Acta Scientiae Veterinariae
ISSN: 1678-0345
ActaSciVet@ufrgs.br
Universidade Federal do Rio Grande do Sul
Brasil

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Acta Scientiae Veterinariae, vol. 36, núm. 2, 2008, pp. 141-147
Universidade Federal do Rio Grande do Sul
Porto Alegre, Brasil

Disponível em: http://www.redalyc.org/articulo.oa?id=289021836008

Acta Scientiae Veterinarie. 36(2): 141-147, 2008.

ORIGINAL ARTICLE
Pub. 777

ISSN 1678-0345 (Print)
ISSN 1679-9216 (Online)

Uso de oócitos bovinos como citoplasma receptor na produção de embriões por transferência nuclear de célula somática interespécie (NTSCi)

Use of bovine oocytes as recipient cytoplasm in the production of embryos through nuclear transfer of interspecies somatic cells (NTSCi)

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ABSTRACT

Interspecies embryo clones have been produced by research groups with relative success in some species. Bovine oocytes matured *in vitro* and enucleated by micromanipulation were used in three experiments as recipeient cytoplasm in nuclear transfer of ovine, caprine and porcine fibroblasts. The fibroblasts were cultivated until the third passage before being frozen and used. The electrofusion was induced by an application of a 20V pulse during 45 ms. The activation was done with 5 mM ionomycin and subsequently 2 mM 6DMAP. NTSC bovine embryos, NTSCi caprine and ovine embryos were cultivated in SOF medium and NTSCi porcine embryos were cultivated in NCSU23 medium. The fusion rates of the reconstructed complexes with bovine cells did not differ from those observed with ovine cells (88.2%), caprine cells (74.1%) and porcine cells (79.4%). The cleavage rates in ovine (60.3%), caprine (68.4%) and porcine (57.1%) NTSCi groups did not differ from the control group NTSC bovine. The blastocyst rate observed in the group of NTSCi ovine embryos (10.3%) was similar to the group of NTSC bovine embryos (12.7%). In NTSCi caprine embryos, 5.3% of the embryos developed up to the blastocyst stage, while in the NTSCi porcine group there was no development up to the blastocyst stage. In conclusion, the bovine cytoplasm was able to support the embryo development in NTSCi up to the blastocyst stage using ovine and caprine fibroblasts as donor cells.

Keywords: Embryo, Clones, Interspecies, Nuclear Transfer, *In vitro*.

RESUMO

Embriões clones interespécie vêm sendo produzidos por diferentes grupos de pesquisa, com relativo sucesso em algumas espécies. Oócitos bovinos maturados *in vitro* e enucleados por micromanipulação foram utilizados em três experimentos como citoplasma receptor na transferência nuclear de fibroblastos ovinos, caprinos e suínos. Os fibroblastos foram cultivados até a terceira passagem antes de serem congelados e utilizados. A eletrofusão foi induzida pela aplicação de um pulso de 20 V durante 45 ms. A ativação foi realizada com 5 mM de ionomicina e 2 mM de 6DMAP. Embriões NTSC bovinos, NTSCi caprinos e ovinos foram cultivados em meio SOF, e embriões NTSCi suínos foram cultivados em NCSU23. As taxas de fusão dos complexos reconstruídos com células bovinas não diferiram daquelas observadas com células ovinas (88,2%), caprinas (74,1%) e suínas (79,4%). As taxas de clivagem nos grupos NTSCi ovino (60,3%), caprino (68,4%) e suína (57,1%) não diferiram dos grupos controles NTSC bovino. A taxa de blastocisto observada nos embriões NTSCi ovinos (10,3%) foi semelhante à taxa observada no grupo NTSC bovino (12,7%). No grupo NTSCi caprino, 5,3% dos embriões chegaram ao estádio de blastocisto, enquanto que no grupo NTSCi suíno não houve desenvolvimento até o estádio de blastocisto. O citoplasma bovino foi capaz de suportar o desenvolvimento de embriões NTSCi até o estádio de blastocisto utilizando-se núcleo de fibroblatos ovinos e caprinos.

Descritores: Embrião, Clones, Interespécie, Transferência Nuclear, *In vitro.*

Received: February 2008 www.ufrgs.br/favet/revista Accepted: April 2008

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INTRODUCTION

Since Dolly's birth [28], clone embryos have been produced through nuclear transfer of somatic cells (NTSC) with relevant success. One of the NTSC applications is the production of interspecies clone embryos (NTSCi), that is, the use of oocytes of domestic species, as recipient cytoplasm for the development of somatic cell nucleus of another species like non-domestic species or domestic were is difficult to obtain viable oocytes [5]. In this sense, bovine oocytes are being used in NTSCi for *in vitro* production of many species of embryos, such as ovine [5,6,9], porcine [5], buffalo [2,13,17], saola [3], bongo antelope [14], human [4,10] african buffalo [18], elande antelope [18], pet dog [19], yak [15,16,19,26], *takin* [15] and goral [21]. Products born from *Bos gaurus* [27] and *Bos javanicus* (banteng) [11] have been already obtained by this methodology.

However, there is no consensus in literature, because many authors did not achieve *in vitro* development up to blastocyst stage of some species, as in caprine [25], *bontebok* [18] and mice [1,12]. Thus, the aim of this paper were to establish at the Laboratory of Embryology and Biotechniques of Reproduction (FAVET/UFRGS) the technique of interspecies nuclear transfer, using bovine oocytes as recipient cytoplasm and ovine, porcine and caprine as donor cells and at the same time determine the *in vitro* fusion and development rates up to blastocyst stage of reconstructed embryos.

MATERIALS AND METHODS

Culture media and reagents

All chemicals were from Sigma Chemical Co. (St. Louis, MO, USA), unless stated otherwise. All solutions were prepared using water from a Milli-Q Synthesis System (Millipore, Bedford, MA, USA).

Donor cell nucleus

The bovine, ovine and caprine fibroblasts used as donors cells were obtained from fragments of recovered ovaries after the *slicing*. The porcine fibroblasts were obtained by auricular biopsy and cultured through the explantation technique. The cells were cultured in DMEM [8] medium until the third passage and frozen with 10% etylene glycol (EG, E9129) in bovine fetal serum (BFS, Nutricell, Campinas, SP, Brazil). For the freezing, the cells were tripsinized, centrifuged and resuspended in BFS. EG was added so that the final concentration was 10%. The cells were then loaded in 0.5 mL straws and put under 4°C refrigeration for 1 hour. After this period, the straws were transferred for into liquid nitrogen vapor during 15 minutes and then plugged into N_2L. The straws were stored in cryogenic containers until they were thawed, 3-5 days before their use. Each straw had enough cells to confer confluence of 80-90% in 3 days of culture in 4-wells dishes (Nunclon®, Nunc, Roskilde, Denmark). The donors cells before use were tripsinized and then resuspended in SOF [7] medium buffered with HEPES (HSOF) containing 20.0 mM of HEPES (H6147) and supplemented with 2 mg/mL BSA (Gibco-BRL, 11018-017).

Source of bovine oocytes

Bovine ovaries obtained from slaughterhouse were collected from the females immediately after slaughter and transported to the laboratory in thermic recipient containing physiologic solution maintained as 30-32°C. The *cumuli*-oocyte complexes (COCs) were obtained through ovarian cortex scarification (*slicing*) in modified PBS – Dulbecco's. The selected COCs were maintained in TCM-HEPES (M2520) medium, containing 50 mg/mL gentamicin (G1264), 0.2 mM sodium pyruvate (P4562), 2.4 mM $NaHCO_3$ (S5761) supplemented with 1 mg/mL BSA (Gibco-BRL, 11018-017) until be transferred to the maturation medium.

In vitro maturation and oocyte selection for micromanipulation

The oocytes groups (35-40 each) were matured into 500 mL of maturation medium [22] composed by TCM199 (M2520) supplemented with 50 mg/mL gentamicin (G1264), 0.2 mM sodium pyruvate (P4562), 26 mM $NaHCO_3$ (S5761), 0.5 mg/mL FSH (Folltropin, Vetrepharm, Belleville, ON, Canada), 0.03 UI/mL hCG (Profasi, Serono, Brazil), 1 mg/mL estradiol (E8875) and 10% inactivated oestrus cow serum (ECS), for 17 hours in 39 °C in humidify atmosphere containing 5% CO_2 in air.

After maturation, the *cumulus oophorus* cells were removed by pipetting using HSOF medium. The stuctures were then transferred and maintained into drops of the same medium, under mineral oil, at 37 °C (heated table), until the end of micromanipulation procedure. The oocytes selected were the ones that presented polar body and cytoplasm of uniform aspect. Some fresh oocytes which were not used for

NT were preserved in these drops during the manipulation period and later activated parthenogenically, as an intrinsic experiment control.

Enucleation

The enucleation was done under optic microscope equipped with epifluorescence system (UV light and G365 filter) and micromanipulator. The oocytes were incubated for 15 minutes in HSOF supplemented with 7.5 mg/mL cytochalasin B (C6762) and 5.0 mg/mL Hoechst 33342 (B2883). The oocytes were held with a *holding* pipette and with the help of a 25 ìm diameter enucleation pipette the metaphasic plate and the polar body were removed.

Reconstruction

The donor cells were transfered to the perivitelline space of the enucleated bovine oocytes, in the presence of 7.5 mg/mL cytochalasin B in HSOF through micromanipulation.

The reconstructed complexes (RC) were fused using electrodes connected to a micromanipulation system under stereomicroscope. First, the donor cell and the recipient cytoplasm were mechanically aligned for the fusion. The electric current applied was of 20 V for 45 ms, using the electrofusion equipment ECM 2001, (BTX, Holliston, MA, USA). The fusion medium was composed by of 0.25 M mannitol (M1902), 0.1 mM $MgSO_4$ (M2643) e 0.5 mg/mL BSA (Gibco-BRL, 11018-017), with osmolarity of 260 mOsm. After the fusion procedure, the RCs were transferred into HSOF and the fusion rate was evaluated 30 minutes after the electrofusion. The non-fused RCs were submitted to a second electrical stimuli of same intensity and length.

Chemical activation

The reconstructed fused complexes were activated by exposition to 5 mM ionomycin (I0634) in HSOF during 5 min and subsequently incubated into SOF medium supplemented with 2 mM of 6-dimethylaminopurine (6DMAP, D2629) during 3h and 30min.

***In vitro* culture**

The bovine-bovine, caprine-bovine and ovine-bovine reconstructed embryos were cultivated in SOF [7] medium supplemented with 0.4% BSA (Gibco, 11018-017) or 10% ECS. On the other hand, the porcine-bovine embryos were cultivated in modified NCSU23 [24] medium, containing 108.73 mM NaCl (S5886), 4.78 mM KCl (P5405), 1.19 mM KH_2PO_4 (P5655), 1.7 mM $CaCl_2$ (C7902), 1.19 mM $MgSO_4$ (M2643), 25.07 mM $NaHCO_3$ (S5761), 2.0 mM glucose (G6152), 2.0 mM sodium lactate (L1375), 0.20 mM sodium pyruvate (P4562), 1.0 mM glutamine (G5763), 7.0 mM taurine (T7146), 5 mM hypotaurine (H1384), 10 mg/mL vermelho de fenol (P5530), 50 mg/mL gentamicine (G1264) and 10% SVE. The glucose was added only 60 h after the beginning of the culture. All embryos were cultivated in drops under mineral oil, in 39 °C, in humidify atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

Parthenogenic activation

In every replicated of experiment, a group of *in vitro* matured bovine oocytes selected for micromanipulation was kept in the same conditions as the reconstructed complexes throughout the procedure as an intrinsic experiment control. At the end of the process, they were chemically activated and these parthenogenic embryos were cultured at the same conditions as the NT bovine embryos.

Parthenogenic embryos were also used in an preliminary experiment, aiming a comparison the culture medium for porcine and bovine, thereunto parthenogenic embryos were cultivated in NCSU23 medium or SOF supplemented with SVE in six replications.

***In vitro* development evaluation**

The cleavage rate was observed 48 hours after the beginning of the CIV and the blastocyst rate was determined on the 7th and 8th Day, considering the moment of activation as Day 0.

Statistic Analysis

The results obtained in the embryonic development were analyzed by the chi-square test, with 5% significance level.

RESULTS

Experiment 1. NTSCi ovine-bovine embryo production

The fusion rate of the interspecies reconstructed embryos was 88.1% (52/59) and did not present a significant difference of the fusion rate of the homospecific group (bovine-bovine) which was 88.2% (67/76) by the chi-squared test ($p>0.05$).

The *in vitro* development rates of NTSCi ovine-bovine embryos did not present any significant

difference when compared to the homospecific bovine-bovine (Table 1), using the same cell type, activation and culture system.

During this experiment, the parthenogenic control group achieved 74.3% cleavage and 41.4 % blastocyst (n=70).

Experiment 2. NTSCi caprine-bovine embryo production

The fusion rates of reconstructed embryos with caprine cells (74.1% – 20/27) and bovine cells (85.3% – 29/34) in bovine cytoplasm did not present significant differences by the chi-squared test (p>0.05).

The *in vitro* development rates of NTSCi caprine-bovine embryos and NTSC bovine are presented on Table 2.

In the control group (parthenogenic embryos), the cleavage rate was 74.5% and blastocyst rate was 25.4% (n=55).

Experiment 3. NTSCi porcine-bovine embryo production

The *in vitro* development rates of parthenogenic activated oocytes which were cultivated in SOF medium or NCSU23 supplemented with 10% ECS are shown on Table 3.

Porcine and bovine fibroblasts were used for the reconstruction of porcine-bovine and bovine-bovine embryos respectively. The fusion rate of the interspecies RC was 79.4% (27/34) and of the homospecific RC was 83.8% (31/37), and there was no significant difference observed by the chi-squared test (p>0,05).

Table 4 presents *in vitro* development rates of NTSCi porcine embryos, cultures in NCSU23 medium and NTSC bovine embryos.

During this experiment the *in vitro* development rates of the bovine parthenogenic embryo control were 69.0% cleavage and 29.6% blastocysts (n=71).

DISCUSSION

Enucleated bovine oocytes were used as receivers for the *in vitro* development of ovine, caprine and porcine clones. The fusion rates did not differ significantly between the NTSCi (74.4% to 88.1%) and NTSC (83.8% to 88.2%) control groups, reinforcing some results observed in the literature, in which the fusion rates between the interspecies and bovine-bovine control groups are similar [13,19,25].

Also, the cleavage rates in all interspecies groups (ovine-bovine, caprine-bovine and porcine-bovine) did not differ from the homospecific groups (bovine-bovine), Tables 1, 2 and 4. These results were expected, since the first cell divisions, before the activation of the embryonic genome, are not controlled by the nucleus [5]. It can be highlighted that the injuries caused by the cloning procedure, as well as the intrinsic quality of the oocytes, were similar

Table 1. Development rates of interspecies clone embryos (ovine-bovine) and homospecific (bovine-bovine), cultivated in SOF means supplemented with SVE.

Donor cell	Cultivated	Cleaved		Blastocysts	
	n	n	%	n	%
Ovine	58	35	60.3	6	10.3
Bovine	63	36	57.1	8	12.7

There was no significant difference by the chi-squared test (p>0.05).

Table 2. *In vitro* embryonic development rates of reconstructed complexes with bovine cytoplasm and caprine or bovine donor cell.

Donor cell	Cultivated	Cleaved		Blastocysts	
	n	n	%	n	%
Caprine	19	13	68.4	1	5.3
Bovine	29	23	79.3	3	10.3

There was no significant difference by the chi-squared test (p>0.05).

Table 3. Rate of embryonic development of parthenogenic embryos cultivated in SOF medium or NCSU.

Culture medium	Cultivated	Cleaved		Blastocysts	
	n	n	%	n	%
SOF	169	130	76.9	66	39.1
NCSU23	165	126	76.4	58	35.1

There was no significant difference by the chi-squared test ($p>0.05$).

Table 4. In vitro embryonic development rates of reconstructed complexes with bovine cytoplasm and porcine or bovine donor cell.

Donor cell	Cultivated	Cleaved		Blastocysts	
	n	n	%	n	%
Porcine	21	12	57.1	0	0.0
Bovine	28	11	39.3	3	10.7

There was no significant difference by the chi-squared test ($p>0.05$).

between the interspecies and homospecific groups.

The ovine-bovine and bovine-bovine embryo blastocyst rates were similar (10.3% and 12.7%, respectively). Likewise, other authors did not observe significant differences between ovine-bovine and bovine-bovine groups, with blastocyst rates of 9.9% and 9.7% respectively [5]. Other authors obtained satisfactory ovine-bovine blastocyst rates (24.7%), even though significantly inferior to those observed in the parthenogenic group [9].

The NTSCi caprine experiment it was possible, the production of one blastocyst after transfer of a fibroblast nucleus to one bovine cytoplasm differently from the literature data in which the production of caprine-bovine clone embryos was not possible [25]. These data show us that the bovine cytoplasm is able to support the development of NTSCi caprine embryos even though the culture conditions can be enhanced. The use of an adequate culture medium for interspecies embryos can be decisive to the success of the technique, which was observed in murines interspecies embryos. Some authors [1,12,23] claimed that the bovine oocyte is not able to support the development of murines embryos, observing that the produced embryos did not develop beyond the 8-cell stage. However, other authors demonstrated that the success of the development of these murine-bovine embryos could be related to the culture system. They compared the *in vitro* development of NTSCi murines-bovine embryos maintained into an adequate culture medium to promote the development of murines embryos (KSOM) with embryos that were cultivated in a means usually used for the culture of bovine embryos (SOF). The system that used KSOM medium showed better *in vitro* development rates and provided the development up to the morula stage, even though none of them reached the blastocyst stage [20]. In this way, the system of *in vitro* the culture seems to be directly related to the success of the NTSCi and the fact that we achieved the development up to the blastocyst of NTSCi caprine-bovine embryos shows that this is possible. Still, the optimization of the *in vitro* culture conditions is necessary to improve the embryo development rates.

When we used porcine cells, the embryos did not develop beyond the 8-cell stage. These data shows that in the condition of the experiment the donor nucleus was not able to assume the embryonic development control, differing from the data obtained by other authors who got 7.8% blastocysts in NTSCi bovine-porcine embryos cultivated in CR1aa medium [5]. It is important that the culture medium be able to support the embryo development after the genome activation of the donor species, however, the medium has to be able to support the first embryo cleavages, coordinated by the recipient cytoplasm. To evaluate whether the NCSU23 medium often used for the

porcine embryo culture would be able to support the initial development of bovine embryos we cultivated parthenogenic activated oocytes in NCSU23 and SOF medium. Parthenogenic embryos cultured in SOF and NCSU23 medium did not present differences in cleavage rates (76.9% and 76.4%, respectively) and blastocysts development rates (39.1% and 35.1%, respectively) suggesting that this medium could be used in the NTSCi porcine embryo culture in bovine cytoplasm. However, no porcine embryo had been produced *in vitro* in our laboratory. So, this culture system may need adjustments to be able to sustain the development of porcine interspecies clone embryos. A small group of NTSCi porcine embryos was also cultivates in SOF (data not presented), yet there was no development up to blastocyst, even though they presented 60% cleavage (n=10). Another explaination of the embryonic development lack is the intrinsic quality of the oocytes used in the experiment, since the cleavage rate of the control group was only 39.3%, unlike the other experiments realized where the cleavage rates of clone embryos and their respective control groups were between 60 and 80%.

CONCLUSION

Enucleated bovine oocytes were able to support the *in vitro* development of ovine and caprine interspecies embryos.

Acknowledgements. The authors thank CNPq for the partial floatation of this Project through master's, doctorate's and research productivity scholarships granted to the authors.

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