BIRTH OF CLONED CALVES PRODUCED WITH ADULT AND FETAL FIBROBLASTS[1]

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ABSTRACT: The present study examined the viability of bovine nuclear transferred embryos. Oocytes were matured in vitro for 17 hours and enucleated after aspiration of the first polar body and the metaphase plate. Fibroblasts from a 10-year-old Nellore cow and 45-day-old male fetus collected from slaughterhouse were used as nuclei donor. Enucleated oocytes were fused with adult and fetal fibroblasts with an electric stimulus. After electrical fusion, the couplets were incubated in TCM199 plus 10% FCS supplemented with cycloheximide and cytochalasin D for 1 hour and then cycloheximide alone for an additional 4 hours. The activated reconstructed embryos were co-cultured with granulosa cells in TCM 199 for 7–9 days. A total of 569 couplets were reconstructed from adult and 668 from fetal fibroblasts. After electrofusion, 181 (adult cells) and 212 (fetal cells) embryos became fused and 30 (16.6%) and 32 (15.1%) reached the blastocyst stage, respectively. After transferring 21 (adult cells) and 18 (fetal cells) blastocysts, pregnancy rates at day 90 were 19% (4) and 16.7% (3), respectively. There was no significant difference ($P<0.05$) between the pregnancy rates. Two pregnancies from adult cells aborted at the 4th and 5th months of gestation. One recipient that received an embryo from adult fibroblasts produced a healthy Nellore female calf weighting 40 kg. The other recipient that received an embryo from adult fibroblasts had hydrallantois and delivered a dead calf at day 290. The first pregnancy from fetal cells produced a healthy male calf, weighting 34kg (MELLO *et al*. 2003). These results indicated that adult and fetal Nellore fibroblasts could be used successfully as nuclei donor, with similar rates of in vitro and *in vivo* development.

Key words: bovine, cloning, fibroblast, Nellore, nuclear transfer.

NASCIMENTO DE BEZERROS CLONADOS PRODUZIDOS COM FIBROBLASTOS ADULTOS E FETAIS

RESUMO: Este trabalho examinou a viabilidade de embriões bovinos obtidos por transferência nuclear. Oócitos foram maturados in vitro por 17 horas e enucleados após a aspiração do primeiro corpúsculo polar e da placa metafásica. Fibroblastos de uma vaca Nelore de 10 anos de idade e de um feto macho de 45 dias coletado em abatedouro foram utilizados como doadores de núcleos. Os oócitos enucleados foram fusionados com fibroblastos de origem adulta e fetal após estímulo elétrico. Após fusão elétrica, as estruturas reconstruídas foram incubadas em TCM199 acrescido de 10%de SFB e suplementado com cicloheximida e citocalasina D por 1 hora e depois mais 4 horas apenas em cicloheximida. Os embriões reconstruídos e ativados foram co-cultivados em TCM199 com células da granulosa por 7-9 dias. Um total de 569 embriões foram reconstruídos com fibroblasto adulto e 668 com fibroblasto fetal. Após a eletrofusão, 181 e 212 embriões oriundos de células adultas e fetais, respectivamente, fundiram e desses 16,6% e 15,1% chegaram ao estágio de blastocisto. Após transferir 21 e 18 blastocistos oriundos de células adultas e fetais, respectivamenet, as taxas de prenhez aos 90 dias foram 19% e 16, 7%. Não houve diferença estatística ($p<0,05$) entre as taxas de prenhez. Duas gestações originadas de células adultas abortaram ao 4º e 5º mês de gestação. Uma receptora que recebeu embrião de fibroblasto adulto pariu uma bezerra Nelore saudável, pesando 40kg. A outra receptora que recebeu embrião de fibroblasto

adulto teve hidroalantóide e acabou parindo uma bezerra morta aos 290 dias. A primeira gestação de células fetais resultou num bezerro macho saudável, pesando 34kg (Mello *et al*. 2003). Esses resultados indicaram que fibroblastos adultos e fetais de Nelore podem ser usados como doadores de núcleo, com taxas semelhantes de desenvolvimento *in vitro* e *in vivo*.

Palavras-chave: bovino, clonagem, fibroblasto, Nelore, transferência nuclear

INTRODUCTION

The Danish scientist Steen Willadsen was the first one to succeed in cloning a mammal (sheep) from embryo cells using the method of nuclear transfer. He fused 8-cell sheep blastomeres with unfertilized eggs and produced 3 lambs (Willadsen 1986). In 1996, Dolly was the first mammal to be successfully cloned from an adult somatic cell. She was cloned at the Roslin Institute in Edinburgh (Campbell et al. 1996).

After Dolly, mammalian cloning by somatic cell nuclear transfer (NT) became a well established methodology around the world (Oback and Wells 2003). The number of species and cell types from which viable offspring have been produced is rapidly increasing (Hill *et al.* 2000). In cattle, adults have been cloned using fibroblasts, cumulus, granulosa, mammary, muscle, oviduct and uterine cells (Edwards *et al.* 2003). It has also been suggested that fetal cells may work better than adult cells for producing live offspring.

The ability to produce viable cloned using somatic cell that can be maintained in culture offers numerous advantages, such as production of large number of identical offspring or genetic modification for production of transgenic animals (Kasinathan *et al.* 2001). Normal development of reconstructed embryos may also be influenced by the quiescent status of donor nuclei.

The production of viable offspring from adult, embryonic and fetal cell lines was reported to be possible when cells were presumably induced to exit the cell cycle and enter the quiescent G0 prior to nuclear transfer (Campbell *et al.* 1996; Wells *et al.* 1998). According to IBGE, Brazil has one of the largest commercial cattle herds in the world with approximately 206 million heads. This population is 80% beef cattle, which are predominantly of the Nellore breed. Therefore, the successfully use of NT in Nellore breed would be economically important for the Brazilian beef cattle industry.

Thus, the aim of present study was to compare the *in vitro* and *in vivo* development of embryos reconstructed with serum-starved adult and fetal Nellore fibroblasts. We also observed the full-term development of a calf cloned from a 10-year-old Nellore cow.

MATERIALS AND METHODS

Preparation of recipient oocytes

All the media and chemicals were purchased from Sigma, St Louis, MO, USA, unless otherwise stated. Ovaries were obtained from slaughterhouse and maintained at 30°C during transport to the laboratory (about 2 hours). Cumulus-oocytes complexes (COCs) were aspirated from follicles measuring 2-8 mm in diameter using a syringe with a 21-G needle. COCs were assessed morphologically and only oocytes that showed compact cumulus cells and homogeneous ooplasm were selected for *in vitro* maturation. The COCs were matured for 17 hours in TCM 199 plus 10% FCS supplemented with 0.5 μg/ml FSH (Folltropin; Vetrepharm, Belleville, Ontario, Canada), 50μg ml^{-1} LH (Chorulon; Intervet, Boxmeer, Holland), 1μg ml^{-1} estradiol, 22μg ml^{-1} pyruvate and 50μg ml^{-1} gentamicin. After maturation, cumulus cells were totally removed by incubating COCs in 0.1% hyaluronidase solution for 10 minutes and by gentle pipetting with a stripping pipette of the same diameter as the oocytes. After removal of cumulus cells, cumulus-free oocytes with the first polar body were transferred to micromanipulation medium (Dulbecco's PBS + 20% FCS) for enucleation. This procedure was carried out by cutting the zona pellucida close to the first polar body using a glass needle and pushing out by compression a very small amount of cytoplasm beneath the polar body. Successful enucleation was confirmed by visualizing the karyoplast that had been pushed out and stained with 5μg ml^{-1} Hoechst 33342 under ultraviolet light. This way, the recipient oocytes were not exposed to ultraviolet light.

Preparation of nuclear donor cells

A primary culture of adult and fetal fibroblasts was established as nuclear donor cells. An ear's skin fragment was surgically removed from a 10-year-old Nellore cow and cultured to establish a primary culture. Fetal fibroblasts were removed from a 45-day-old male fetus. The head and internal organs were removed and the remaining tissue was cut into small pieces with scissor. The pieces of adult and fetal fragments were separately rinsed in Dulbecco's PBS and seeded in flasks containing Dulbecco's Modified Eagles Medium (DMEM) + 10% of FCS, 50µg ml^{-1} gentamicin for 24 hours. After being passaged up to three times in DMEM + 10% of FCS, cells were cryopreserved in DMEM with 20% FCS and 10% dimethylsulfoxide and stored in liquid nitrogen for further use. Two to three days before NT, frozen-thawed fibroblasts were cultured in medium containing 0.5% FCS (serum starvation). Thirty minutes before NT, a cell suspension of adult or fetal fibroblasts was prepared by standard trypsinization (Freshney 1994). In this study, only fibroblasts between the 3rd and 10th passage were used as nuclear donors, since there is no difference in efficiency in those passages (Kato *et al.* 2000; Poehland *et al.* 2007).

Production of Nuclear Transfer embryos

The medium used for reconstruction was the same as the employed for enucleation (Dulbecco's PBS + 20%FCS). An individual fibroblast was introduced into enucleated oocytes through a slit in the zona pellucida and the enucleated oocytes-nucleus donor cell complexes were transferred to the fusion medium (0.3 M Mannitol). After 5 minutes, the complexes were manually aligned in the electrode chamber and fused and activated with a single DC pulse of approximately 4.0KV/cm for 20 µs. After fusion, couplets were incubated in cytochalasin D (2.5µg ml^{-1}) and cycloheximide (10 µg ml^{-1}) for 1 hour and cycloheximide alone for further 4 hours. The fusion rate was determined 1 hour after electrofusion by microscopic examination and before *in vitro* culture.

***In vitro* culture, blastocyst cell counting and embryo transfer**

Nuclear transferred embryos were co-cultured with a granulosa cell layer in a 50µl drop of TCM 199 + 10% FCS overlaid with mineral oil at 39°C in a humidified air with 5% CO_2 (Smith 1991). Cleavage and development to the blastocyst stage were examined after 2 and 7 days of culture, respectively. From day 7 to 9 after fusion, only grade I embryos (embryo evaluation according to Roberts and Nelson 1998) of the same stage of development (hatching blastocyst) were selected. Part of these blastocysts was stained with 5µg ml^{-1} of Hoechst 33342 for counting blastocyst cell numbers while others were transferred to recipients. One or two embryos were transferred nonsurgically to the uterine horn of a recipient cow on day 7 or 8 after natural or induced estrus. Recipients that had corpus luteum were synchronized with a single application of prostaglandin F2 alpha. Estrus detection was performed for all animals. Pregnancy was diagnosed by ultrasound on day 90 of gestation.

Statistical analysis

Results from *in vitro* and *in vivo* development were analyzed by chi-square and fisher exact test. The total number of cells was analyzed with the Statistical Analysis System for Windows V8 (SAS Institute Inc., Cary, NC, USA, 2000). The mean and standard deviation was analyzed by ANOVA for Fisher test. Differences between treatments were considered significant at $P<0.05$.

RESULTS

***In vitro* development**

The *in vitro* development of embryos reconstructed from either adult or fetal cells is summarized in Table 1. The potential of enucleated oocytes to develop to blastocysts was not different between adult and fetal fibroblasts (Table 1). However, the potential to develop to the 2-cell stage was higher in embryos reconstructed with fetal cells. The control group (IVF embryos) showed a blastocyst rate higher than NT embryos. In relation to fusion rates, there was no significant difference between clone groups.

Pregnancy rates

Twenty-one blastocysts from adult cells were transferred into 11 recipients and 18 derived from fetal fibroblasts were transferred into 14 recipients

Table 1. *In vitro* development of NT embryos derived from either adult or fetal fibroblasts or IVF embryos (control)

Donor cell type	Nº of couplets (replicates)	Nº of fused couplets (%)	*Nº of embryos that developed to the (%)	
			2-cell stage	Blastocyst stage
Adult	569 (28)	181 (31.8)a	130 (71.8)a	30 (16.6)a
Fetal	668 (33)	212 (31.7)a	193 (91)b	32 (15.1)a
**Control	1611 (12)	-	1210 (75.1)a	516 (32)b

* Nº based on the number of fused couplets.
** *In vitro* produced embryos.
a, b: values with different superscripts in the same column differ significantly (P<0.05).

(Table 2). On Day 90 of gestation, we diagnosed pregnancy by ultrasonography in 4 recipients for adult cells and in 3 recipients for fetal cells. There was no difference in the pregnancy rates between adult and fetal group; however, both rates were lower than the control group. Although most recipients had received two embryos, all pregnancies consisted of only a single fetus.

From 4 pregnant recipients with embryos derived from adult cells, two of them had spontaneous abortions at the 4th and 5th month of gestation. The placenta and umbilical cord from both aborted calves were edematous and a decreased number of placentomes was observed. However the fetuses were apparently normal. One calf weighing 40 kg was born by Caesarian section at 291 days of gestation and other recipient, who had hydrallantois, delivered a stillbirth at day 290 (normal weight and pregnancy length for Nellore breed, respectively, 31 ± 3.62kg and 290 ± 4.70 days). Concerning pregnancies with embryos derived from fetal fibroblasts, one pregnancy resulted in the birth of one male weighing 34kg. One recipient died of hydrallantois 2 months before term and other delivered a stillbirth at 252 days of gestation. The two cloned calves that were born alive had a good development and never showed any health problems. They presented a normal growth rate at thirty and 60 days of age.

Quality of NT embryos

The mean blastocyst cell numbers were 101.3, 129.3, and 114.3, respectively for adult, fetal and IVF (control) embryos (Table 3). There was no significant difference (P<0.05) in the number of cells in blastocysts between groups.

Table 2. Pregnancies following embryo transfer of NT blastocysts reconstructed with either adult or fetal fibroblasts and *in vivo* produced embryos (control)

Donor cell type	Blastocysts transferred	Nº of recipients	Fetuses at day 90 (%*)	Stillbirth	Live calves 60 days after birth
Adult	21	11	4 (19)a	1	1
Fetal	18	14	3 (16.7)a	1	1
**Control	111	111	62 (55.9)b	0	60

* Nº based on the number of blastocysts transferred.
** *In vivo* produced embryos.
a, b: values with different superscripts in the same column differ significantly (P<0.05).

DISCUSSION AND CONCLUSIONS

This study demonstrated that fetal fibroblasts did not display improved development rates over the adult fibroblasts from a 10 year old Nellore cow. It has been suggested that fetal cells may work better than adult cells for producing live offspring but we did not observe differences in blastocyst and pregnancy rates between these groups. Previous studies have also shown similar blastocyst development of NT bovine embryos derived from adult and fetal fibroblasts. In those studies, the

blastocyst rate for fetal cells ranged from 18 to 43%, while for adult cells ranged from 9 to 49% (CIBELLI *et al.* 1998; WELLS *et al.* 1999; KATO *et al.* 1998; WELLS *et al.* 1998). In another study that compared the NT embryo development rates of adult and fetal cells within the same genotype, the authors also obtained similar blastocyst and pregnancy rates (HILL *et al.* 2000).

In order to clarify the possible reason for lower *in vitro* development rates and higher frequency of abortions in cloned embryos, we compared cell numbers of NT blastocysts with IVF derived embryos. As shown in Table 3, the total cell number in blastocysts derived from adult and fetal cells was similar to IVF embryos (P>0.05). Similar cell numbers in NT blastocysts derived from different types of somatic cells and IVF derived embryos have been shown in previous studies (KASINATHAN *et al.* 2001; KOO *et al.* 2002).

Table 3. Mean cell number of blastocysts reconstructed with adult or fetal fibroblasts or IVF embryos (control)

Donor cell type	No. of blastocysts	Mean cell number ± SD
Adult	6	101.3 ± 25.45a
Fetal	10	129.6 ± 16.98a
**Control	6	114.3 ±21.62a

** *In vitro* produced embryos.
a, b: values with different superscripts in the same column differ significantly (P‹0.05).

Another study showed aberrant allocation of inner cell mass and trophectoderm cells in bovine NT blastocysts (KOO *et al.* 2002). The authors have shown that NT embryos have a higher ratio of ICM:total cells than did IVF or *in vivo*-derived embryos and suggest that placental abnormalities or early fetal losses in the cloning system may be due to aberrant cell allocation. In the present study, although we have similar cell numbers in NT blastocysts and IVF derived embryos, the *in vivo* development rate was lower in NT embryos (Table 2). This result could be explained by probable different ratio of ICM : total cells between NT and in IVF-derived embryos. As shown in a previous study, the ratio of ICM : total cells for bovine blastocyst produced *in vitro* or *in vivo* ranged from 20 to 40%, while in NT embryos, this ratio was 50.1% (KOO *et al.* 2002). The present study, in agreement with KOO *et al.* 2002, hypotheses that the decreased number of trophectoderm cells in NT embryos might explain in part the high incidence of placental abnormalities or early fetal losses in this experiment.

These anomalies may also lead to inappropriate expression of genes essential for early embryonic development. Expression of a few genes that are exclusively expressed in the TE was heavily affected by the cloning procedure (WRENZYCKI *et al.* 2001), supporting the hypothesis that abnormal placentation may be a major cause of fetal loss after transfer of NT embryos.

According to pregnancy rates, the mean percentage of pregnant recipients observed in this study at day 90 (17.9%, 17/39) was lower than in other study (KATO *et al.* 2000). This difference could be due to the fact that the pregnant rate in the present study has been calculated on the number of embryos transferred and not on the number of recipients as done in other study.

The lack of spontaneous parturition and increased prenatal mortality is a consistent finding in animals cloned from somatic cells (KATO *et al.* 1998; WELLS *et al*. 1999; HEYMAN *et al*. 2002). In this experiment, from 9 cows that carried cloned fetuses, 7 did not produce live calves (2 cows with hydrops, 4 had spontaneous abortions between 2nd and 5th month of gestation, and 1 cow delivered a stillbirth at term). The high rate of fetal loss in this experiment might be related to abnormalities of placentation that also have been observed in others studies (HILL *et al.* 1999; Koo *et al.* 2002; HEYMAN *et al.* 2002). DNA methylation plays an important role in the regulation of gene expression during embryonic development.

A recent report has shown abnormal methylation patterns in cloned embryos and offspring as compared with those derived by *in vitro* fertilization (CIBELLI *et al.* 2002; Maalouf *et al.* 2008; Niemann *et al.* 2008). Another study showed that the NT process affects gene expression patterns in the trophectoderm- and inner cell mass-derived tissues to different extents (DINDOT *et al.* 2004). These authors demonstrated loss of imprinting (LOI) of the *XIST* locus within the chorion of all pregnancies (n = 3) generated and additionally revealed improper reprogramming of the satellite I and epidermal cytokeratin promoter in the chorion. In conclusion, it has been suggested that during nuclear reprogramming, somatic cell nuclei are improperly

reprogrammed during early embryonic development and potentially induce the placental abnormalities that are prevalent in cloned animals.

The present NT procedures have proved to be repeatable, but are very inefficient, ranging between 1% and 4% of reconstructed embryos developing to adulthood (WILMUT *et al.* 2003). The inefficiency is the accumulated effect of failure at all stages of development which might be related to incomplete reprogramming of the somatic nucleus by egg cytoplasm (TAMADA *et al.* 2004). Before somatic cell NT is applied to practical animal breeding, further investigations into increasing survival of NT embryos, fetuses and offspring should be done to increase the efficiency of NT technique. In conclusion, although much work is yet to be done, our results are in accordance with other publications (CAMPBELL *et al.* 1996) that it is possible to clone animals of advanced age. The present study showed that fibroblasts from a 10 year old Nellore cow can be successfully reprogrammed using the NT technique to produce viable offspring. These results also showed that fetal fibroblasts do not improve the *in vitro* and *in vivo* rates of development of NT embryos.

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