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RESEARCH ARTICLE

Production of Somatic Cell Nuclear Transfer derived cloned calves in the United Arab Emirates

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ABSTRACT

Despite the tremendous advancement in somatic cell nuclear transfer (SCNT) in camels, the generation of cloned calf using SCNT in cows is yet to be reported in the United Arab Emirates (UAE). The present study intended to produce cloned calves in the local environment of the UAE. We surgically collected ear tissue from two high-performance Holstein cows (average production of 71 liter/day) and established the fibroblast cell line to perform SCNT. Fifty-seven (18.48%) blastocysts were derived from 330 fused oocytes for cell donor A, and 51 (19.14%) blastocysts were generated from 289 fused oocytes for cell donor B. We used 102 recipients for embryo transfer; among those, 53 and 49 recipients received embryos reconstructed with cell donor A and B, respectively. A single blastocyst in transfer media was transferred deep into the horn of the uterus of natural cycling recipient cows by trans cervical method on Day 7; where estrus was considered as Day 0. Non-return to estrus (up to 42 days), early pregnancy, and the live birth rate did not differ significantly for the two donor cells. Four (26.67%) and 1 (7.69%) live calves were produced for cell donor A and B, respectively. Microsatellite analysis of 18 bovine-specific loci confirmed the perfect match of cell donors and cloned calves. This is the first reported case of cloned calves using SCNT in the UAE. Our results could benefit the UAE dairy industry by supporting the locally produced high-performance replacement heifers.

Keywords: Bovine; Cloning; *In vitro* maturation; Oocytes; UAE.

INTRODUCTION

The dairy industry is transforming and reorganizing rapidly in the United Arab Emirates (UAE). The expanding population size, coupled with people's increased concerns about living a healthy lifestyle and the nutritional value offered by milk and other dairy products, has boosted the local dairy industry in the UAE. Market research conducted by Dijik (2021) reported that dairy products had a market size of US$ 1.66 billion in 2020, which may reach US$ 2.47 billion by 2026, with an annual growth rate of 6.89%. This increased production requires a smooth supply of replacement heifers to support continued and increased milk production. Bolstering local heifer production and incorporating indigenous traits and developmental adaptation to the local environment may achieve this goal compared to the importation of foreign replacements. Advanced genetic and reproductive technologies can also aid in selecting and propagating livestock adapted to local conditions.

Among the various reproductive tools, somatic cell nuclear transfer (SCNT) draws intense scientific interest in animal breeding, principally to enable the multiplication of individuals with outstanding performance. Besides the breeding program, it has versatile uses, such as producing transgenic animals and conserving endangered animals. The groundbreaking success of cow cloning was announced by ABS Global Inc. in 1997 when they declared the first-ever cloned calf, Gene (Arthur, 1998). Following the successful cloning of elite bulls, the production of cows with superior milk yield and even the conservation of an endangered breed of cows named Enderby Island was reported (Wells et al., 1998; Yonai et al., 2005; Hoshino et al., 2009). In addition, Cibelli et al. (1998) reported the production of the first transgenic

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cows, which opened the door to using cows in human biomedical research.

Nuclear transfer technology has successfully cloned several mammalian species, but cows are probably the most extensively studied animals for SCNT (Oback and Wells, 2007; Ross and Cibelli, 2010). Hundreds of healthy cloned calves from different breeds, such as Holstein, Jersey, etc., had been born and reached adulthood. The reproductive performances of cloned animals were reported to be normal; their descendent also had similar productive and reproductive parameters to the age-matched non-cloned animals (Enright et al., 2002). Despite tremendous advances in SCNT in various animal species, there is no report of cow cloning from any Middle Eastern countries, except for one popular article from the Royan Institute of Iran (Anonymous, 2009). Many factors influence the success of SCNT, such as oocyte quality, oocyte reconstruction, fusion and activation condition and culture condition of embryos, estrus synchronization of recipients, and embryo transfer procedure (Suzuki et al., 2009; Sugimura et al., 2012; Akagi et al., 2014; Su et al., 2014). Besides these biological and technical factors, the environmental condition also influences the overall performance of cows (Morton et al., 2007; Wolfenson and Roth, 2018). The unique dry and hot environment of the UAE may limit the success of SCNT. Accordingly, our aim was to produce cloned calves from two elite Holstein cows in the local environmental condition of the UAE. We also evaluated the feasibility of using *in vitro* matured oocytes, the most expedient oocytes source, for producing cloned calves in the UAE.

MATERIALS AND METHODS

UAE Biotech Research Centers' Ethics Committee carefully reviewed all the procedures related to animal usage and approved the experiment protocol (Approval No.: UAEBRC-B02).

Chemicals and media

We purchased most of the chemicals and reagents from Sigma. However, the chemicals and reagents purchased from other sources were mentioned accordingly.

Management of animals

Before enrolment in the experiment, cows were checked for their general and reproductive health. Only healthy cows without any reproductive abnormalities were used as surrogates. The cows were fed with standard feed, and adequate drinking water was provided. The age of selected cows was 3 to 6 years and body weight ranged between 400 to 550 kg. The experiments were performed from February to May 2021. Pregnant cows were kept in the research facility until the delivery of calves.

Preparation of donor cells

Biopsy was performed to collect the ear skin from two high-performance Holstein cows (Average Daily production: 71 liter/cow). Primary culture and subcultures of the collected skin were done using our standard protocol (Jang et al., 2006).

In vitro maturation, SCNT, and embryos culture

We used a nearby slaughterhouse to collect cow ovaries and brought them to the laboratory without any delay (usually within 2 h) in lukewarm 0.9% (v/v) NaCl solution supplemented with 1% antibiotic–antimitotic (Thermo Fisher Scientific). We followed our laboratory standard protocols (IVM: Son et al., 2022a; SCNT: Jang et al., 2006). After reconstruction and activation of oocytes, we cultured 7 to 9 oocytes in a culture drop. We used BO-IVC to make the drop; the drop was 30 μL in size and covered by light mineral oil (IVF Bioscience, Falmouth, U.K.). We used CO_2 and O_2 at 5% concentration in a humidified atmosphere at 38 °C. Various embryo developmental stages, such as cleavages, morulae, and blastocysts were observed and recorded at 48h, 96 h, and 168 h after culture.

Embryo transfer and pregnancy detection

A single blastocyst in BO-transfer media was transferred deep into the horn of the uterus of natural cycling recipient cows by trans cervical method on Day 7 (estrus = Day 0).

Fifty-three blastocysts were transferred to 53 recipients for cell donor A, and 49 blastocysts were transferred to 49 recipients for cell donor B. All recipients were monitored for estrus behavior. Non-return to estrus cows were examined per rectum on Day 42 post estrus (35 days after ET) in order to confirm pregnancy. All pregnant cows were closely monitored, and pregnancy was detected at regular intervals by rectal palpation.

Parentage analysis

Thermo Scientific Bovine Genotypes Panel 3.1 was used to amplify 18 STR loci. All the routinely used 12 STR loci for bovine parentage testing and identification were included in our selected 18 STR loci (Manufacturers' manual). We isolated the genomic DNA from the two donor cells, the umbilical cord of all cloned calves, and the venous blood of surrogate mothers using a commercial Kit (Qiagen, Germany).

Statistical analysis

Statistical Package for Social Science (version 15) was used for statistical analysis. Student's t-test was used to compare

the embryo development rate and the average number of embryos transferred per recipient (Son et al., 2022b). Pearson's Chi-square test, followed by Fisher's exact test, was used to compare the pregnancy rate between groups. A P value less than 0.05 is considered significant.

RESULTS

Culture of reconstructed embryos

Five hundred seventy oocytes were reconstructed with cell donor A, whereas 431 were reconstructed with cell donor B. The fusion rate (61.66 ± 4.06 vs. 65.62 ± 2.80), cleavage rate (64.92 ± 2.17 vs. 62.17 ± 3.42), and blastocyst rate (18.48 ± 2.04 vs. 19.14 ± 2.01) were similar (P>0.05) for both the cell lines (Table 1).

Pregnancy outcome and parentage of cloned calves

The data for non-return to estrus rates, pregnancy rates at 42 and 90 days, and full-term development rates are presented in Table 2. The non-return to estrus rate at 42 days after estrus (up to 35 days after ET) was 28.30% and 26.53% in Groups A and B, respectively. On rectal palpation after 35 days of ET, 15 cows (28.30%) were found pregnant in Group A, and 12 (24.49%) were found pregnant in Group B. Four (26.67%) and 1 (7.69%) live births were observed in Groups A and B, respectively (P>0.05). Parentage analysis of 18 bovine loci confirmed that cloned offspring and their somatic cell donors have the same genome (Table 3 and Table 4).

The pattern of pregnancy loss

Among the 15 pregnant cows in Group A, 8 cows (53.33%) suffered from early pregnancy loss (within the first trimester of pregnancy), and 3 cows (20.0%) suffered from second to third trimester pregnancy loss, and 4 gave live birth. Among the 12 pregnant cows in Group B, early pregnancy loss (within the first trimester) was observed in 7 cows (58.33%) and 4 cows (33.33%) suffered from third-trimester pregnancy loss, and 1 gave live birth.

DISCUSSIONS

SCNT is an excellent reproductive tool as it can reproduce genetically valuable individuals within a shorter period. Here, we have reported the production of multiple cloned calves from two high-performance Holstein cows in the local environmental condition of the UAE. Our result revealed that cloned embryos could be successfully produced using *in vitro* matured oocytes, and viable offspring could be obtained from these embryos.

The availability of good-quality matured oocytes is the key factor in the commercial utilization of SCNT technology. Oocyte quality has a very crucial role in embryonic growth and pregnancy rate after ET (Polejaeva and Campbell, 2000; Miyoshi et al., 2003). Although *in vivo* matured oocytes has a greater chance to give birth of live offspring than *in vitro* matured oocytes (Rizos et al., 2002; Son et al., 2008) considering the effort and cost, IVM oocytes are desirable for biotechnological usages. Likewise, *in vitro* matured oocytes are considered the primary source of oocytes for bovine cloning experiments. IVM is well developed in bovine, and around 70% maturation rates are reported elsewhere. Using the oocyte pick-up method, we collected immature oocytes from naturally cyclic and hormone superstimulated Holstein cows and reported a 72.62 to 80.81% metaphase II rate (Son et al., 2022a). Maturation of oocytes is a multifaceted process and is influenced by numerous factors such as holding temperature and transport time of collected ovaries to the laboratory (Yang et al., 1990), size of the antral follicles present in the ovaries (Blondin and Sirard, 1995; Pavlok et al., 1992) culture media composition (Lonergan et al., 1997), various supplement to the media such as hormones, energy substrate, antioxidant and serum (Zuelke and Brackett, 1990; Avery et al., 1998). However, we have standardized our laboratory protocol for bovine IVM using a commercially available culture media with manufacturer recommendations. We observed a 62.0% maturation rate from the slaughterhouse ovaries.

Table 1: Developmental competence of bovine embryos produced by somatic cell nuclear transfer using *in vitro* matured oocytes (Mean±SE)

Group	Nuclear transfer			
	No. of oocytes			
	Reconstructed oocytes	Fused oocytes (%)	Cleaved oocytes (%)	Blastocysts (%)
Cell Donor A	570	330 (61.66±4.06)	214 (64.92±2.17)	57 (18.48±2.04)
Cell Donor B	431	289 (65.62±2.80)	180 (62.17±3.42)	51 (19.14±2.01)

Table 2: Pregnancy and live birth rates following transfer of bovine cloned blastocyst

Groups*	Number of recipients	Pregnancy rate			Live birth rate
		Non-Return to estrus up to 42 days	Pregnancy detection at 42 days	Pregnancy detection at 90 days	
Cell Donor A	53	15 (28.30)	15 (28.30)	7 (46.67)	4 (26.67)
Cell Donor B	49	13 (26.53)	12 (24.49)	5 (38.46)	1 (7.69)

Table 3: Microsatellite analysis of somatic cell donor-A, cloned calves and surrogates

Markers	Donor cells	Cloned calf	Surrogates
TGLA227	81/91	81/91	89/91, 91/93, 91/98, 83/93
BM2113	123/131	123/131	123/131, 123/131, 123/123, 131/131
TGLA53	158/158	158/158	160/160, 160/160, 158/160, 160/174
ETH10	215/219	215/219	215/219, 215/219, 213/215, 215/219
SPS115	248/254	248/254	248/258, 246/246, 248/248, 248/256
SPS113	295/295	295/295	297/297, 295/297, 295/295, 285/297
RM067	88/88	88/88	88/88,96/96, 88/88, 88/88
TGLA126	111/111	111/111	113/113, 113/113, 111/115, 111/113
TGLA122	139/139	139/139	145/145, 145/145, 139/139, 159/179
INRA23	204/208	204/208	204/212, 202/208, 202/208, 202/212
BM1818	258/262	258/262	258/258, 258/258, 260/262, 258/262
ETH3	125/127	125/127	125/127, 127/127, 123/127, 125/127
ETH225	142/142	142/142	144/146, 146/146, 138/146, 146/146
BM1824	180/190	180/190	178/188, 178/178, 178/188, 178/178
CSRM60	94/102	94/102	102/102, 92/102, 92/96, 92/100
MGTG4B	145/155	145/155	151/155, 141/147, 141/153, 135/141
CSSM66	185/187	185/187	187/191, 191/195, 189/191, 187/191
ILST006	294/298	294/298	290/296, 296/296, 290/290, 290/294

Microsatellite analysis was performed on genomic DNA from cloned offspring as well as surrogate and donor cells. The values of all markers were confirmed to be identical in all cloned offspring. Values represent base pairs of the amplified microsatellite DNA markers in each sample

Table 4: Microsatellite analysis of somatic cell Donor-B, cloned calves and surrogates

Markers	Donor cells	Cloned calve	Surrogates
TGLA227	91/93	91/93	81/101
BM2113	123/131	123/131	123/131
TGLA53	158/158	158/158	160/164
ETH10	215/221	215/221	205/213
SPS115	248/254	248/254	248/256
SPS113	289/299	289/299	285/291
RM067	88/88	88/88	88/88
TGLA126	113/115	113/115	111/113
TGLA122	139/139	139/139	179/179
INRA23	202/212	202/212	202/212
BM1818	262/266	262/266	258/262
ETH3	113/113	113/113	113/125
ETH225	138/142	138/142	144/146
BM1824	182/190	182/190	178/190
CSRM60	102/102	102/102	100/102
MGTG4B	141/141	141/141	151/155
CSSM66	187/187	187/187	185/189
ILST006	294/296	294/296	290/296

Microsatellite analysis was performed on genomic DNA from cloned offspring as well as surrogate and donor cells. The values of all markers were confirmed to be identical in all cloned offspring. Values represent base pairs of the amplified microsatellite DNA markers in each sample

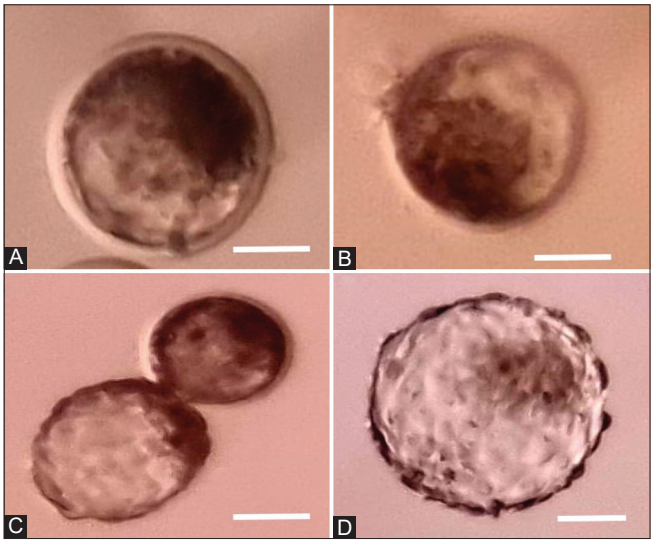

Fig 1. Representative photographs of blastocysts produced by SCNT. (A) Early stage blastocyst (B) Expanding blastocyst (C) Hatching blastocyst (D) Hatched blastocyst. Scale bar = 50 μM.

The development of oocytes into a transferrable good-quality blastocyst is a crucial step in the SCNT program. In bovine, both the SCNT and IVF-derived embryos showed a similar blastocyst formation rate (Heyman et al. 2002; Li et al. 2006; Iager et al. 2008). We observed a similar blastocyst development rate for both the donor cells, 18.48 and 19.14% for donor cells A and B, respectively. Akagi et al. (2003) reported a 17.0 to 45.0% blastocyst formation rate following SCNT in bovines. We have observed an overall lower maturation and blastocyst formation rate. We postulated that the unique environmental condition of the UAE might have an effect on the fewer oocyte maturation and blastocyst formation rate. Pavani et al. (2015) collected postmortem ovaries of Holstein cows from a slaughterhouse for one-year time for IVM and reported a significantly lower nuclear maturation (Metaphase II) rate and embryo developmental rate during the hot seasons of the year compared to the cold months of the year. The inherent quality of oocytes could be compromised due to metabolic changes in the follicular environment in response to warm weather, which may lead to lower embryonic development.

Embryo transfer is the final step in the SCNT process to get the live offspring. We observed 26 to 28% non-return to estrus up to 35 days following ET (42 days from the last observed estrus), which we considered the initiation of

pregnancy. Pace et al. (2002) reported a 24.7% pregnancy initiation rate after SCNT-derived blastocyst transfer. More than 85% of non-return to estrus recipients were found pregnant per rectum palpation on 35 to 42 days. Both the donor cells have similar initial pregnancy and live birth rates. The mean live births were 26.67% and 7.69% for donor cells A and B, respectively. This cloning efficiency is comparable to other published reports. Most of the large-scale bovine cloning data reported below 20.0% live-born rate. Watanabe and Nagai (2011) compiled over a decade of SCNT data and reported 12.6% live calving, and Pace et al. (2002) reported 19.04% calving from 535 confirmed pregnant recipients. Akagi et al. (2014) reported a higher calving rate of 25 to 50%; however, his experiment had few recipient animals. The reproduction of cows is influenced by environmental temperature (Dash et al., 2016). Holstein cows show efficient reproductive performances when they maintain at 5–25°C ambient temperature (McDowell, 1972). Holstein cows are exotic to the UAE, where the average minimum and maximum temperature is 26.32°C and 40.90 °C, respectively (https://weather.com) in spring. Despite a relatively high temperature, the pregnancy rate following cloned embryo transfer was comparable with other published reports.

The most limiting factor for bovine SCNT is the low live birth rate for embryonic loss, fetal loss, abortion, or stillbirth due to structural and functional abnormalities of the placenta (Bauersachs et al., 2009). Indeed, Heyman et al. (2002) reported that embryos derived by SCNT or IVF procedures had similar initial pregnancy rates (55.06 vs. 62.7 for SCNT and IVF, respectively); however, significant differences were observed by 70 days (14.3% vs.49.0% for IVF and SCNT, respectively) and at calving (6.8% vs. 49%). Watanabe and Nagai (2011) compiled over a decade of data on SCNT in Japan and reported a 9.2% calving rate. We observed a higher rate of pregnancy loss, irrespective of cell donor; 73 to 91% of transferred embryos failed to give live birth. We observed pregnancy losses throughout the gestation period, but most of the losses were observed around the first trimester of pregnancy. Among the 19 pregnancy losses in the present study16, pregnancies were lost within the second trimester. Inferior or abnormal embryos, defective or fewer placentae, and changes in the maternal uterine environment are the leading causes of early fetal losses (Bauersachs et al., 2009).

CONCLUSIONS

This was the first attempt of bovine cloning in the UAE, which produced 5 healthy calves from 2 distinct donor individuals. Further work is needed to maximize the IVM rate of oocytes and embryo production in a hot environment and minimize fetal losses. However, current techniques are adequate to produce clone cows, thus, opening new possibilities for commercializing SCNT in bovine to support the local dairy industries in the UAE.

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Conflicts of Interest

No conflict of interest was declared by the authors.

Authors contributions

Mohammad Shamim Hossein design the experiment, collect and analysis the data; Young-Bum Son and Mina Kang carried out the investigation and data collection; Yeon Ik Jeong, Per Olof Olsson, Huijeong Kim, Yura Bae, Han Seock Kim, Ji Young Noh and Kyung Ik Hwang performed experiments; Romeo Abad Angeles did the embryo transfer, Woo Suk Hwang design and supervised the experiments.

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