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Expression pattern of *OCT 4, NANOG* and *HSP 90 β* gene in oocytes and various stages of *in vitro* produced sheep embryos

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ABSTRACT

The study was conducted to assess the expression pattern of pluripotent genes *(OCT 4* and *NANOG)* and stress response gene *HSP 90 β* by quantitative real time polymerase chain reaction (qRT-PCR) in immature and mature oocytes and 2 cell, 4 cell, morula stages of *in vitro* fertilized (IVF) and parthenogenetically activated (PA) preimplantation ovine embryos to investigate the similarity between them in turn for the establishment of embryonic stem cells (ESCs) from parthenote embryos. The pluripotent related genes showed almost similar pattern of expression between IVF and PA embryos at all the developmental stages which indicated that PA embryos could be used as an alternate source for the production of embryonic stem cells. The stress response gene showed increased levels after *in vitro* maturation and non significant differences between IVF and PA embryos, thus, indicating no significant differences between the quantum of stress experienced by the 2 groups of embryos which would not affect the development of embryo quality and in turn affect the parthenote embryonic stem cells (PESCs).

Key words: Genes, *In vitro* fertilization, Parthenogenetic activation, Quantitative real time PCR, Sheep

Parthenogenesis has attracted wide attention due to the potential of deriving pluripotent lines. The attractive perspective of deriving stem cells from the alternative embryo source would be from the parthenogenetic embryo. The advantage for IVF embryos and limitations in PA embryos are mainly due to the absence of paternal genes for the embryonic development. The complete exact gene expression pattern in PA embryos has not yet been explored. Hao *et al.* (2009) revealed the similarity of gene expression between PA and IVF or *in vivo* derived blastocysts, but the developing potential of pESCs is still under investigation. Genes that differ between IVF and PA preimplantation embryos would serve as potential candidates for further research. In this view, the present study analyzed the expression pattern of panel of genes related to pluripotency and stress response in oocytes and various stages of IVF and PA preimplantation embryos.

MATERIALS AND METHODS

Retrieval of cumulus oocyte complexes (COCS): Sheep ovaries were obtained from local abattoir in medium containing 0.9% normal saline (NS) with penicillin (100 IU/ml) and streptomycin (50 mg/ml) at 30–35°C within 2 h of slaughter. Ovaries were washed 2–3 times under running tap water and rinsed 5 times in 0.9% normal saline at 37–38°C. Cumulus oocyte complexes (COCs) were retrieved by slicing technique and graded as grade A, B and C based on their cumulus cells investment and cytoplasm homogeneity (Wani *et al*. 2000).

Brilliant cresyl blue (BCB) test: Developmentally competent COCs were selected based on brilliant cresyl blue (BCB) test as oocytes with a blue cytoplasm or grown oocytes (BCB + ve) and oocytes without a blue cytoplasm (colourless) or growing oocytes (BCB - ve) as outlined by Rodriguez-Gonzalez *et al.* (2002).

In vitro maturation: Only BCB + ve COCs were subjected to *in vitro* maturation medium supplemented with hormones and growth factor and *in vitro* matured for 24 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air (Wani *et al*. 2000). The matured oocytes were graded as described by Kobayashi *et al*. (1994). *In vitro* matured oocytes were randomly and equally assigned for *in vitro* fertilization (IVF) and parthenogenetic activation (PA) groups.

In vitro fertilization (IVF): Approximately, 2–5 µl of the motile sperm suspension obtained by swim up technique (Parrish *et al.* 1988) was used to inseminate the oocytes in IVF droplets (10–15 oocytes/75 µl droplet) to achieve the final concentration of 2 million sperm/ml and co-incubated for 24 h.

Parthenogenetic activation (PA): Matured oocytes were activated as per Bebbere *et al.* (2010). Briefly, oocytes were exposed for 5 min in 5 µM ionomycin in TCM 199

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Table 1. Primer sequence

Product	Sequence	Size (bp)	Accession No
YWHAZ	FP: GCATCCCACAGACTATTTCCRP: GCAAAGACAATGACAGACCA	120	BM446307
GAPDH	FP: TTCCACGGCACAGTCAAGGRP: CGTACTCAGCACCAGCATCA	118	NM_001190390.1
SDHA	FP: CAGCAGAAGAAGCCGTTTGARP: CACAGTCGGTCTCGTTCAAA	127	DQ386895.1
OCT 4	FP:GAGGAGTCCCAGGACATCAARP: CCGCAGCTTACACATGTTCT	204	HQ907734.1
NANOG	FP: GATCTGCTTATTCAGGACAGRP: TGCATTTGCTGGAGACTGAG	203	FJ970651.1
HSP 90 ß	FP: TGGAGATCAACCCTGACCARP: GGGATCCTCAAGCGAGAAG	142	NM_001079637.1

supplemented with 10% FBS followed by 3 h in 2 mM 6-dimethylaminopurine (6-DMAP) in TCM 199 supplemented with 10% FBS.

In vitro culture (IVC): In both the IVF and PA groups, the presumptive zygotes were transferred separately into pre-equilibrated 50 µl IVC droplets (10–15 zygotes/droplet) containing 2-step synthetic oviduct fluid (SOF) medium and developmental competence of embryos at various stages, viz. 2-, 4–, 8–16 cell, morula and blastocyst were recorded in both IVF and PA groups.

Gene expression analysis: Expression pattern of genes related to pluripotency (*OCT 4* and *NANOG*) and stress responsive gene (*HSP 90* ß) along with standard house-keeping genes *YWHAZ, GAPDH, SDHA* were analyzed (Goossens *et al*. 2005) using quantitative real time polymerase chain reaction (qRT-PCR) in both immature and mature oocytes and at various developmental stages (2-, 4– cell and morula) of preimplantation embryos produced under IVF and PA groups. The primers sequence used are given in Table 1. The gene expression status of all the genes analyzed was subjected to 2-way ANOVA with Bonferroni multiple comparison test in graph pad prism 5 software.

RESULTS AND DISCUSSION

The panel of genes related to pluripotency (*OCT 4* and *NANOG*) and stress response (*HSP 90 ß*) were analyzed against the standard reference genes *(GAPDH, YWHAZ, SDHA)* in both immature and mature oocytes and at various preimplantation embryo stages (2-, 4- cell and morula) derived from both IVF and PA groups in triplicate using SYBR green chemistry in quantitative real time PCR and are presented in Fig. 1. The "Ct" value for each gene was calculated at a threshold of 40 fluorescence units. Negative controls were performed for each gene as a control.

In the present study, less significant difference of *OCT 4* expression was observed between IVF and PA embryos at 2- and 4-cell stages. (Fig. 1). The uniform expression of *OCT 4* (Kumar *et al.* 2012) between 2 groups of embryos indicated that the embryos had undergone proper genome activation and there was no difference in pluripotency of IVF and PA embryos. Hence, the PA embryos could serve as an alternative source of IVF embryos for the ESC isolation. Indeed, the ESCs could be isolated only from the embryos that expressed *OCT 4* and *NANOG*. Furthermore, the present observations of morula embryos were in contrast to the findings of Bebbere *et al*. (2010) with decreased expression of *OCT 4* in case of PA embryos than IVF embryos.

The important maternal factor *NANOG* played a critical role in formation and maintenance of pluripotency. The detection of *NANOG* in the oocytes also confirmed the pluripotency of embryos. The *NANOG* gene worked in association with other key pluripotent factors such as *OCT 4* and *SOX 2* to control the set of genes regulating the pluripotency. The expression pattern of *NANOG* showed no significant differences between IVF and PA embryos at 4-cell and morula stages except with minute increase in transcripts of 2-cell stage IVF embryos (Fig. 1). Our results

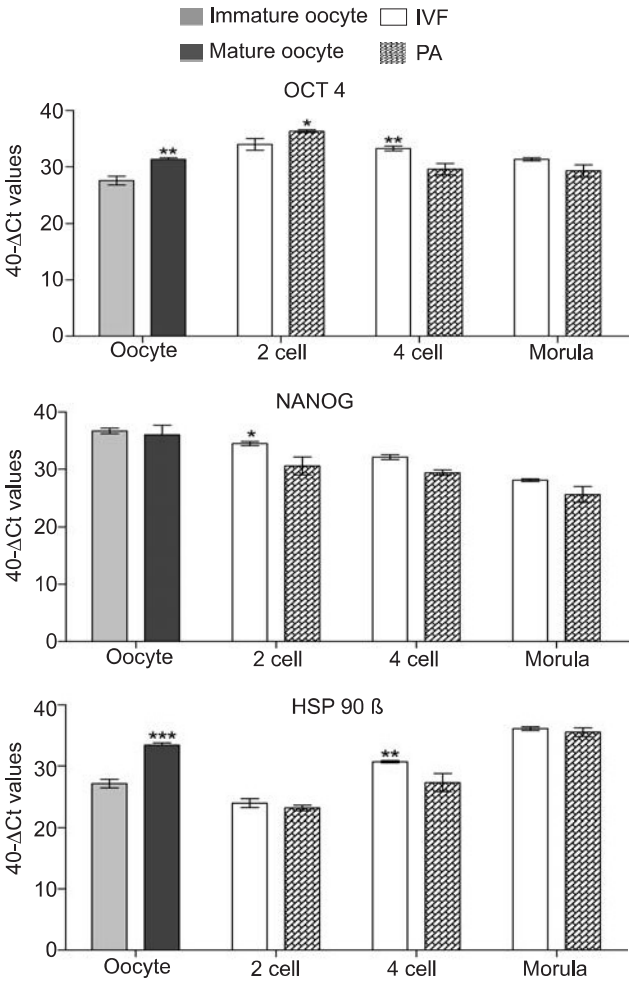

Fig. 1. Relative abundance (mean±SD) of *OCT 4, NANOG* and *HSP 90 beta* in oocytes, *in vitro* fertilized (IVF) and parthenogenetically activated (PA) sheep embryos at various developmental stages.

are in accordance with the findings of Bebbere *et al*. (2010) who reported a similar expression pattern of *NANOG* gene in IVF and PA embryos at morula stage and Liu *et al.* (2010) who observed no differences in pluripotent markers which included *OCT 4, NANOG, SOX 2* and *Myc* between the IVF and PA blastocysts by using microarray analysis. Based on the pluripotency of *NANOG* gene expression pattern in IVF and PA embryos of the present study, PA embryos could be used as an alternative source for IVF embryos in ESC isolation.

The RA of *HSP 90 ß* showed similar expression pattern between IVF and PA preimplantation embryos at all developmental stages except an up regulation in oocytes after IVM and increased transcripts in 4 cell stage IVF embryos (Fig. 1). The *HSP 90 ß* started expressing and the embryonic genome activation in ovines occurred over several cycles from 1-cell to 16 cell stages. Similarly, Bebbere *et al*. (2010) also observed no significant differences in the expression pattern of *HSP 90 ß* in 2-cell, morula and blastocysts stages of IVF and PA embryos. It could be inferred from our results that there were no significant differences between the quantum of stress experienced by IVF and PA groups of embryos which would affect the development of embryo quality and in turn affect the parthenote ESCs.

The pluripotent related genes *(OCT 4* and *NANOG)* showed similar pattern of expression between IVF and PA embryos at morula stage indicating that PA embryos could be used as an alternate source for the production of embryonic stem cells. The stress response gene *(HSP 90 ß)* showed increased levels after IVM and not much differences between IVF and PA groups of embryos. Based on the results, it could be inferred that even though significant difference observed at the earlier developmental stages of embryos, there were no significant differences between the quantum of stress experienced by the 2 groups of embryos at morula stage which would not affect the embryo quality and in turn parthenote ESCs.

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