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GENE EXPRESSION STUDY IN BOVINE *IN VITRO* PRODUCED TWO CELL EMBRYOS

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ABSTRACT

The objective of this study was to examine expression of certain developmental genes in bovine 2- cell stage embryos. Bovine oocytes were recovered from abattoir originated ovaries and subjected for maturation and fertilization *in vitro*. mRNA extraction and subsequent reverse transcriptase PCR was done to evaluate expression of GJA1, ACTB, PAPOLA and POU5F1 genes. Expression of these genes in 2- cell stage embryos might be due to its importance in the subsequent development and embryonic genome activation.

KEYWORDS: Bovine oocytes, PCR, GJA1, ACTB, PAPOLA, POU5F1.

INTRODUCTION

Pre implantation embryo development requires a well-orchestrated expression of genes derived from the maternal and embryonic genome (Kidder, 1992).The early embryonic growth phase includes a series of modulations of transcripts and proteins within the post fertilized oocyte, which are necessary for the embryo to achieve its own independent developmental competence quality. The oocyte supplies mRNAs that sustain embryonic development up to the stage of maternal-embryonic transition and a few mRNAs persist throughout development until the blastocyst stage. The oocyte content of mRNA are related to the developmental competence of oocytes in later stages of reproduction. In particular, the post fertilized oocyte contains transcription factors involved in the minor and major activation of the embryonic genome at the 2-4 cell stage and 8 cell stage respectively (Viuff *et al*., 1996). Bovine 2-cell embryos are already transcriptionally competent and active although major embryonic genome activation occurs at the eight-cell stage (Memili *et al.*, 1998).

Initiation of EGA is a species-specific time point, which occurs at the two-cell stage in mouse (Schultz, 1993), at the four-cell stage in human (Braude *et al*., 1988), and at the late eight-cell stage in bovine embryos (Memili and First, 2000). EGA is considered to be the most critical event for viability during early development (Meirelles *et al.*, 2004), and is associated with early differentiation events, successful embryo implantation, and fetal development (Niemann and Wrenzycki, 2000). Various studies have shown the occurrence of minor EGA as early as the two-cell stage before the activation of embryonic genome at the eight-cell stage in bovine species (Memili and First, 2000). This transition is crucial in genome reprogramming and acquisition of totipotency by the embryo (Baroux *et al*., 2008). Epigenetic modifications are involved in gene expression regulation in the embryo and play a crucial role in controlling reprogramming events during early embryogenesis (Li, 2002).

The objective of this study was to evaluate expression of four developmentally important genes (GJA1, ACTB, PAPOLA and POU5F1) in 2 cell embryo. Growth and differentiation of embryo is dependent on intercellular communication via gap junctional protein GJA1. Gap junctions act as passage for sharing low molecular weight metabolites and second messengers. Beta actin (ACTB) is a key component of cytoskeleton, with critical roles in a wide range of cellular processes, including cell migration, cell division, and the regulation of gene expression. These functions are attributed to the ability of actin to form filaments that can rapidly assemble and disassemble according to the needs of the cell. For its ubiquity nature in all cell types it is considered as housekeeping gene (Brevini-Gandolfi *et al*., 1999; Bunnell *et al.*, 2011). Translational activation of mRNAs derived from the mother is important for expression of genes during oocyte maturation and this requires Poly A Polymerase- α (PAPOLA) which in turn synthesizes poly A tail in the mRNA. POU5F1 is a member of the POU family of transcriptional activators which contain the DNA-binding POU domain (Ryan and Rosenfeld, 1997). The POU5F1 gene is essential for the maintenance of totipotency / pluripotency in embryonic stem cells and primordial germ cells, a progressive loss of POU5F1 has been associated with loss of pluripotency (Okamoto *et al*., 1990; Yeom *et al*., 1996; Gendelman *et al*., 2010).

MATERIALS & METHODS

***in vitro* embryo production:** Ovaries were collected from local cattle slaughter house and processed within 2-3 hours as per standard protocol. Isolation of oocytes, grading of cumulus oocyte complexes were performed as per Dutta *et al.* (1998). Good quality oocytes were considered for subsequent *in vitro* maturation and *in vitro* fertilization study. *In vitro* maturation (IVM) and *in vitro* fertilization

(IVF) was done as per Dutta *et al.*, (2013) and Raj *et al.*, (2016). Briefly, bovine ovaries were collected from local slaughter house and transported in 0.9% NaCl solution. Oocytes were collected by aspiration following slicing technique for maximum recovery into a collection medium (Medium 199, BSA and 75 µg/mL Gentamicin). Morphologically good quality cumulus oocyte complexes (COCs) were selected and *in vitro* matured in Medium 199 containing 200 mM L-glutamine solution, 10% FBS. The maturation status was observed by examining degree of expansion of cumulus oophorus and extrusion of first polar body. *In vitro* matured oocytes were co-incubated with capacitated sperm in Bracket and Oliphant medium (Bracket and Oliphant, 1975) at 38.5°C, 5% CO_2 in air and saturated humidity for 20-22 hours. The fertilized oocytes were cultured in mCR2aa media containing 5% FBS and supplemented with 2% essential amino acids (v/v), 1% non-essential amino acids (v/v), 1% α- glutamic acid and 0.3% BSA at 38.5°C upto 2- cell stage embryo.

Isolation of mRNA from embryos

mRNA was extracted from approximately 50 numbers of 2- cell stage embryos using Oligotex direct mRNA minikit (Qiagen, 72022). RNA quality and quantity was assessed by Nanodrop spectrophotometer.

Reverse transcription

For a 20µl reaction of first strand cDNA synthesis the following components are mixed: 1 µl oligo(dT)12-18, 1 µl dNTP mix(10mM each), 5 µl mRNA sample were heat mixed to 65°C for 5 min and quick chill on ice. Then 4 µl 5X first strand buffer, 2 µl 0.1M DTT and 1 µl RNase inhibitor was added and incubate at 42°C for 2 min. Then 1 µl (200U) of Superscript II RT was added and incubated for 42°C for 50 min followed by inactivation at 70°C for 15 min.

PCR amplification

For a 25 µl reaction the following components are mixed: 12.5 µl OneTaq Hot Start 2X master mix with standard buffer, 0.5 µl 10 µM forward primer, 0.5 µl 10 µM revese primer, 5.0 µl cDNA template and 6.5 µl nuclease free water. PCR was carried out in an automated thermal cycler using the following conditions: initial denaturation at 94°C for 30 sec, 32 cycles of 94°C for 15 sec., 63°C for 30 sec. and 68°C for 30 sec and final extension at 68°C for 5 min.

RESULTS & DISCUSSION

In the present study the PCR product of GJA1, PAPOLA, ACTB and POU5F1 genes, upon 2% agarose gel electrophoresis revealed an expected amplicon of 143bp, 252 bp, 154 bp and 123 bp respectively. Expression of these genes in 2- cell stage embryo in the current study was consistent with previous observations (Wrenzycki *et al.*, 1996; Mishra *et al.*, 2010; Massicotte *et al.*, 2006; Niakan and Eggan, 2013) supporting the expression of these genes in bovine early embryos.

In cattle, GJA1 was detected in COCs, immature, *in vitro* matured oocytes, *in vitro* produced zygotes, 2-4-cell embryos, 8-16-cell embryos and morulae (Wrenzycki *et al.*, 1996, Mishra *et al.* 2010). Similarly, PAPOLA gene expression was detected in all embryonic stages (Wrenzycki *et al.*, 2005; Mishra *et al.*, 2010). However, Lonergan *et al.* (2003) suggested the presence of GJA1 in all stages of developing embryos including blastocyst stages. In mouse embryos, GJA1 mRNA and protein are detected from the four-cell stage onwards. Transcription levels of GJA1 at day 2 and 3 were higher in faster-developing embryos independently of culture conditions. At day 4 however, a higher GJA1 transcription was observed in *in vivo* produced embryos independently of the embryonic developmental speed (Rizos *et al.*, 2002; Gutierrez-Adan *et al.*, 2004).

TABLE1: Primer details of different mRNA transcripts of bovine embryo

Gene Name	mRNA Accession no	Primers (5' - 3')	Amplicon size (base pairs)
Poly(A) polymerase alpha (PAPOLA)	NM_176647.2	GTTTCCTCGGTGGTGTTTCCTGGGCTATGC TGGAGTTCTGTTGTGGGTATGCTGGTGTAA	252
gap junction protein alpha 1 (GJA1)	NM_174068.2	TGTTAGGGATAGGCGAGTGG GGGTGTGTGGGAAAGAAAAA	143
Beta actin (ACTB)	NM_173979.3	GCTGCGTTACACCCTTTTTC CACCTTCACCGTTCCAGTTT	154
POU class 5 homeobox 1 (POU5F1)	NM_174580.2	TGCAGCAAATTAGCCACATC AATCCTCACGTTGGGAGTTG	123

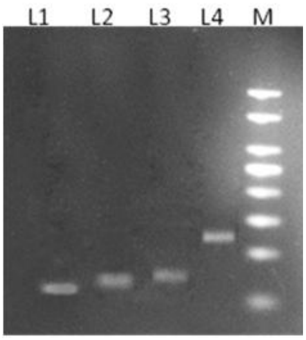

FIGURE 1: Agarose gel electrophoresis of PCR amplified genes, L1: 123 bp POU5F1, L2: 143 bp GJA1, L3: 154 bp ACTB and L4: 252 bp PAPOLA genes. M indicates 100 bp DNA marker.

In the bovine, ACTB has been frequently used as a housekeeping marker (Robert, 2002; Bilodeau-Goeseels and Schultz, 1997) and is well characterized in the bovine oocyte. The amount of ACTB mRNA varies across oocyte maturation and the embryo preimplantation period (Robert, 2002; Bilodeau-Goeseels and Schultz, 1997; Brevini-Gandolfi *et al*., 1999). However, the length of the poly(A) tail is stable during maturation up to the 2-cell embryo, suggesting that the translation of ACTB should be relatively constant (Brevini-Gandolfi *et al*., 1999). ACTB is translated continuously during oocyte maturation and early embryo development to the maternal to embryonic transition stage (Massicotte *et al*., 2006) and the levels decrease regularly as the embryo develops until it reaches the eight-cell stage followed by a sharp increase at the blastocyst stage (Bunnell *et al*., 2011; Bettegowda *et al*., 2006). The mRNA of different housekeeping genes like b-actin, GAPDH, ubiquitin, lamin B, tubulin, histone H2A, cytochrome b and histone H3 are present throughout oocyte maturation and embryonic development as determined by semi-quantitative and quantitative PCR (Robert, 2002; Bilodeau-Goeseels and Schultz, 1997).

Expression of POU5F1 in bovine IVF embryos was reported in all the developmental stages, starting from immature and mature oocytes, two-cell, four-cell, eight- to 16-cell, morula, and blastocyst stages (Van Eijk *et al*., 1999; Daniels *et al*., 2000; Khan *et al*., 2012; Niakan and Eggan, 2013). In pig, POU5F1 transcripts were high in the oocyte followed by reduction at the four- to eight-cell stage, and then leading to an increase at the blastocyst stage in both IVF and cloned embryos (Lee *et al*., 2006; Brevini *et al*., 2007). Zygotic POU5F1 mRNA and protein are initially expressed in all cells of mouse embryos from the 4-cell (Nichols *et al*., 1998) and 8-cell stage, respectively (Liu *et al*., 2004; Palmieri *et al*., 1994).

Early embryos possess an mRNA population very similar to that of the oocyte and early eight-cell embryos and display an mRNA profile comparable with that found in the blastocyst (Natale *et al*., 2000; Vigneault *et* al., 2009). Jiang *et al*., (2014) reported that between the oocytes and the 2-cell embryos, 166 of the 324 differentially expressed genes were down-regulated indicating rapid degradation of the maternally stored transcripts. Gene ontology analysis indicated significant over-representation of elements involved in cell cycle and mitosis II suggesting that the 2-cell embryos reprogrammed its cell cycle regulation from that of an arrested state to an active mode of cell division through changes of gene expression (Jiang *et al*., 2014).

CONCLUSION

GJA1, ACTB, PAPOLA and POU5F1 genes which are considered as developmentally important genes were amplified from the *in vitro* produced bovine 2-cell embryos. It can be concluded that expression of these genes in early embryos might have important role in subsequent embryonic developmental competence *in vitro*.

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