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Expression patterns of *OCT-4* and *NANOG* genes in buffalo (*Bubalus bubalis*) embryos produced by *in vitro* fertilization or parthenogenetic activation

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ABSTRACT

The correct gene expression in early cleavage stage embryos is essential to sustain embryo development. The precise regulation of genes involved in pluripotency like *OCT-4* and *NANOG* is crucial to the formation of inner cell mass and trophoblast cells. Therefore, the present study was focused on the expression of *OCT-4* and *NANOG* genes in buffalo embryos produced by *in vitro* fertilization (IVF) or parthenogenetic activation (PA). For gene expression studies, a group of 5 embryos at every developmental stage of 2-cell-, 4-cell-, 8-to 16– cell, morula and blastocyst generated through IVF and PA were collected on second, third, fourth, fifth, and seventh or eighth day post-insemination or activation respectively. The transcripts of *OCT-4* were detected in embryos at all the stages examined, starting from the 2-cell stage through the hatched blastocyst stage in both IVF and PA embryos. Whereas, the expression of *NANOG* was observed to begin at the morula stage and was also present in the blastocysts stage of embryos produced through either protocol. These results indicated that *OCT-4* and *NANOG* used as a marker for pluripotency of ESCs in several species are expressed in early stage of buffalo embryos and continue to be expressed in blastocysts stage.

Key words: Buffalo, Embryo, Parthenotes, Pluripotent gene, Transcription factor

In vitro embryo development is a dynamic developmental process acknowledged by changes in the transcript type, quantity and cell number (Goossens *et al.* 2005, Chauhan *et al.* 1998, Totey *et al.* 1996). In the *in vitro* studies, during early embryonic development 10–20% of oocytes subjected to *in vitro* maturation (IVM) reached blastocyst stage after *in vitro* fertilization (IVF, Palta and Chauhan 1998, Gasparrini *et al.* 2003) in buffalo. This rate is 30–40% lower than that in cattle (Yang *et al.* 1998). However, in contrast other methods of embryo production like parthenogenesis, which undergo several cycles of cell division after oocyte activation, but embryos never proceed to term, as they are arrested at different stages of development, depending on the species (Loi *et al.* 1998, Fukui *et al.* 1992). Thus the, explanation of early embryonic development is critical for the refinement of assisted reproductive technology.

The transcription factors *OCT-4* and *NANOG,* required for propagation of undifferentiated embryonic stem (ES) cells in culture, are evolutionarily conserved and also play key roles in cell fate specification in many organisms (Hombria and Lovegrove 2003). *OCT-4* checks the expression of genes activated during differentiation and it is normally found in the pre-gastrulation stage of embryos, including oocytes, early cleavage-stage embryos, and the inner cell mass of the blastocyst (Rosner *et al.* 1990). The precise regulation of *OCT-4* and *NANOG* is crucial to the formation of ICM and trophoblast cells. Therefore, keeping this in view, the present study focuses on the expression of *OCT-4* and *NANOG* genes in buffalo embryos produced by IVF or parthenogenesis.

MATERIALS AND METHODS

Collection of oocytes and in vitro *maturation*

Buffalo ovaries were obtained from abattoir and transported to the laboratory in an insulated container in 0.9% normal saline containing antibiotics 400 IU /ml penicillin and 500 mg/ml streptomycin at 32–37°C within 4–5 h. Oocytes were collected by aspiration of surface follicles (2–8 mm diameter) with a 19-gauge disposable needle attached to a 10 ml syringe in the medium consisting of TCM-199 and 0.6% (w/v) bovine serum albumin (BSA). For oocyte maturation, compact cumulus oocyte complexes (COCs) with an unexpanded cumulus mass having <4 layers of cumulus cells and with homogenous evenly granular ooplasm were selected. The oocytes were washed 4 times with the washing

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medium (TCM-199 + 10% FBS + 0.81 mM sodium pyruvate + 50 µg/ml gentamycin sulphate) and then twice with the IVM medium (TCM-199 + 10% FBS + 5 µg/ml porcine FSH + 1µg/ml estradiol-17α + 0.81mM sodium pyruvate + 5% buffalo follicular fluid + 50 µg/ml gentamycin sulphate). The washed COCs were placed in 80 µl droplets (15–20 oocytes/ droplet) of the IVM medium, covered with sterile paraffin oil, in a 35 mm petri-dish and cultured for 24 h in a CO_2 incubator (5% CO_2 in air, 90–95% relative humidity) at 38.5°C. *In vitro* matured oocytes with expanded cumulus cells were subjected to IVF or parthenogenetic activation (PA).

In vitro *fertilization and parthenogenetic activation*

In vitro matured oocytes were divided in 2 groups for IVF (group A) or PA (group B).

IVF (group A): A group of *in vitro* matured oocytes was fertilized as per Kumar *et al.* (2007).

PA (group B): A group of *in vitro* matured oocytes were denuded of cumulus cells by incubation in 0.2% hyaluronidase in DPBS for 2 min.

The denuded oocytes with a prominent polar body were activated by exposure to 7% ethanol for 7 min followed by incubation with 2 mM 6-dimethyl aminopurine in mCR2aa medium for 3.5 h in a CO_2 incubator (5% CO_2 in air, 90–95% relative humidity) at 38.5°C.

In vitro *embryo development and gene expression analysis*

IVF and PA zygotes were transferred separately and cultured for 8 days in petri-dishes containing mCR2aa medium containing 0.6% BSA and 10% FBS (Kumar *et al.* 2007) under mineral oil, in maximum humidified atmosphere with 5% CO_2 in air at 38.5 °C. For gene expression analysis, a group of embryos at 2 cell-, 4 cell-, 8- to 16- cells, morula and blastocyst stage generated through IVF or PA were collected on second-, third-, fourth-, fifth- and seventh- or eighth- day after post-fertilization or activation respectively. Three replicates of each group contained 5 embryos at every developmental stage were used for gene expression studies.

Isolation of mRNA and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from different stages of embryos using the "cells-to-cDNA kit-II" as per manufacturer's instructions. Briefly, the embryos were washed with ice cold PBS after which 30 µl of cold cell lysis buffer was added and the reaction mixture was incubated in a thermal cycler at 75°C for 10 min. The cell lysate was treated with DNase-I at 37°C for 30 min to degrade genomic DNA and then heated at 75°C for 5 min to inactivate DNase-I. The 10 µl cell lysate + 2 µl random decamer primer + 4 µl dNTPs were mixed and incubated at 78°C for 1 min after which 2 µl of 10 X RT buffer, 1 µl RNAse inhibitor and 1 µl moloney murine leukaemia virus (MMLV) mix were added and the mixture was incubated at 42°C for 60 min and then at 95°C for 10 min. The PCR cycle included heating to 94°C for 2 min, followed by repeated cycles of 94°C for 30 sec, annealing temperature 55–60°C for 30 sec, and extension at 72°C for 45 sec. cycle was repeated 33–35 times with final extension for 72°C for 10 min. The following primers were used for PCR: primers specific for OCT-4 (accession no. AF022987), 5'-TTCTCTTTGGAAAGGTGTTC-3' and 5'-ACACTCGGACCACGTCTTTC-3'; for *NANOG* (accession no. DQ487022), 5' GGGAAGGGTAATGAGTCCAA-3' and 5'-AGCCTCCCTATCCCAGAAAA-3'; for 18S-rRNA (accession no. AF176811), 5'-GAGAAACGGCTACCACATCC-3' and 5'-GGACACTCAGCTAAGAGCATCG-3'; for ACTIN (accession no. K00622/BC016624), 5'-TGAACCCTAAGGCCAACCGTG -3' and 5'-TGTAGCCACGCTCGGTCAGG -3'. A –ve RT reaction (i.e. parallel RT reaction but without MMLV enzyme) was set with every batch of cDNA preparation to check genomic DNA contamination. For *OCT-4* and *NANOG* gene expression studies, *actin* and *18s rRNA* were used as internal controls respectively. Amplifications yielded products of 341 bp (*OCT-4*), 211 bp (*NANOG*), 337 bp (*18S rRNA*) and 268 bp *(actin*).

RESULTS AND DISCUSSION

In the present study, we have analyzed the expression of *OCT-4* and *NANOG* genes, which are known to be associated with developmental competence, regulation of transcription and cell pluripotency. Expression of *OCT-4* and *NANOG* were examined in 2-, 4-, 8- to 16-cell, morula and blastocyst stage embryos generated through IVF or PA. The transcripts of *OCT-4* were detected in embryos at all the stages examined, starting from the 2-cell stage through the hatched blastocyst stage in both IVF and PA embryos (Fig. 1). *OCT*-4 is a transcriptional regulator of genes involved in maintaining the undifferentiated pluripotent state and it also prevents expression of genes activated during differentiation. It is normally found in the pluripotent stem cells of pre-

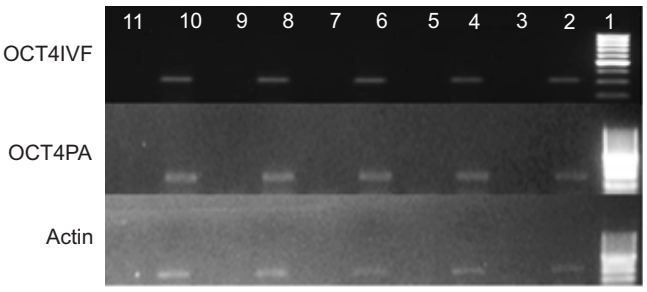

Fig. 1. Expression of *OCT-4* and *Actin* (house keeping) gene in different embryonic stages of *in vitro* fertilized or parthenogenetic activated buffalo embryos.

Lane 1, Marker (100 bp); 2, 2 cell; 3, -ve; 4, 4 cell; 5, -ve; 6, 8-16 cell; 7, -ve; 8, morula; 9, -ve; 10, blastocyst; 11, –ve.

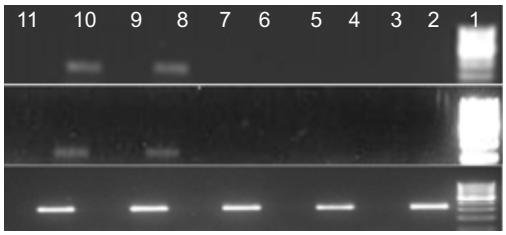

Fig. 2. Expression of *NANOG* and *18s-rRNA* (house keeping) gene in different embryonic stages of *in vitro* fertilized or parthenogenetic activated buffalo embryos.

Lane 1, Marker (100 bp); 2, 2 cell; 3, –ve RT; 4, 4 cell; 5, –ve RT; 6, 8-16 cell; 7, –ve RT; 8, morula; 9, –ve RT; 10, blastocyst; 11, –ve RT.

implantation embryos, including early cleavage-stage embryos, and the ICM of the blastocyst (Rosner *et al.* 1990). In earlier studies also, the expression of *OCT-4* was widely detected in early embryos, and was found to be restricted to the ICM at the blastocyst stage (Pesce and Scholer 2001, Boiani *et al.* 2002). The important role of *OCT-4* was also demonstrated by the gene knockout experiments of *OCT-4* in which *OCT-4*-deficient embryos developed to the blastocyst stage but the ICM cells lost their pluripotency and embryos could differentiate only along the extra-embryonic trophoblast lineage (Nichols *et al*. 1998), resulting in embryonic lethality. Several different laboratories have now shown that expression of *OCT-4* gene starts from early development stages i.e., from the 2-cell stage and continues up to the blastocyst stage in pigs, cattle and goats (van Eijk *et al.* 1999, Kirchhof *et al.* 2000, He *et al.* 2006). Oct-4 protein was also detected at all the development stages from 2-cell through the blastocyst stage in bovine (Daniels *et al.* 2000) and buffalo (Anand *et al.* 2011) embryos produced through *in vitro* system. Our results of gene expression are in agreement with the previous studies.

In the present study, expression of *NANOG* was observed to begin at the morula stage and was also present in the blastocysts stage of embryos produced through either protocol (Fig. 2). Although in previous studies, the expression of *NANOG* was detected at the 8- to 16-cell stage and increased levels were observed at the morula and blastocyst stages in goat (He *et al.* 2005) and pig (Brevine *et al.* 2007) whereas it was strongly down regulated in the trophectoderm cells. Similar expression patterns of *NANOG* were found in mouse and bovine pre-implantation embryos (Hart *et al.* 2004, Gandolfi *et al.* 2006) as well. However, in a few contrasting reports, *OCT-4* and *NANOG* expressions were detected both in the ICM and trophectoderm of blastocyst-stage in goat (He *et al*. 2006), porcine and bovine embryos (Kirchhof *et al*. 2000). Our observation of expression pattern of *NANOG* gene in early developmental stage of buffalo embryos derived from IVF or PA was partially similar to the reported observations.

A continuous expression pattern of *OCT-4* gene in early development stages and in blastocysts suggests that this transcription factor may play a crucial role in early embryonic development whereas the initiation of *NANOG* expression at a still later stage may be an indicative of its important complementary role in association with *OCT-4*. Our results suggested that, *OCT-4* and *NANOG* which are used as a marker for pluripotency of ESCs in servral of species are expressed in early stage of buffalo embryos and continue to be expressed in blastocysts stage.

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