



Comparative expression profile of hexokinase gene in pre-implantation *in vitro* embryos in buffalo

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The expression patterns of glucose metabolism genes could be a valuable indicator of the development potential of oocytes and embryos (Kumar *et al.* 2015). Expression pattern of glucose metabolism gene during oocytes maturation and early embryonic development showed developmental competence of oocytes or embryos (Kumar *et al.* 2013). Therefore, the present study was conducted to test whether hexokinase—an enzyme in glycolysis pathway—can be used as a marker of totipotent stem cell derived from 8-16 cell stage IVF derived buffalo embryos which can distinguish from pluripotent one.

Buffalo ovaries of unknown reproductive status were collected from local slaughter house and carried to the laboratory in normal saline solution (0.85% NaCl). The oocytes were aspirated from all the visible non atretic surface follicles by a sterile 18 G needle into syringe containing oocyte collection media and searching of oocyte were carried out under stereo zoom microscope. The good quality of cumulus oocyte complexes (COCs) having evenly distributed granulated cytoplasm and more than three cumulus oocyte complex layers were cultured in maturation media and kept in CO₂ incubator with an atmosphere of 5% CO₂, 37°C temperature in humidified air for 24 h. After prescribed period, the matured COCs were serially washed in sperm TALP and fertilization TALP media and finally transferred to 50 µl droplet of fertilization TALP media. To this, 20 µl of capacitated spermatozoa were added and the droplet was covered with warm mineral oil and finally incubated in CO₂ incubator at same atmospheric conditions. After 18–20 h of sperm-oocyte co-incubation, the presumptive zygotes were taken out of fertilization drops and washed several times in modified synthetic oviductal fluid (mSOF) (Walker *et al.* 1996) and further cultured in 100 µl droplet of the same media. The embryo developments were assessed by observing the cleavage rate after 48–72 hours of *in vitro* culture (IVC). The embryos of 8–16 cell stage were shifted to proteinase-K drops for thinning or

dissolution of zonal layer and the resulted clumped blastomere was washed in stem cell culture media and put onto inactivated buffalo fibroblast monolayer and kept in CO₂ incubator for development of primary stem cell clone. Developed primary embryonic stem (ES) cell clone was characterized by using molecular stem cell specific markers for expression of pluripotent genes like Oct4, Nanog, Klf4 and Foxd₃ using real time PCR. The relative expression of hexokinase gene was also analyzed in immature and mature oocytes (n=40), pooled blastomeres of 2–4 cell (n=10), 8–16 cell (n=10), 32 cell stage embryos (n=10) as well as primary ES cell clones derived from 8–16 cell stage embryos. The relative expression of gene was calculated as outlined by Ingle *et al.* (2010) with suitable modifications.

All the experimental data including relative expression of pluripotent genes were analyzed by Statistical Package for Social Sciences (SPSS-16.0, SPSS Inc., Chicago, IL, USA) using one way ANOVA.

The relative expression of hexokinase gene in immature and matured oocytes, 2–4 cell, 8–16 cell, 32 cell stage embryos and primary embryonic stem (ES) cell clone generated from 8-16 celled embryos are presented in Table 1 and Fig. 1. The expression of different pluripotency gene in primary ES cell clone has also been presented in Fig. 2. Blastomere from 8-16 cell embryos expressed

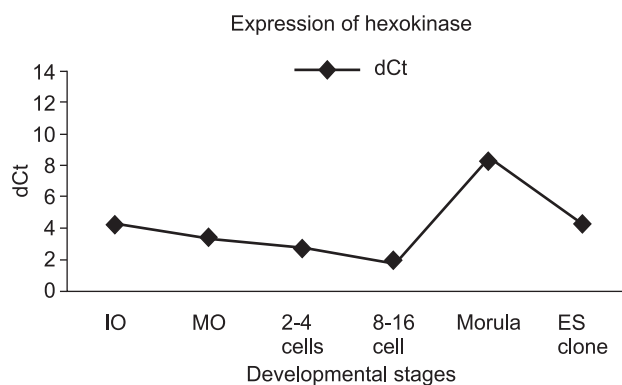


Fig.1. Expression of hexokinase gene in different developmental stages of oocytes, blastomeres derived from various stages of embryos and primary ES cell clone.

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Table 1. Relative expression of hexokinase gene in oocytes, different developmental stages of embryos and primary embryonic stem cell clone in buffalo

IO	Different developmental stages				
	MO	2-4 cell blastomeres	8-16 cell blastomeres	Morula (32 cell)	ES cell clone (P ₀)
4.2 ^d ±0.05	3.39 ^c ±0.05	2.74 ^b ±0.08	1.89 ^a ±0.03	8.29 ^e ±0.03	4.35 ^d ±0.04

IO, Immature oocyte; MO: Matured oocyte; ES, Embryonic stem cell. abcde, means bearing different subscripts in a column differ significantly***($P<0.005$).

significantly ($P<0.005$) lower concentration of hexokinase compared to all other developmental stages of embryos studied (Table 1, Fig. 1). The expression pattern indicated a linear decrease from immature oocyte to 8-16 cell stage, then a sharp increase at 32 cell embryo followed by a decrease in ES cell clone (Fig. 1). The expression in primary embryonic stem cell clone was considerably higher than the expression of 8-16 cell stage blastomere but was significantly lower than 32 cell stage embryos. Similar pattern of hexokinase expression was observed in bovine embryos by Lequarre *et al.* (1997). They also reported that there was significant drop in the hexokinase mRNA level starting at the four-cell stage and reaching a minimal value at the 8- and 16-cell stages followed by a sharp rise at the morula stage. A similar dramatic increase of this enzyme activity at the morula stage has been reported in mouse (Hooper and Leese 1988). The metabolic requirement for mammalian embryo changes as development proceeds from the zygote to the blastocyst stage. Evidence from a number of species indicates that before activation of the embryonic genome, human and other mammalian embryos have a preference for oxidizable energy substrates, particularly pyruvate, non-essential amino acids and glutamine. The temporal pattern of hexokinase shows a sharp decrease from the four-cell stage up to the 16-cell stage, followed by a strong increase at the morula stage coincident with the second increase in glucose consumption (Rieger *et al.* 1992). Glucose metabolism of the bovine embryo is low during the first cleavages and increases sharply after the major resumption of the genome in 8–16 cells (Lequarre *et al.* 1997). Kumar *et al.* (2013) found that high expression of lactate dehydrogenase [LDH] and pyruvate dehydrogenase indicated that in absence of glucose, embryos try to use available pyruvate and lactate sources.

Marcello Rubessa *et al.* (2011) demonstrated that the energy substrate during *in vitro* culture affects both the production and the viability of later stage embryo.

In the present study, developed primary embryonic stem cell clone had significantly higher amount of hexokinase expression than 8-16 cell stage blastomere indicating that the ES cell clone developed could not maintain totipotency (Fig. 1). Therefore, further modification of culture condition is required to make a totipotent stem cell line. In conclusion, it can be stated that hexokinase gene expression can be used as a totipotent stem cell marker in buffalo and may be in other animals too. Further, based on the expression of hexokinase, it can also be concluded that stem cell clone derived from totipotent embryos did not maintain totipotency and progress towards pluripotency. This warrants further modification of culture condition for making totipotent stem cells in this species.

SUMMARY

Comparative expression pattern of hexokinase gene in various developmental stages of oocytes and embryos was studied to exploit it as a totipotent stem cell marker. Gene expression was compared with immature, mature oocytes, pooled blastomeres from 2-4 cell, 8-16 cell, 32 cell stage IVF embryos and primary embryonic stem cell clones. The expression pattern indicated a linear decrease from immature oocytes to 8-16 cell stage, then a sharp increase at 32 cell embryo followed by a decrease in embryonic stem cell clones. On the basis of gene expression pattern, it can be concluded that hexokinase gene may be used as a totipotent stem cell marker which should be the lowest one as compared to pluripotent stem cells developed from morula or inner cell mass.

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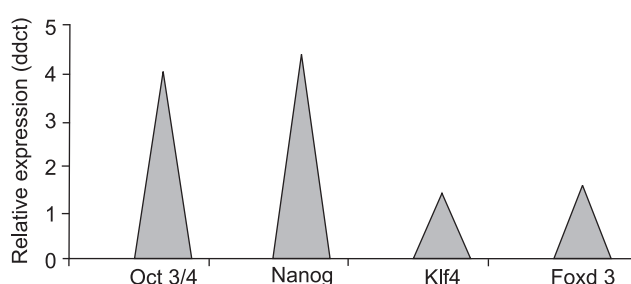


Fig. 2. Expression of pluripotent genes in primary embryonic stem cell clone derived from 8-16 cell stage embryos of buffalo.

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