pathway) and Tcf3 (transcriptional factor activated by the Wnt pathway) can bind to the promoters of OCT4, NANOG, and SOX2 and regulate expression of these three genes to ensure the maintenance of pluripotency [43,44]. This confirms that BMP signaling could have a direct effect on pluripotency gene expression. Considering that OCT4, NANOG, and SOX2 play an essential role in the specification of the inner cell mass and trophectoderm lineages [38,45–47], the changes in the expression of these three genes in embryos treated with BMP5 and the positive effect of BMP5 on the blastocyst rate allow us to suggest that BMP signaling could contribute to activation of genes later involved in the early lineage specification, facilitating the first step of differentiation necessary for blastocyst formation.

It is important to note that although BMP5 was supplemented during the initial 2 days of the IVC, a long-term effect was observed 5 days later not only by the increase in the proportion of developed blastocysts but also by the changes in the gene expression profile of those blastocysts. It would be interesting to determine if these long-term effects could be associated with some kind of epigenetic control mechanism exerted by BMP5. It is known that the early embryo during its transit through the oviduct undergoes epigenetic changes, including DNA methylation and histone modifications, that can be altered by microenvironmental conditions and have a significant impact on further embryonic development [48,49]. Considering that the oviductal fluid and the coculture with bovine oviductal epithelial cells have shown to induce changes in the expression levels of DNA methylation genes in the early embryo [50,51], it would be interesting to explore whether maternal proteins such as BMP5 can contribute to epigenetic regulation during preimplantation embryo development.

In conclusion, the results of the present study show that (1) BMP5 is not expressed during preimplantation development of IVF bovine embryos, reinforcing previous studies that provide evidence of its maternal origin; (2) mRNAs for BMP receptors (BMPR1A, BMPR1B, BMPR2) are detected throughout early development; however, the higher relative abundance of these transcripts at two-, four-, and eight-cell stages respect to blastocyst stage suggests that the embryo could be target of BMPs during its transit through the oviduct; and (3) the early supplementation of BMP5 to embryo culture medium improves the proportion of cleaved embryos reaching the blastocyst stage and increases the relative mRNA abundance of developmentally important genes, suggesting a possible embryotrophic action. Although this study is only focused on mRNA expression analysis without evaluation of protein expression, it provides new evidence to suggest that BMP signaling could have a potential role in the preimplantation development of bovine embryos, particularly at early stages of development, when the embryo is in transit through the oviduct. Further studies to elucidate the specific mechanisms by which BMP5 promotes bovine embryonic development are currently in progress.

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Competing Interests

The authors declare that they have no competing interests.

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