Changes in the gene expression of mRNA transcripts for insulin like growth factor (IGF-I), their receptor (IGF-IR) and facilitative glucose transporter (Glut-I) in IVM oocytes and preimplantation embryos of Buffalo derived from Somatic Cell Nuclear Transfer

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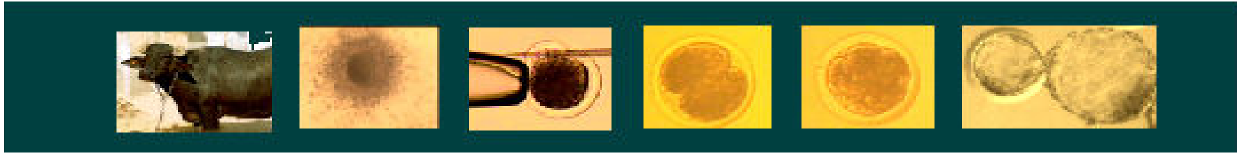


Introduction

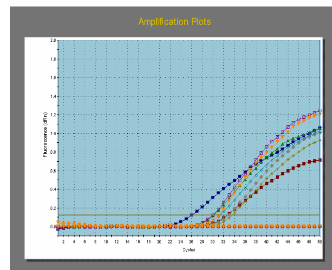
Cloning of bovine embryos via SCNT employing fetal or adult somatic donor cells has made great progress during the pas few years. However, the efficiency of cloning is still low and the offspring born from NT embryo are generally affected by multiple abnormalities. Analysis of mRNA transcripts levels related to oocyte/ embryos development is considered as an indirect way of quality assessment. Ligands IGF-I and its receptor IGF-IR both are expressed in the bovine ovary and the level of IGF-I production is not affected by antral follicle development. The presence of IGF-I in maturation medium has been shown to promote the progression of meiosis in bovine oocytes Glucose transport across plasma membrane in mammalian cells is mediated by passive energy transport via facilitative glucose transporters (Glut). The objective of the present study was to investigate the relative gene expression of IGF-I, IGF-IR and Glut-I mRNA transcript during oocyte maturation and preimplantation developmental stages up to blastocyst in somatic cell nuclear transfer (SCNT) cloned embryo of buffalo from cultured skin fibroblast cells.

Materials and Methods

Preparation of donor Karyoplast: The skin tissues (1 cm^2) were collected by ear biopsy from two young female Bhadawari buffaloes. The cell cultures were established in DMEM + Ham's F12 culture medium containing 15% FBS at 37°C, 5% CO2 and 95% humidity. The primary fibroblast cells were harvested using Trypsin + EDTA protocol. The cells were passaged up to 115th passage and two cell lines MF32 and MF82 were developed.

Production of nuclear transfer embryos: Recipient buffalo oocytes were matured in TCM-199 +10% FBS, supplemented with 0.2 mM pyruvate, 0.5 µg/ml LH, 0.1 µg/ml FSH and 1µg/ml Estradiol for 26h at 38.5˚C with 5% CO_2 in air, after that cumulus cells were completely removed from the oocytes by manual pipetting in the presence of 2 µg/ml hyaluronidase. The polar body and MII plate were removed by enucleation pipette and from the same slit one donor cell was placed into the perivitelline space of each enucleated oocyte by using a transfer pipette. NT couplets were fused with electrodes in fusion medium (Mannitol). After the fusion couplets were chemically activated in 5 µM ionomycin for 10 min, 6-DMAP, CHX + Cyto-B for 3.30 h, followed by co-culture on feeder layer of granulosa cell for 9-10 days.

Preparation of cDNA samples: The cDNA sources were created from the oocytes after 12h and 24h of maturation and embryos at 2 cell, 4 cell, 8 cell, 16 cell, morula and blastocyst stages. Matured oocytes and embryos were centrifuged at 1200g and washed with Phosphate buffer saline (PBS) by centrifugation for 5 min at 4˚C at 1200g. To make a cell lysate, 100 µl of the cell lysis buffer was added to pelleted oocyte/ embryo, vortexed for 5 min in order to mix the cells and then heated using a block type thermal cycler system. Cell lysate were supplemented with 0.2 µl (final concentration 0.4 U/µl) of DNase I and the mixture was incubated at 37˚C for 15 min immediately after the incubation; each tube was heated for 10 min at 75˚C to inactivate the DNase.



To synthesize the first strand of cDNA 5µl cell lysate, 4 µl dNTP mix, 2 µl oligo dT and 5 µl RNase free water were assembled in a RNase free 0.5 ml tube, then heated for 3 min at 70˚C. After the mixture was cooled on ice, 2 µl 10X RT buffer, 1 µl M-MLV reverse transcriptase and 1 µl RNase inhibitor were added to the reaction tubes. Reverse transcription was carried out for 1h at 42˚C, followed by incubation at 95˚C for 10 min. RT minus product with all the reaction components except of the reverse transcriptase were produced for each sample, and were then employed for real time PCR in order to demonstrate that the template for the PCR product was cDNA, not genomic DNA.

Real time PCR analysis of gene expression: The cDNA samples from all stages were analyzed by real time PCR with SYBR Green chemistry. PCR reactions were run as triplicates in a total volume of 25 µl which consisted of cDNA equivalent to 1 embryo, 2X PCR SYBR Green master mix (Stratagene), 10 nM each of sequence specific primers and 2 µl RT product. The basic protocol for real time PCR was an initial incubation at 95˚C for 10 min to activate modified Taq Polymerase. Fifty five cycles of PCR were carried out with denaturation at 95˚C for 10S, then at annealing temp for 10S, and then extended at 72˚C for 15S. After amplification a melting curve was generated by heating the sample to 95˚C for 5S, followed by cooling at 55˚C for 15S, to verify specific amplifications. Amplified PCR products were analyzed by using light cycler software. After analysis the target gene expression was normalized to 18SrRNA.

Results and Discussion

Somatic Cell Nuclear Transfer: The developmental rate of preimplantation embryonic development was 54.0%, 46.2%, 38.64%, 34.42%, 22.36% and 16.8% in embryos at 2-cell, 4-cell, 8-cell, 16-cell stages, morula and blastocyst respectively. These results are in consistency with earlier reports.

Gene expression profile of mRNA transcript: IGF-I transcript expression was visible only in matured oocytes. It was highest at 12h culture and then declined at 24hrs of in vitro maturation of buffalo oocytes. To our knowledge, there is no published data on IGF-I expression in buffalo oocytes. Our findings are consistent with the previously published results indicating presence of mRNA for IGF-I in bovine IVP embryos (Lonergan et al., 2000).

Expression of IGF-IR transcript showed decrease from one cell zygote to eight cell stage and then increased up to the blastocyst production. At 8-cell stage the mRNA transcript level was lowest and down regulated but these differences were reduced after the 16-cell stage. Earlier studies (Yaseen

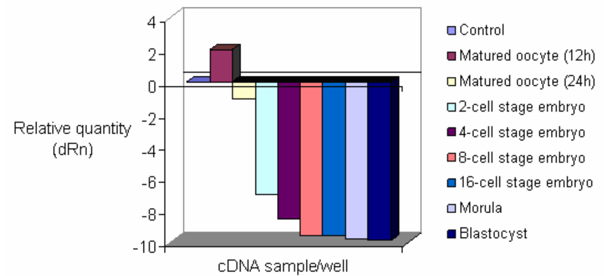


Fig. 1. Relative abundance of IGF-I transcript in buffalo oocytes and SCNT embryos

et al., 2001) reported the similar expression of IGF-IR gene in *in vitro* matured oocytes as well as in all stages of pre-implantation embryonic development in cattle.

The presence of transcripts for IGF-I and IGF-IR in all preimplantation buffalo NT embryos from matured oocyte to the blastocyst stage indicated that maternal transcripts occur in the oocytes and that these transcripts persist at cleavage stage before activation of the embryonic genome.

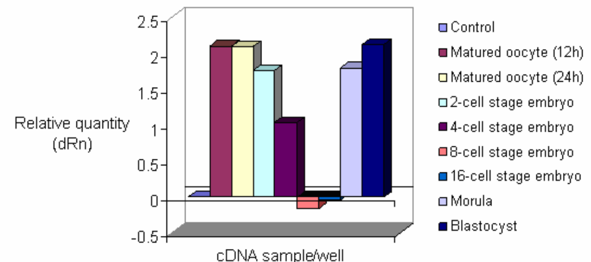


Fig. 2. Relative abundance of IGF-IR transcript in buffalo oocytes and SCNT embryos

Transcripts of Glut-I were found in matured oocytes and all embryonic stages studied with continuous up-regulation throughout preimplantation development. This shows that expression of Glut-I transcript plays an important role in the development of preimplantation embryos. This finding was consistent with other reports of Glut-I expression in development of IVF bovine embryos (Wrenzycki et al., 1998).

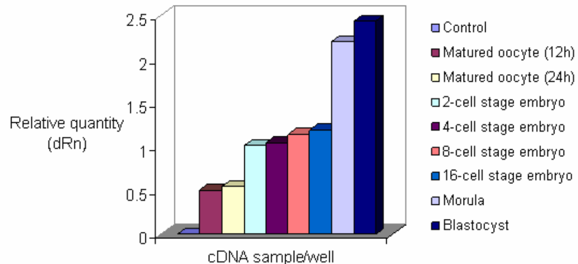


Fig. 3. Relative abundance of Glut-I transcript in buffalo oocytes and SCNT embryos

Conclusion

From the preliminary data, it is clear that the expression pattern of IGF-I, IGF-IR and Glut-I play important role in maturation of primary oocytes and embryonic development after cleavage. The studies would be of immense value in understanding the reprogramming of SCNT derived embryos and improving the efficiency of cloning.

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