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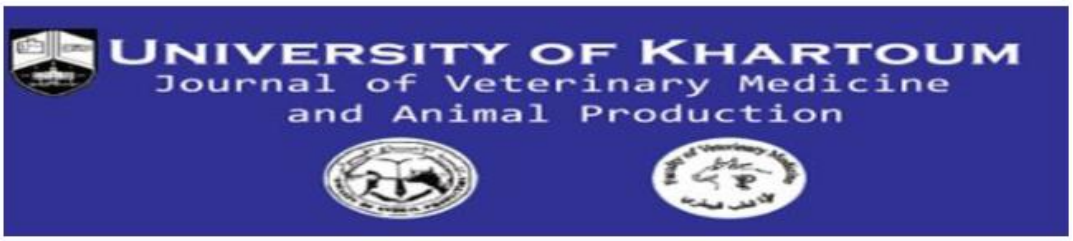

Quality of bovine preimplanted somatic cell nuclear transfer embryos compared to in vitro fertilized embryos

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Abstract

The *in vitro* preimplantation developmental potential and the quality of blastocysts produced by somatic cell nuclear transfer (SCNT) were examined compared to *in vitro* fertilized embryos (IVF). SCNT embryos were reconstituted from small and medium size donor cumulus cells synchronized at the G0-G1 cell stage by serum starvation and electrically fused to metaphase II arrested enucleated oocytes. The cell cycle phase of the donor cells was confirmed at the G0-G1 by flow cytometric analysis. *In vitro* fertilized embryos were produced by incubating the mature cumulus oocyte complexes with motile spermatozoa for 18 h at 39°C under a humidified air with 5% CO_2. Presumptive zygotes were cultured to the blastocyst stage in modified synthetic oviduct fluid medium. The cleavage rate and the development to blastocyst were the same for both types of embryo. SCNT blastocysts were morphologically similar to the IVF ones without significant difference in their cell number.

Key words: Cloning, nuclear transfer, somatic cells, cell cycle

المستخلص

أجري هذا البحث لقياس مقدرة الأجنة المنتجة من خلايا جسدية بإستخدام تقنية نقل الأنوية (SCNT)على النمو والتطورخلال مرحلة ما قبل الإنغراس و ذلك بالمقارنة مع الأجنة المنتجة بإستخدام تقنية إنضاج البويضات و تلقيحها فى المعمل (IVM, IVF). تم استخدام الخلايا الركامية كخلايا جسديه لإنتاج الأجنة. الخلايا الركامية المُستجدمة تمت زراعتها في وسط بتركيز السيرم (0.5%) وذلك بغرض إحداث تزامن في دورة إنقسام الخلية. تم دمج هذه الخلايا مع بويضات ناضجة منزوعة المادة الوراثية وتنشيطها بإستخدام صدمات كهربائية لمواصلة الإنقسام والتطور. الأجنة المنتجة من البويضات الناضجة الملقحة خارجياً تم الحصول عليها بزراعة البويضات الناضجة مع الحيوانات المنوية تحت درجة حرارة 39°م وضغط جوي مع إضافة 5% من ثاني أكسيد الكربون. تم رصد مراحل تطور نوعي الأجنة إبتداءً من بداية الإنقسام وحتى مرحلة تكوين الفقاعة الجنينية وعدد الخلايا في كل منها. لم يلحظ أي فرق معنوي في المعايير التي تم رصدها بين نوعي الأجنة مما يدل على مقدرة الأجنة المستنسخة على النمو والتطور بنفس مستوى الأجنة المنتجة بإستخدام تقنية إنضاج البويضات و تلقيحها فى المعمل (IVM, IVF).

الكلمات المفتاحية: استنساخ، نقل الأنوية، الخلايا الجسدية، دورة الخلية

Introduction

The biotechnology of reproduction in cattle started with the development of artificial insemination (AI) around 1950. During the nineteen- seventies the technique of embryo transfer was introduced. More recently, bovine cloning from somatic cells using the nuclear transfer technique. Production of cloned embryos by nuclear transfer from adult somatic cells is a novel and promising technique in animal biotechnology. After the first successful report of live offspring in sheep following nuclear transfer from adult somatic cells (Wilmut, *et al*., 1997) several encouraging studies in cattle using somatic cells as donor nuclei resulted in full term development (Cibelli, *et al*., 1998; Kato, et al., 1998; Wells, *et al*., 1999; Sangalli, *et al*., 2014; Saini, *et al*., 2018). In spite of numerous success reported in cattle and other species, the efficiency of the technique remains very low and some problems encountered such as embryonic and fetal mortality during pregnancy and peri-natal life, high birth weight and abnormal placentation (Schnieke, *et al*., 1997; Cibelli *et al*., 1998; Kato *et al*., 1998; Palmieri, *et al*., 2008). Very few studies tried to investigate the possible causes (Mohamed Nour & Takahashi, 2000; Alexopoulos, *et al*., 2008; Mrowiec, *et al.*, 2021), however the precise causes are still unknown. In this study we investigated the pre-implantation developmental potential of the nuclear transfer embryos compared to in vitro fertilized embryos (IVF). IVF embryos were reported of having far less problems (Behboodi, *et al*., 1995; Kruip & Dendaas, 1997) and more closely related to in vivo embryos. With regard to nuclear transfer embryos, two factors are known to affect the development, these are, the coordination between the cell cycle of the donor cell and recipient cytoplast (Campbell, *et al*., 1996; McLean, *et al*., 2021) and the size of the donor cells (Boquest, *et al*., 1999) as more percentage of cells reported to be in the G0/G1 phase. Therefore, in this study somatic cells were synchronized in G0/G1 phase by serum starvation and both small and medium cells in size were fused to metaphase II ooplasm to fulfill the appropriate cell cycle coordination between donor nuclei and recipient cytoplast. IVF embryos were produced as described earlier (Mohamed Nour & Takahashi, 1999). The developmental potential and quality of SCNT embryos produced were evaluated reference to IVF embryos in terms of development to the blastocyst and the number of cells in the developed blastocyst.

Materials and methods

Oocyte collection

Bovine oocytes were collected from slaughterhouse ovaries as described previously (Takahashi & First, 1993). Briefly, cumulus-oocyte complexes (COCs) were aspirated from small antral follicles (2-7 mm in diameter) with an 18-gauge needle attached to a 10 ml syringe. Oocytes surrounded by three or more layers of the cumulus cells with homogenous or slightly coarse granulated ooplasm were selected (Mohamed Nour, *et al*., 2004).

Oocyte maturation

In vitro oocyte maturation was conducted in HEPES-buffered TCM 199 (Gibco laboratories, Grand Island, NY, USA) supplemented with 10% FCS (Gibco), 0.02 units/ml FSH (from porcine pituitary, Sigma), 1 µg/ml estradiol-17ß (Sigma), 0.2 mM sodium pyruvate, and 50 µg/ml gentamycin sulfate (Sigma) (Takahashi, *et al*., 1996). Oocyte were then cultured in this maturation medium under a humidified atmosphere of 5% CO_2 in air at 39°C for 20 h.

In vitro embryo production:

In vitro fertilization and culture of fertilized embryos were performed as described previously (Takahashi *et al*., 1996). Briefly, frozen semen from a single ejaculate of a Holstein bull was used. Motile spermatozoa were separated using 45 and 90% Percoll gradient solution. The cumulus oocyte complexes were co-incubated with spermatozoa fertilization drop containing 3 mg/ml fatty acid-free bovine serum albumin (BSA) (Sigma) and 2.5 mM theophylline (Sigma) for 18 h at 39°C under a humidified air with 5% CO_2. Presumptive zygotes were cultured in modified synthetic oviduct fluid medium (Takahashi & First, 1992) supplemented with 1 mM L-glutamine (Sigma), essential amino acids for basal medium Eagle (Sigma), nonessential amino acids for minimum essential medium (Sigma), 1 mM glucose and 3 mg/ml fatty acid-free BSA.

Recipient cytoplast preparation

After 20 h of maturation culture, cumulus cells were removed by vortexing the COCs in 0.1% hyaluronidase (Type 1-S, Sigma) in Ca^{2+}- and Mg^{2+}-free TALP-HEPES. Denuded oocytes were examined under an inverted microscope (Diaphot-TMD, Nikon, Tokyo, Japan) to determine the extrusion of the first polar body. Denuded oocytes with the first polar body were enucleated by removing the polar body and the adjacent cytoplasm presumably containing the nuclear material (Prather, *et al*., 1987) in a 40 µl micromanipulation drop of TALP-HEPES supplemented with 10% FCS and 5 µg/ml cytochalasin B (Sigma). After enucleation, cytoplasts were incubated in TALP-HEPES containing 5 µg/ml Hoechst 33342 (Sigma) for 15 min at 39°C. Enucleation was confirmed by exposing the oocytes to ultraviolet light for a few seconds under an inverted microscope (Diaphot-TMD) equipped with an epifluorescence and UV-2A filter block.

Donor cell preparation

Donor cell preparation was conducted as described previously (Mohamed Nour, *et al*., 2000), a primary cell line was established from the cumulus cells collected 18-20 h after the start of maturation culture. The cumulus cells were separated and then washed several times in DMEM/F12 (Gibco). Viable Cells were cultured (8-9x10^4 live cells/ml) in DMEM/F12 supplemented with 10% FCS in 35x10 mm dishes (Falcon 3801) at 37°C under a humidified atmosphere with 5% CO_2. Cultured cells were allowed to multiply for 3-4 days followed by another 3-4 days of culture in DMEM/F12 + 0.5% FCS to induce quiescence. After the designated culture period, the cells were disaggregated by trypsinization and used as donor nuclei.

Characterization of donor cells

Disaggregated cells from serum-starved cultures were characterized in terms of cell size, cell-cycle phases of different cell sizes. Cell size was measured using the ocular scale under an inverted microscope (x400), and cells were categorized into small, medium and large size (Mohamed Nour *et al*., 2000).

The cell cycle phase distribution and the effect of cell size on the distribution of cells in the various phases of the cell cycle was determined by flow cytometry according to the method previously described (Mohamed Nour *et al*., 2000).

Production of nuclear transfer embryos

Trypsinized cumulus cells were inserted individually in the perivitelline space of the recipient cytoplast. Manipulated couplets were placed between two electrodes (0.5 mm apart), overlaid with 0.3 M mannitol solution containing 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$. Cell fusion was induced by 2 DC pulses of 0.9 kv/cm for 40 µsec, 1 sec apart delivered to the chamber using a BTX Electro Cell Manipulator 2001 M (BTX, San Diego, CA, USA). Successfully fused

couplets were incubated in the embryo culture medium supplemented with 10 µg/ml cycloheximide (Sigma) under a humidified atmosphere of 5% CO_2 in air at 39 C for 5–6 h. They were then thoroughly washed and subsequently cultured in the embryo culture medium (mSOFai) (Takahashi *et al*., 1996) supplemented with1 mM glucose and 3 mg/ml fatty acid-free BSA(Sigma) under 5% CO_2, 5% O_2, and 90% N_2. The cleavage rate was determined at 33 h after fusion. Development to blastocysts and the cell count (Takahashi & First, 1992) were checked 174 h post-fusion.

Statistical analysis

Data were analysed using One-way ANOVA of SPSS 21.0 for Windows. Significant means were separated by Duncan's test at a 5% significance level.

Results

As shown in Table 1, the computer-analyzed histograms showed that the percentages of nuclei existing in the G0/G1 phase for the small and medium cell populations were significantly higher than those for large ones under serum starvation culture conditions ($P< 0.05$). However, more than 83% of the large cells still had their nuclei in the G0/G1 phase. The percentages of cells in the S and G2/M phases for large-sized cells were higher than those for small- and medium-sized ones ($P<0.05$).

Table 1. Cell cycle distribution of cultured cumulus cells after serum starvation

Cell size	Cell cycle phase (%)		
	G0/G1	S	G2/M
Small	98.7±0.4[a]	0.7±0.5[a]	0.3±0.2[a]
Medium	95.6±0.8[a]	2.0±0.6[a]	2.2±0.3[a]
Large	83.4±6.9[b]	4.8±1.6[b]	11.5±3.9[b]

Concerning the developmental potentials of the *in vitro* fertilized and both nuclear transfer groups reconstituted from small and medium sized somatic cells, there were no significant differences in the cleavage rate, development to blastocysts (Table 2)., and blastocyst cell number (Table 3). No significant difference was detected in the fusion rate when both small and medium sized somatic cells were fused with recipient cytoplast.

Table 2. Development of bovine *in vitro* fertilized (IVF) embryos, nuclear transfer embryos reconstituted from serum starved small donor cells (NT-small) and serum starved medium size donor cells (NT-Medium)

Type of embryos	No. of oocytes used	%*[1] of		
		Fused	Cleaved*[2]	Blastocysts*[2]
IVF	186	____	91.0±5.3	41.3±7.6
NT- Small	157	46.2±10.1	84.0±7.2	37.2±5.6
NT-Medium	131	48.1±5.7	88.8±9.3	39.3±8.1

*[1] Values are means±SD of 5 replicates.

*[2] Based on the number of oocytes fused.

Table 3. Number of cells counted for *in vitro* fertilized (IVF) embryos, nuclear transfer embryos reconstituted from serum starved small donor cells (NT-small) and serum starved medium size donor cells (NT-Medium)

Type of Embryos	Blastocyst cell number (No.)
IVF	192.7±56.7(28)
NT- Small	176.0±76.6(28)
NT-Medium	188.4±65.3(23)

*[1] Values are means±SD of 5 replicates.

Discussion

In the present study, embryos generated by either in vitro-production (IVP) or SCNT, were compared in terms of first developmental progress (cleavage), development to the blastocyst and the number of cells in the blastocysts to detect any significant developmental abnormalities. In the previous studies of SCNT very few authors considered the size of the donor somatic cells (Mohamed Nour *et al*., 2000; Mohamed Nour & Takahashi, 2000). In bovine embryonic cell lines cultured under non-serum-starvation conditions, it is generally accepted that small cells have divided more recently, and therefore are earlier in the cell cycle (G1 phase) (Stice, *et al*., 1996). In another study, pig cells derived from cycling cultures of fetal fibroblasts *(Boquest et al*., 1999) and mammary glands (Prather, *et al*., 1999) were mostly in the G2/M phase, and that serum-starved and confluent cultures had large cells containing higher percentages of G0/G1-phase nuclei compared to the cycling ones. Therefore in this study, the effect of cell size on the distribution of cells in the various phases of the cell cycle was determined using forward light scatter to separately gate on small, medium, and large cells and subsequent calculation of G0/G1, S, and G2/M percentages within different gates (Boquest *et al*., 1999). The unique accuracy of gating for different cell sizes was achieved guided by the microscopically measured cell-size plotted histograms.

Another factor considered regarding construction of SCNT embryos in this study was the cell cycle stage of recipient oocyte, and that was by transferring synchronized donor cells to MII cell stage cytoplast. Rates of development to the blastocyst stage in vitro of SCNT embryos that were reconstructed from activated cytoplast were very low, suggesting that exposure of donor nuclei to unactivated recipient cytoplasts is beneficial for reprogramming of somatic nuclei *(Shiga, et al*., 1999; Mohamed Nour & Takahashi, 2000; Tani, *et al*., 2001).

The quality of embryos reconstituted from cumulus cells (G0/G1 small + M combination) and (G0/G1 medium + M combination) were examined by comparing to *in vitro* fertilized embryos. The rates of cleavage and development to blastocysts were the same for all 3 sets of embryos. All experimental groups produced morphologically normal blastocysts containing the same cell number. These results revealed no clear detectable abnormalities due to nuclear transfer procedure. Similar blastocyst development rates obtained in SCNT embryos also seem to indicate their ability to overcome some of the early difficulties of embryonic development at the same rate as *in vitro* fertilized embryos. In previous study (Mohamed Nour & Takahashi, 2000), the only abnormality detected at the

preimplantation in vitro growth period was the shorter time taken from the first cleavage to blastocoel formation in embryonic and somatic cell nuclear transfer embryos compared to *in vitro* fertilized embryos.

Blastocyst cell numbers obtained for both *in vitro* fertilized and nuclear transfer embryos in this study were equivalent to day 8 *in vivo* derived ones (Lindner & Wright, 1983), and were higher than those in the previous reports (Heyman, *et al*., 1994; Westhusin, *et al*., 1996; Wells *et al.*, 1999). High blastocyst cell numbers may relate to the culture conditions used in which more than 60% of the blastocysts obtained were categorized between the expanded and hatching blastocyst stages.

In the present study, no clearly detectable abnormalities were noticed due to the nuclear transfer procedure as compared to the *in vitro* fertilized one. In this respect, the author suggests further studies using techniques such as the time-lapse cinematography, immunohistochemistry, and transmission electron microscopy to investigate any other developmental milestones.

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