The objective of this study was to examine consequences of somatic cell nuclear transfer after fusion of somatic cell with maternal (autologous) or non-autologous ooplasts. To this end, granulosa cells from Jersey cows were fused with enucleated oocytes from the same Jersey cows (autologous ooplasts) or from Holstein, Hereford, and Angus cows (non-autologous ooplasts). The ability of cloned embryos to cleave and develop to morula or blastocyst was assessed on days 4 and 7 post-activation, respectively. Compact morulae and blastocysts were transferred to synchronized recipients and pregnancy confirmed by ultrasound 30-32 days post-estrus. Data were analyzed using Fisher’s Exact Test. Development to the 8 to 16-cell stage was greater for clones constructed with non-autologous ooplasts. However, morula and blastocyst development did not differ. Four pregnancies progressed beyond 200 days but fetal death occurred in three due to placental insufficiency. The one clone that progressed to term was constructed with a non-autologous ooplast, highlighting that the use of autologous ooplasm does not always improve somatic cell nuclear transfer outcomes.

**Keywords:** Somatic cell nuclear transfer, Bovine, Autologous ooplast, Clone, Pathologies

**INTRODUCTION**

Somatic cell nuclear transfer (SCNT) typically involves the transfer of a somatic cell nucleus into an ooplast (enucleated oocyte) obtained from a nondescript female. This results in embryos with nuclear DNA from the somatic nucleus but mitochondrial DNA (mtDNA) from the “foreign”, non-autologous oocyte cytoplasm (Plante et al., 1992; and Evans et al., 1999). Thus, the resulting embryo is not a “true” clone like identical twins; rather it is chimeric at the mitochondrial level (i.e., mtDNA heteroplasmy).
The significance of maternal inheritance of oocyte mitochondria for embryo development is largely ignored in SCNT procedures despite decreased physical performance and growth rates when nuclear and mitochondrial genomes were mismatched (Nagao et al., 1998). Yang et al. (2006) reported improved embryo development and birth rates when SCNT embryos were constructed with cumulus cells and oocytes from the same donor Holstein (autologous SCNT) rather than oocytes obtained from a different Holstein.

Subsequent efforts by the same laboratory demonstrated that when embryos were constructed from Holstein ear fibroblasts and oocytes with the same mtDNA haplotype (i.e., homotype A-A or B-B), embryo development, pregnancy rates, and live births were improved (Yan et al., 2010). This strongly suggests that mtDNA and/or mitochondria play an important role in the development of clones.

The objective of this study was to determine the repeatability of constructing SCNT embryos from autologous or non-autologous ooplasm using a different cattle breed and cell type than previously examined. To this end, autologous ooplasm was derived from the same Jersey cows (n = 6) using an ultrasound-guided transvaginal probe as previously described (Lawrence et al., 2005). Immediately after collection, any cumulus-oocyte complexes were removed from the aspirate to maximize the likelihood of establishing a primary culture of granulosa cells. The cells were washed in Dulbecco's Phosphate-Buffered Saline (DPBS) before culture in Dulbecco's modified Eagle medium containing 10% FBS (v/v), 4.5 g/L glucose, 2 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin at 38.5 ºC in 5.5% CO₂ in humidified air. Cultured cells, which were kept separate according to somatic cell donor, were frozen in FBS containing 10% dimethyl sulfoxide (v/v) and stored in liquid nitrogen. Before nuclear transfer, cells were thawed and cultured in 0.5% FBS (passage = 3; confluence 70-75%).

**Somatic Cell Nuclear Transfer**

The day before nuclear transfer, ovaries were surgically removed from Holstein, Angus and Hereford cows (non-autologous, n = 10) or Jersey cows (n = 6) from which the ovarian/granulosa cell lines were previously derived (autologous). Cumulus-oocyte complexes...
(COC) were freed from ovarian follicles by slicing and agitating in oocyte collection medium (M199 with Hanks salts supplemented with 4.2 mM NaHCO₃, 10 mM 1 M HEPES, 2% FBS (v/v), 2 mM L-glutamine, 50 U/mL penicillin, and 50 µg/mL streptomycin with 50 µg/mL heparin to prevent clotting) and kept separate according to origin for maturation in oocyte maturation medium (OMM; Medium-199 containing Earle’s salts supplemented with 10% FBS (v/v), 50 µg/mL gentamicin, 5 µg/mL FSH, 0.3 µg/mL LH, 0.2 mM sodium pyruvate, and 2 mM L-glutamine). After 18 and 20 h in OMM, oocytes were denuded of cumulus and examined for polar body extrusion. Oocytes with polar bodies were Hoechst stained (5 µg/mL No. 33342) in medium containing cytochalasin B (5 µg/mL) for 5 to 10 min. Polar body and metaphase II plate were removed in 5 to 10% of the ooplasm volume using a glass micropipette (10 µm internal diameter; Figure 1). Removal of the polar body and metaphase II plate was confirmed by exposing micropipette content to ultraviolet light. Enucleated oocytes were washed thoroughly and held in OMM at 38.5 ºC in 5.5% CO₂ in humidified air.

Autologous clones were constructed using an ooplasm and somatic cell derived from the same Jersey cow. Non-autologous clones were constructed with somatic cells from Jersey cows but with ooplasm from Holstein, Angus, or Hereford cows. Granulosa cells were kept separate according to donor and were washed in DPBS without Ca²⁺ or Mg²⁺ before trypsinization to obtain a single cell suspension. A single granulosa cell was then positioned in contact with the ooplasm using a glass micropipette (10 µm internal diameter) and the couplet placed between electrodes in fusion medium (0.26 M mannitol containing 0.1 M MgSO₄; Figure 1). Couplet fusion was induced by exposure to an electrical pulse of 2.2 kV/cm for 40 µsec (Electro Cell Manipulator ECM 2001, BTX/Harvard Apparatus; Holliston, MA). Cloned embryos were activated 28 to 32 h post-maturation using ionomycin (5 µM for 4 min) and 6-dimethylaminopurine (2 mM for 4 h; Susko-Parrish et al., 1994). Activated embryos were transferred to KSOM (Biggers et al., 2000) supplemented with 0.5% bovine serum albumin (w/v) and cultured in an atmosphere of 7% O₂ and 5.5% CO₂. Cleavage (ability to divide into more than 1-cell) and morula or blastocyst development were assessed on days 4 and 7 post-activation, respectively (Figure 1).
Embryo Transfer
Compact morulae and blastocysts were transferred into the uterine horn ipsilateral to the corpus luteum of synchronized recipients (n = 37 Angus, Hereford, or crossbred heifers) injected intramuscularly at transfer with 10 cc flunixin meglumine (Banamine; Schering-Plough, Kenilworth, NJ). Establishment of pregnancy was confirmed 30-32 days after estrus by presence of an embryonic heartbeat using ultrasound (Figure 1). Established pregnancies were monitored weekly to days 99-101.

Statistical Analysis
Data were analyzed using Fisher’s Exact Test using SAS (version 8.0, SAS Institute, Inc., Cary, NC, USA). Significance was set at P < 0.05.

RESULTS AND DISCUSSION
In Vitro Development of Cloned Embryos
The proportion of oocytes recovered after denuding (93.2 vs. 95.4% for nonautologous vs. autologous cows; P = 0.27) and extruding a polar body (58.9 vs. 58.4% for nonautologous vs. autologous cows; P = 0.93) did not differ between groups. Electrically-induced fusion of granulosa cell/cytoplast couplets was similar regardless of whether they were constructed using non-autologous or autologous cytoplasm (66.0 vs. 63.1%, respectively; P = 0.69). Significantly more couplets constructed with non-autologous ooplasm lysed compared to those constructed with autologous ooplasm (33.0 vs. 13.1%, respectively; P = 0.0002). Other than possible differences in lipid content of non-autologous oocytes compared to autologous (Jersey) oocytes (Weathers and Prien, 2014), the underlying basis for increased lysis of non-autologous couplets is unclear. However, because of the overall low numbers of fused couplets, this physiological difference is questionable.

Development of cloned embryos to the 8 to 16-cell stage by day 4 was greater (P = 0.0052) for those constructed with non-autologous versus autologous cytoplasm (Table 1). This is in contrast to others who saw similar (Yan et al., 2010) or improved (Yang et al., 2006) development to the 8-cell stage for SCNT embryos constructed with autologous cytoplasm. The lack of a beneficial effect of autologous cytoplasm on development 8 to 16-cell embryos as described herein may be due to limited mitochondria-nuclear interaction in early cleavage-stage embryos (Tarazona et al., 2006). Nonetheless, by day 7 a similar proportion of both groups had developed to the morula and blastocyst stages (Table 1). This may indicate a

<table>
<thead>
<tr>
<th>Cytoplasm</th>
<th>Number</th>
<th>8 to 16-cell (%)</th>
<th>M/B (%)</th>
<th>ET</th>
<th>No. of Recips</th>
<th>Pregnant 30-32 d (%)</th>
<th>Pregnant 73-75 d (%)</th>
<th>Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-autologous</td>
<td>68</td>
<td>52 (76.5)</td>
<td>16 (23.5)</td>
<td>10</td>
<td>10</td>
<td>2 (20)</td>
<td>2 (100)</td>
<td>1</td>
</tr>
<tr>
<td>Autologous</td>
<td>99</td>
<td>54 (54.6)</td>
<td>24 (24.2)</td>
<td>16</td>
<td>16</td>
<td>5 (31.3)</td>
<td>2 (40)</td>
<td>0</td>
</tr>
</tbody>
</table>

P-value 0.005 1.00 0.67 0.43

Note: * Number of reconstructed embryos; b Total number of morulae and blastocysts; c Total number of cloned embryos transferred to recipients.

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heightened ability of autologous cytoplasm to support development of embryos to morula and blastocyst stages compared to non-autologous cytoplasm as reported by others (Yang et al., 2006; and Yan et al., 2010). As early embryogenesis proceeds, mitochondrial activity remains low in non-competent embryos but increases in developmentally-competent embryos (Tarazona et al., 2006). The ability of a greater proportion of 8 to 16-cell autologous embryos to reach the morula and blastocyst stages compared to non-autologous 8 to 16-cell embryos suggests that matched mitochondrial-nuclear genome interactions are important for in vitro embryo development and that autologous embryos were more able to upregulate mitochondrial activity.

Establishment and Maintenance of Pregnancy

Establishment of pregnancy after embryo transfer on days 30 to 32 was similar between groups (Table 1). Four pregnancies progressed beyond 70 days. At days 208 and 236 of pregnancy, fetal death occurred in two recipients because of placental insufficiency associated with hydralantois. A caesarean section was performed on a third recipient at 241 days of pregnancy due to hydramnii and hydralantois. However, fetal death with aspiration of meconium and amniotic squames had occurred prior to delivery. This high incidence of hydrallantois with placentome reduction and compensatory intercotyledonary placental hyperplasia is similar to other reported SCNT embryo transfers attempts in cattle (Heyman et al., 2002; and Edwards et al., 2003). Hydralantois alters the placenta resulting in reduced fetalmaternal interactions to induce fetal stress and death due to impaired gas and nutrient exchange across fetal membranes (Farin et al., 2001). The presence of meconium and amniotic squames in the lungs of late term fetuses in our study suggests fetal distress prior to death. In addition, specific placental abnormalities including altered protein profiles (Kim et al., 2005) and abnormal fetal fluid biochemical composition (Li et al., 2005) have been reported which may also play a role in fetal death of clones. While it is tempting to speculate that mitochondrial heteroplasmy may play a role in the development of this condition, the occurrence of hydralantois in pregnancies with clones derived from both autologous and non-autologous ooplasts does not support this notion.

Only one clone constructed with non-autologous cytoplasm progressed to term and was delivered by caesarean section at 275 days, suggesting that use of autologous ooplasm does not always improve SCNT efficiency. One possible reason for the lack of a beneficial effect may be the apparent elimination of nuclear donor mitochondria by the 16-cell stage in cloned bovine embryos (Do et al., 2001). Furthermore, donor mtDNA (i.e., mtDNA heteroplasmy) may persist without being detrimental to embryo viability. Heteroplasmy of mitochondrial genomes has been reported in certain strains of mice (Meirelles and Smith, 1997 and 1998) with no apparent negative impact on health. In the bovine, donor mtDNA may persist in the blastocyst stage embryos (Do et al., 2002), live offspring (Takeda et al., 1999; and Steinborn et al., 2000) and adult clones (Hiendleder et al., 1999). Such persistent heteroplasmy in SCNT-derived offspring may result from fusion of cytoplasmic and somatic cell donor mitochondria (Meirelles and Smith, 1997 and 1998) or incorporation of donor mtDNA by endogenous mitochondria (King and Attardi, 1988 and 1989). Mitochondrial genotype was not
assessed in this study, precluding further speculation as to influence of maternal lineage on observed embryo and fetal development.

The surviving calf was of normal birth weight (34 kg; Andersen and Plum, 1965) and developed normally up to six months of age at which time she began to lose weight and develop diarrhea. She was diagnosed with rumen stasis and acidosis. A rumenotomy with transfaunation was performed to restore rumen function. The calf subsequently developed peritonitis resulting in humane euthanization at seven months of age. This complication has been reported previously in cloned cattle (Wells et al., 1998).

The low live birth rate is similar to that previously obtained in our laboratory (Lawrence et al., 2005) and others (Wells et al., 1998; and Meirelles et al., 2001) suggesting this rate is not laboratory dependent. However, previous cloning efforts by our laboratory using MHC class I homozygous or heterozygous donor cell lines demonstrated improved efficiency when donor cells were MHC class I homozygous (Davies et al., 2004). Specifically, homozygous cell lines had a term birth rate of 21.3% (23/108 transferred) vs 1.9% (1/53 transferred) for heterozygous cell lines (Davies et al., 2004). Other investigators have suggested SCNT efficiency may be dependent upon cell line (Powell et al., 2004) or related to maternal mtDNA haplotype (Bruggerhoff et al., 2002; and Jiao et al., 2007). Specifically, maternal lineage affected the number of transferable embryos and numerically increased the proportion of established pregnancies (Bruggerhoff et al., 2002).

Construction of SCNT embryos with autologous rather than non-autologous cytoplasm did not improve embryo development or the establishment and maintenance of pregnancy. These results are in contrast with three other reports from the same laboratory (Yang et al., 2006; Yan et al., 2010; and Yan et al., 2011). Discrepancy is difficult to explain but may be multi-factorial including differences related to breed and procedures.

**CONCLUSION**

The use of autologous cytoplasm for SCNT is the most direct approach to address concerns regarding incompatibility between donor and host nuclear and mitochondrial genomes. The results described herein demonstrate a similar inefficiency of SCNT whether using autologous or non-autologous cytoplasm to construct cloned embryos. Poor pregnancy outcomes when using SCNT embryos are likely related to other factors in addition to mismatches in nuclear-mitochondrial communication.

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