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Necdin and neuronatin genes expression among diploid parthenogenetic, IVF and *in vivo* derived female sexed embryos during preimplantation development in goat

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ABSTRACT

The present research work was undertaken to compare the expression of necdin (*Ndn*) and neuronatin *(Nnat)* genes among diploid parthenogenetic (DIP), female sexed *in vivo* and female sexed *in vitro* produced embryonic cell colony. The good quality caprine oocytes were matured in presence of cytochalasin B (CCB) and activated by ethanol (7%) for 5 min followed by incubation with 2mM 6-dimethyl amino purine (DMAP) for 4 hrs for DIP embryos production. Embryonic cell colony were developed from 8–16 and morula stage DIP, IVF and *in vivo* derived embryos and these colonies were used for studying the *Ndn* and *Nnat* gene expression. There was no expression of *Ndn* gene in both the stages of DIP embryos, while this gene was expressed almost similarly in IVF and *in vivo* derived 8–16 cell stages but down regulated significantly in morula of IVF compared to *in vivo* derived embryonic cell colony. The *Nnat* gene expression was absent in *in vivo* derived 8–16 cell and morula stage embryos, while it was expressed in both the stages of DIP and IVF embryos. Further, it was observed that the expression of this gene was significantly lower in DIP embryos of both the stages as compared to IVF one. There was no significant difference of this gene expression between 8–16 cell and morula of DIP but morula of IVF showed significantly higher expression than 8–16 cell stage.

Key words: Cytochalasin B, Diploid embryos, Developmental gene, *Ndn*, *Nnat*

Parthenogenesis, a reproductive strategy, that leads to a combination of new genes originated by mutation to remain as such due to absence of segregation by crossing over and meiosis. Parthenogenetic embryo is a good tool to study the genes important for the development and growth of the early embryo. Preimplantation embryo development is characterized by compaction, cavitation and blastocoel expansion, which require well orchestrated expression of genes derived from the maternal and/or embryonic genome. *In vitro* produced embryos gene expression starts at the 2 to 4 cell stage. Some important genes related to embryo development are desmocolin I, II, III, desmogelin 1, plakophilin, connexin 43 Hsp70.1, oct4, nanog, Glut–1, trophoblast protein genes (TP), polymerase (Wrenzycki *et al.* 1997) Peg1/Mest, H19, Igf-2, Xist, Ndn, Nnat, $P57^{kip2}$, Grb10, Igf2r etc. (Perecin *et al.* 2009). The normal mammalian development requires balanced expression of both maternal (*H–19, $P57^{kip2}$, Igf2r, Grb10, Gnas, Mash/ Hash, Xist* etc.) and paternal (*Igf2, Peg1/Mest, Peg3, Dlk1, Ndn, Nnat* and *Sgce* etc.) as well as other development related genes (Barton *et al*. 1984). Therefore, the fundamental reason of failure of parthenogenetic embryos to live birth in mammals was found associated with lack of or perturbed expression of these genes (Barton *et al.* 1984, Singh *et al.* 2013, 2014).

The expression of early embryonic developmental as well as paternal and maternally imprinted genes was reported in pre implantation embryos in domestic animal like cattle, sheep, pig etc (Ruddock *et al.* 2004, D'Cruz et *al.* 2008, Feil *et al.* 1998). There are sporadic studies on comparative expression of early embryonic developmental, paternal and maternal genes among parthenogenetic, IVF and *in vivo* derived embryos mostly in mice (Perecin *et al.* 2009), but no such information is available in caprine. Further, in most of these studies, the comparison was made in IVF of both sexes or *in vivo* derived embryos. The true comparison would be when parthenogenetic embryos are compared with IVF or *in vivo* derived female sexed embryos.

Therefore, we studied the comparative gene expression profile of *Ndn* and *Nnat* gene among DIP, *in vitro* produced and *in vivo* derived female sexed embryonic cell colony.

MATERIALS AND METHODS

Ethics: All the experiments were approved by ethics committee of the Indian Veterinary Research Institute,

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In vitro *and parthenogenetic embryos production:* The cumulus oocyte complexes (COC) were isolated, evaluated, graded and matured in maturation media (TCM–199 with HEPES media, 10% FBS, 10% goat follicular fluid, 0.5µg/ml FSH, 10µg/ml LH, 0.5µg/ml estradiol). After maturation, the oocytes were washed 4–5 times with fertilization TALP media for removing expanded cumulus cell mass. Then oocytes were transferred to 50µl drop of fertilization TALP media and 50µl capacitated spermatozoa collected from experimental shed from elite buck, were added to this. After 18 h of sperm co-incubation with oocyte, the presumptive zygotes were washed several times in mSOF supplemented with 0.8% BSA and essential and non-essential amino acids and cultured in 100µl of same media in CO_2 incubator at 37°C, 5% CO_2, and 95% relative humidity until assessment to determine cleavage rates.

For parthenogenetic diploid (DIP) embryo development, isolated oocytes were cultured in maturation media supplemented with 15µg/ml of CCB (Ranjan *et al.* 2013a). The matured oocytes were activated by ethanol (7%) for 5 min followed by incubation with 2mM DMAP for 4 h and washed 4–5 times in mSOF and further cultured in same media until assessment to determine cleavage rates.

Surgical collection of embryo: For superovulation, healthy female goats of 2–2.5 years old were selected and feeding and management was done as per standard practice. The goats were monitored for cyclicity at least for 3 consecutive cycles. For this, Pluset® @ 20 IU per goat was administered intramuscularly in a constant dose schedule (2.50 IU) at 12 h intervals over 4 consecutive days starting from 11th day of estrous cycle. Treated goats were checked for estrus and mated with a buck of proven fertility. Surgical embryo collection was done from 10 goats as per standard protocol (Agrawal *et al.* 1982). Embryos (42) of different stages (2 cell: 22; 4 cell: 16 and 8 cell: 4) along with 12 oocytes were collected. Some of the embryos were further cultured to get morula stage embryo in mSOF supplemented with 0.8% BSA.

Culture of embryonic cells isolated from early stage embryos: The DIP, IVF and *in vivo* derived embryos of 8–16 cells and morula were zona lysed with proteinase-K (0.02%) and clumped blastomere were washed in 4–5 drops of stem cell culture media [Knockout DMEM supplemented with 10 % FBS, LIF (40ng/ml), ITS (0.3%), NEAA (0.5%), SCF (20ng/ml), bFGF (4ng/ml), IGF–1 (100ng/ml)]. After washing, the clumped blastomere were cultured in wells on mitomycin C inactivated goat fetal fibroblast monolayer at 37°C, 5% CO_2 and 90% relative humidity in a CO_2 incubator and the formation of cell colonies was observed. When the clumped blastomere of individual embryos were divided and make a colony, these cells were eluted by 0.25% trypsin EDTA solution.

Karyotyping of parthenogenetic embryo: Karyotyping was done as per Ranajn *et al*. (2013a). A small number of cells from the colony developed from individual parthenogenetic embryos were used for karyotyping to check ploidy, and rest of the cells of the colony was stored at –80°C. After karyotyping, the colonies (10) of DIP embryos of cultures were used for gene expression studies. A normal DIP blastomere in goat shows 30 pairs (2n=60) of chromosome.

Sexing of embryonic cell colony: Similarly, colonies developed from individual IVF and *in vivo* derived embryos of 8–16 and morula stage embryos were divided into 2 parts. One part was immediately kept in –80°C and another part was used for genomic DNA isolation for sexing. After sexing, the colonies developed from female embryos were used for gene expression studies. Genomic DNA was also isolated from blastomere colony by Pure LinkTM genomic DNA mini kit. The sex related SRY gene expression was analysed by real time PCR using genomic DNA (Ranjan *et al.* 2013b).

RNA extraction and reverse transcription: For gene expression analysis, embryonic cell colony generated from 8–16 and morula stages of DIP (36 and 37), IVF (20 and 22) and *in vivo* (6 and 5) derived female sexed embryonic cell colonies were picked up from the culture media separately and washed with 1X PBS. Then the embryonic cell colony was separately transferred in 2 ml centrifuge tube for RNA isolation. The total RNA was isolated using a kit. cDNA was synthesized using iScript select cDNA Synthesis kit. After preparing cDNA, O.D of cDNA was maintained at 500ng/µl and used for real time PCR study.

Real time PCR: Relative quantification of different genes was performed using a real time polymerase chain reaction

Table 1. Primers used for real-time PCR

Primer	Sequence (10 pmol/µl)	Annealing temp (°C)	Product size (bp)
ß-actin	F 5´TCATCACCATCGGCAATG3´ R 5´CCAATCCACACGGAGTAC3´	60	286
Gapdh	F 5´GGAGAA ACC TGCCAAGTATG3´ R 5´TGAGTGTCGCTGTTGAAGTC3´	60	126
Nnd	F 5´GTCAAGGACCAGAAGAGG3´ R 5´CAGGATCATCAGCAGGAG3´	60	255
Nnat	F 5´TCGCCACAGGAGCACTTG3´ R 5´GATCTCTTGTTGTCGTCATTGTC3´	60	85
Sry	F5´TGAACGAAGACGAAAGGTGGCTCT3´ R 5´ATCAGGGACTGTGAGCGGCATAAT3´	60	342

method by using BioradCFXManager™ Software and EvaGreensupermix. For amplification of genes, primers were designed in beacon software. The real time PCR thermocycling conditions were: an initial denaturation step at 95°C for 30 sec followed by 40 cycles of 95°C for 5 sec, annealing for 15 sec. Transcript level of all genes were quantified using the relative quantification method based on comparative threshold cycles values (Ct). The abundance of gene was determined relative to the abundance of the housekeeping gene ß-actin and GAPDH. The detail of primers is given in Table 1.

Statistical analysis: The data of embryonic cell colony formation among different stages and types of embryos were analyzed by applying one way ANOVA using the SPSS 16 computer program as different variables and results are presented as mean±SE. The relative expression of gene in fold changes was calculated by $2^{-\Delta\Delta Ct}$ method (Pfaffl *et al.* 2002) and analysed by one way ANOVA using SPSS 16.

RESULTS AND DISCUSSION

Paternal genes, *Ndn* and *Nnat*, were analyzed in the present study. There was no expression of *Ndn* gene in both the stages of DIP embryos, while this gene was expressed almost similarly in IVF and *in vivo* derived 8–16 cell stages but down regulated significantly (P<0.05) in morula of IVF compared to *in vivo* (Figs 1, 2) derived embryos. There was no significant difference (P<0.05) in expression between 8–16 and morula of *in vivo* and IVF derived embryonic cell colony (Fig. 1). *Ndn* encodes the protein necdin, which in the mouse, is expressed only from differentiated neurons in the brain, and in the human, is expressed in many tissues with highest levels in the brain and placenta (McDonald and Wevrick 1997). Ndn expression was found in 1 of 4 morulae and 1 of 4 human blastocysts tested (Salpekar *et al.* 2001). Ndn mRNA is expressed following the maternal-to-zygotic transition (MZT) during bovine pre implantation development (8–16-cell onwards) (Ruddock *et al.* 2004). In our study, we could not find any *Ndn* expression in parthenogenetic derived 8–16 and morula stage embryonic cell colony. The earlier worker also reported the same type of observation (Salpekar *et al.* 2001, Ruddock *et al.* 2004). In fact, the expression of this gene started only after 16 cell stage onward and sometime the expression was absent in 8–16 cell and morula stage embryos in human and bovine (Salpekar *et al.* 2001, Ruddock *et al.* 2004).

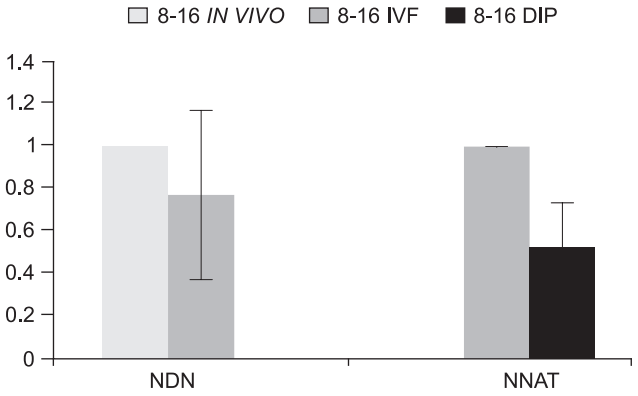

Fig. 1. Relative expression of *Ndn* and *Nnat* genes in embryonic cell colony derived from 8–16 cell stages of DIP, *in vivo* and IVF derived embryos in caprine (mean±SE).

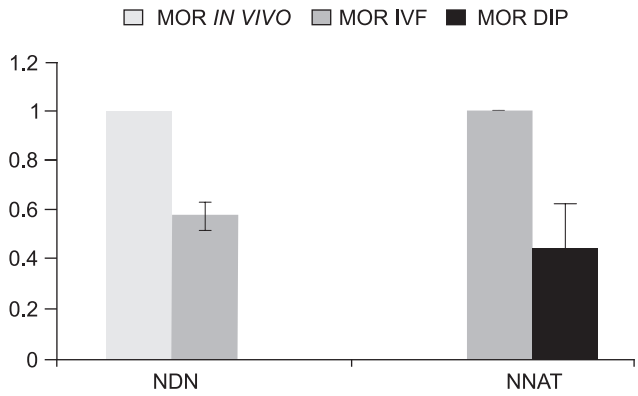

Fig. 2. Relative expression of *Ndn* and *Nnat* genes in embryonic cell colony derived from morula stages of DIP, *in vivo* and IVF derived embryos in caprine (mean±SE).

The *Nnat* (*Neuronatin*) gene expression was absent in *in vivo* derived 8–16 cell and morula stage embryos, while it was expressed in both the stages of DIP and IVF embryos (Figs 1–2). Therefore IVF derived 8–16 cell and morula stage embryonic cell colony were used as control. The expression of this gene was significantly lower in DIP embryos of both the stages as compared to IVF one (Fig. 2). There was no significant difference (P<0.05) of this gene expression between 8–16 cell and morula of DIP but morula of IVF showed significantly higher (P<0.05) expression than 8–16 cell stage (Fig. 2). The Nnat mRNA has been shown to be expressed in IVF and not in parthenogenetic blastocysts, suggesting that it is imprinted by this stage of development in bovine (Ruddock *et al*. 2004). Its transcripts were present in the oocyte and off by the 8-cell stage, then reappeared at the blastocyst stage, with stronger expression in day 8 than in day 7 blastocysts. Interestingly, the ß isoform of this gene was the predominant form in early embryos, and the α isoform was stronger in the blastocysts. In porcine, a similar expression pattern was observed in preimplanation embryos (Park *et al.* 2011). In the present study, we have also observed *Nnat* expression in DIP and IVF embryos and but no expression could be detected in *in vivo* derived embryos. This difference may be due to subtle differences in the *in vitro* culture or to the variation in the activation protocols used (D'Cruz *et al.* 2008). There are reports which indicated that *in vitro* culture condition affects gene expression (Khosla *et al.* 2001, Doherty *et al.* 2000). Therefore, the changes of relative expression of these genes in DIP and IVF embryos in the present study might be due to the effect of *in vitro* culture condition.

Several *in vitro* developmental defects have been identified in parthenogenetic embryos, viz. delayed development, reduced total cell number, fewer cells in the inner cell mass of blastocysts compared with fertilized

embryos (Loi *et al.* 1998). In our earlier experiment we also observed early fetus death due to lack of formation of placenta or perturbed developmental gene expression (Ranjan *et al.* 2013c).

In conclusion, the present study demonstrated the comparative expression profile of some important paternal expressed genes which seems responsible for proper growth of fetus among DIP, IVF and *in vivo* derived pre implantation embryos. The paternally expressed genes Ndn was not expressed by the DIP embryos of both the stages. The results further pointed out that in DIP embryos, the expression profile of paternally expressed genes were perturbed, which may be responsible for developmental failure of parthenogenetic embryos in this species and warrants further modification of protocol for generation of DIP embryos having normal gene expression as compared to *in vivo* and IVF one.

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