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Cloning of a Deceased High-Performance Milking Camel Using Somatic Cell Nuclear Transfer

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Abstract

Somatic cell nuclear transfer (SCNT) has the unique ability to replicate animals of high genetic merit, making it an exquisite tool in animal breeding. In this study, we successfully produced four cloned calves from a deceased high-performance milking camel. A total of 95 open female camels were involved, with 33 serving as oocyte donors and 62 as recipients. Cumulus oocyte complexes (COCs) were collected from super-stimulated camels using ultrasound-guided ovum pick-up (OPU). Single, double, or 3-4 blastocysts at various stages of development were transferred to synchronized recipients. Pregnancy was confirmed through progesterone assays and ultrasonographic scanning, while parentage of the cloned calves was verified via microsatellite analysis. We collected 476 COCs from 614 follicles, achieving an average recovery rate of 76.9 ± 10.5%. Of these, 410 were at the metaphase II stage, resulting in an average maturation rate of 87.8 ± 12.6%. The average fusion, cleavage, and blastocyst rates were 84.3 ± 11.1%, 73.2 ± 10.6%, and 32.8 ± 10.4%, respectively. The four clones were born from two camels that received single embryos and two camels that received two embryos, all of which continued to three months of pregnancy. No live births were observed in the group that received 3-4 embryos. The unhatched and hatching blastocyst groups produced one and three calves, respectively, while no live births were observed from hatched blastocysts. We recommend transferring two cloned unhatched or hatching blastocysts per recipient to increase pregnancy rates and reduce pregnancy losses in dromedary camels.

Keywords: Resurrection; Somatic cell nuclear transfer; Pregnancy rate; Pregnancy loss; Camel

Introduction

Somatic cell nuclear transfer (SCNT) is an important breeding tool as it could multiply animals with superior genetic traits. Lately, cloning champion camels from different utilities such as racing, beauty, or milking camels have gained much popularity in Arabian Penisula [1]. Additionally, we produced cloned camels from a decade-old, vitrified skin tissue collected from a deceased camel [2]. However, aside from the technological difficulties, it involves many other challenges, such as establishing a healthy donor cell line, acquiring superior-quality metaphase II oocytes, producing transferrable blastocysts, estrus synchronization of donor and recipient camel, etc [3]. Camel milk is traditionally popular in Middle Eastern countries because of its significant health benefits. However, camels typically produce relatively low

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quantities of milk, compared to cattle, with an average daily production of 6.0 ± 0.12 kg [4]. Cloning high-performance milking camels is a groundbreaking advancement in animal breeding, crucial for meeting the rising global demand for camel milk due to its unique nutritional benefits. Cloning addresses these challenges by replicating elite camels, ensuring consistent milk production and preserving valuable genetic traits [1]. This technology enhances productivity, accelerates herd improvement, and supports the sustainable growth of the camel milk industry. Donor cells are crucial to the success of SCNT. Typically, a skin biopsy is performed on the camel selected for cloning, and a fibroblast cell line is established from this sample as a source of diploid cells. However, complications arise when skin tissue must be collected from a cadaver, especially if a significant amount of time has passed between the animal's death and autopsy [2]. In the current study, a high milk-producing camel died, and 46 hours after burial, it was exhumed. Ear tissue was collected, and a fibroblast cell line was successfully established. Some early studies showed that the embryo stage and the number of transferred embryos per recipient are important factors in pregnancy establishment and maintenance [5]. Camels are notably susceptible to pregnancy losses, particularly within the first three months following embryo transfer [6]. However, mid- to late-term pregnancy losses are also frequently observed [7]. Various factors were investigated, including the source and quality of oocytes used for embryo production, culture conditions, embryo size and shape, the number of embryos transferred per recipient, embryo transfer protocols, donor-recipient synchronization, and environmental influences [5, 6, 8]. However, more detailed studies are needed to reliably determine the impact of these factors on pregnancy rates and to enhance production efficiency. This manuscript reports the successful production of a high milk-producing female camel using SCNT, highlighting the effects of embryonic stage and the number of embryos per recipient on pregnancy and live birth rates in dromedary camels.

Materials and Methods

Ethics statement

All experiments involving animal usage were conducted strictly following the guidelines approved by the Management of Scientific Center and Presidential Camels Ethics Committee (Accession No: PC4.1.5). These guidelines are in accordance with the ARRIVE guidelines.

Media, Chemicals and reagents

Unless otherwise specified, all media, chemicals, and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Donor and recipient camels

A total number of 95 non-lactating open local female camels (Age: 5-12 years old; Weight: 442 ± 27 Kg; Parity: 2.1 ± 1.5), 33 of which were oocyte donors and 62 were recipients. All the camels were free from reproductive tract abnormalities and any obvious diseases. The camels were housed in two spacious pens, each measuring 60x60 meters. Their diet consisted of 1.5 kg wheat bran, 0.8 kg crushed maize, 0.4 kg crushed barley, 4.0 kg Rhodes grass, and 2.0 kg dried alfalfa per head per day. Only after embryo transfer and during the first three months of pregnancy, the recipient camels were fed only on 2.0 kg wheat bran, 6.0 kg Rhodes grass, and 0.5 kg dried alfalfa per head per day. All camels had unrestricted access to water and mineral blocks and received an oral supplement of 30 grams per head per day of a mineral and vitamin premix (NeoMix Super®, Neofarma Co, Italy). Procedures, including ovum pick-up (OPU), SCNT, and embryo transfer (ET), were conducted from October 2020 to February 2021, the main breeding season for camels in the UAE. However, pregnant camels were monitored carefully and maintained in the research facility until parturition.

Estrus synchronization and ovarian super-stimulation

For each working session, a group of 7 to 9 females camels were examined by transrectal ultrasonography and those having one or more ovarian follicle of 10 -16 mm in diameter were treated as shown in Figure 1. Briefly, these females were injected intramuscularly with 20 µg GnRH (Buserelin acetate, Fertiline®, Vetoquinol, Cedex, France) at Day 0. The females were randomly assigned as donors and recipients based on the experimental requirements and the number of super-stimulated ovarian follicles needed. On Day 4, 3000 IU and 2000 IU eCG (Novormon, Syntex S.A, BUENOS AIRES, Argentina) were injected into the donor and recipient, respectively. On Day 13, all the females received another dose of IM injection of 20 µg of Buserelin acetate (GnRH) for the final maturation of oocytes. On Day 14, cumulus oocyte complexes (COCs) were collected from the donors, and on Day 21, embryos were transferred to recipients.

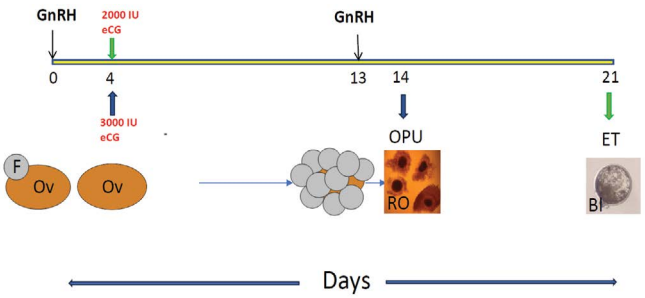

Figure 1: Synchronization and super-stimulation protocol of the donors and recipients' female camels

: Recipients; Donors; Ov: ovary; F: ovarian follicle; OPU: ovum pick-up; RO: Recovered oocytes; ET: embryo transfer; Bl: cloned blastocyst

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Collection of in-vivo matured oocyte

Camels with at least 5 follicles larger than 10 mm in diameter were attempted for oocyte collection using our standard protocol [5]. Briefly, camels were tranquilized by intravenous injection of xylazine 2% (Alfasan, The Netherland) at a dose of 0.1-0.2 mg/kg and an epidural injection of 5 ml 2% lidocaine HCL (Ilium, Troy Laboratories, Australia). Oocytes were collected transvaginal by aspiration of follicular fluid using an Aloka ultrasound unit (Aloka, Tokyo, Japan) with a 5 MHz convex transvaginal probe mounted with a needle guide (Aloka, Tokyo, Japan). The OPU needle and a 50 ml capped storage tube filled with 2 ml OPU Media (IVF Bioscience, Agtech Inc, USA) were channelized using a pipe (IVF Bioscience, Falmouth, UK). The suction pressure of 90 mmHg was controlled by a regulated vacuum pump. COCs were collected from aspirated follicular fluid under a stereomicroscope.

Donor-cell preparation

A high milk producer Pakistani dromedary female (~18 L/day in the first six months post-partum) had died during July 2017 and it was buried under sand in a desert area. Forty-six hours after burial, the carcass was excavated according to a request from the owner and ear tissue was collected. In our laboratory, a fibroblast cell line was established, and the donor cells were kept at a concentration of $1X10^6$ cells in a cryovial immersed in liquid nitrogen at Passage 1. To apply SCNT in 2020, cells were thawed at 38°C and cultured in DMEM supplemented with 10% Fetal Bovine Serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA), 1% non-essential amino acids (Thermo Fisher Scientific, Waltham, MA, USA), 0.1% β-mercaptoethanol (Thermo Fisher Scientific, Waltham, MA, USA) and 1% antibiotic–antimycotic at 38 °C, in 5% CO_2 in humidified air. The culture media was changed every two days until confluence reached 80% and passaged using 0.25% trypsin EDTA solution. Cells were cultured for 72 h and 4 - 5 passaged cells were washed twice by centrifugation and used for SCNT.

Somatic cell nuclear transfer

SCNT was performed according to our standard protocol [2]. Briefly, COCs were denuded by repeated gentle pipetting in DPBS containing 0.1% (w/v) hyaluronidase. After denuding, oocytes with the first polar body (MII phase) were stained with 5 µg/ml bisbenzimide to detect genetic materials. The first polar body and a small volume (less than 10%) of cytoplasm were removed from the oocytes using a bevelled glass pipette (16 µm, inner diameter). A single diploid nucleus of a donor cell was microinjected into the perivitelline space of each enucleated oocyte. The reconstructed oocyte couplets were fused in a fusion medium comprising 0.26 M mannitol, 0.1 mM $MgSO_4$, 0.5 mM HEPES, and 0.05% (w/v) BSA with two DC pulses of 1.8 kV/cm for 15 µs using BTX Electro Cell Manipulator 2001 + (BTX Inc, San Diego, CA, USA) and activated by treatment to 5 µM ionomycin for 3 min followed by 2.0 mM 6-dimethylaminopurine (6-DMAP) in BO-IVC (IVF Bioscience, Falmouth, UK) under a humidified atmosphere of 5% CO_2 at 39 °C for 4 h. Activated embryos were cultured in groups of 6 to 8 per 30 µl droplet covered with mineral oil for 7 days at 38 °C in a humidified atmosphere of 5% CO_2 and 5% O_2. On Day 2 of culture, the cleavage rate and on Day 7, the blastocyst stage were evaluated.

Embryo transfer (ET)

On Day 7 of culture, the blastocyst was loaded into an embryo-loading straw and transferred into the left uterine horn of the synchronized recipients using the recto-vaginal technique, given that over 98% of pregnancies in camel occur in the left horn [9]. We generally transfer a single blastocyst to each recipient; however, when there was a shortage of synchronized recipients, 2-4 blastocysts of similar developmental stages were transferred into the remaining recipients. After embryo transfer, recipients were separated from non-pregnant females and kept together in an isolated pen.

Pregnancy diagnosis

Serum progesterone levels of recipients were measured on the day of 18 after embryo transfer using a commercial P4- ELISA assay (Ridgeway Science, Alvington, UK). The recipients were considered pregnant when the P4 concentration was ≥ 1.0 ng/mL [10]. At 1, 2 and 3 months, the pregnancy was confirmed by trans-rectal ultrasonography. Early pregnancy loss (EPL) was diagnosed when the embryo with the surrounding fetal fluids was not detected, or the

Table 1: Microsatellite loci used for STR analysis to confirm the cloned camels.

ID #	Locus	Allele range (bp)	Reference
1	YWLL08	129-175	Lang et al, 1996
2	VOLP67	145-208	Obreque et al, 1998
3	LCA90	234-246	Penedo et al, 1998
4	VOLP10	240-269	Obreque et al, 1998
5	LCA 33	135-169	Penedo et al, 1998
6	LCA18	221-229	Penedo et al, 1998
7	VOLP03	144-176	Obreque et al, 1998
8	LCA63	198-232	Penedo et al, 1998
9	LCA 66	224-242	Penedo et al, 1999
10	YWLL44	86-120	Lang et al, 1996
11	CVRL1D	188-253	Mariasegaram et al, 2002
12	CVRL05	155-185	Mariasegaram et al, 2002
13	CVRL07	270-300	Mariasegaram et al, 2002
14	LGU49	224-260	Sarno et al, 2000
15	LGU75	184-230	Sarno et al, 2000
16	P 149	256-284	Munyard et al, 2009
17	PCTD 17	172-204	Munyard et al, 2009

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embryo was detected without a heartbeat. After 3 months of pregnancy till the parturition, pregnant camels were monitored regularly for the tail curling test in the presence of a camel male [11].

Genetic confirmation of clones

The genetic constituents of cloned calves were confirmed with somatic cell donor cells and recipients using the standard procedure of short tandem repeat (STR) profiling of 17 camelid-specific microsatellites (Table 1). The genomic DNA was isolated from the venous blood of cloned calves and tissues of cell donors and recipients using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Data regarding the short tandem repeat (STR) results is given in Table 2.

Table 2: Microsatellite analysis of donor cells, cloned calves, and recipients.

Markers	Donor cells	Cloned 1	Recipient	Cloned 2	Recipient	Cloned 3	Recipient	Cloned 4	Recipient
129-175	167/169	167/169	**148/171**	167/169	**163/165**	167/169	**148/155**	167/169	**169/169**
145-208	153/155	153/155	**188/190**	153/155	**184/194**	153/155	**176/178**	153/155	**147/180**
234-246	238/238	238/238	**240/240**	238/238	**238/240**	238/238	**240/240**	238/238	**240/240**
240-269	251/261	251/261	**249/261**	251/261	**259/261**	251/261	**249/259**	251/261	**249/251**
135-169	147/155	147/155	**137/145**	147/155	**149/153**	147/155	**145/157**	147/155	**147/149**
221-229	223/229	223/229	**225/229**	223/229	**225/225**	223/229	**225/229**	223/229	**225/229**
144-176	144/171	144/171	**144/144**	144/171	**167/171**	144/171	**144/144**	144/171	**144/167**
198-232	216/216	216/216	**214/222**	216/216	**216/220**	216/216	**216/216**	216/216	**214/214**
224-242	238/238	238/238	**238/238**	238/238	**236/236**	238/238	**240/242**	238/238	**234/240**
86-120	105/113	105/113	**105/107**	105/113	**105/113**	105/113	**105/113**	105/113	**105/105**
188-253	202/234	202/234	**226/246**	202/234	**202/234**	202/234	**202/234**	202/234	**234/234**
155-185	159/169	159/169	**169/179**	159/169	**159/169**	159/169	**159/169**	159/169	**159/159**
270-300	277/285	277/285	**281/281**	277/285	**277/285**	277/285	**277/285**	277/285	**273/281**
224-260	221/242	221/242	**223/239**	221/242	**221/242**	221/242	**221/242**	221/242	**221/223**
184-230	188/190	188/190	**188/204**	188/190	**188/190**	188/190	**188/190**	188/190	**192/208**
256-284	260/284	260/284	**260/260**	260/284	**260/284**	260/284	**260/284**	260/284	**268/268**
172-204	184/184	184/184	**188/188**	184/184	**184/184**	184/184	**184/184**	184/184	**188/188**

Statistical analyses

Rates of pregnancy, early pregnancy loss (EPL), live births, and abortions and/or stillbirths were calculated according to the following formulas:

To test the effect of the number of transferred blastocysts on the pregnancy rate and EPL, the data of the recipients that received 3-4 embryos were combined due to the low overall tested number of recipients that received 3-4 embryos. Also, only the data of ET of single and double embryos of the same developmental stage were combined when the effect of the blastocyst developmental stage was tested. The pregnancy rate and/or EPL were considered as binomial occurrences (pregnant = 1 and non-pregnant = 0) followed by logistic

$$\text{Rate of pregnancy} = \frac{\text{Number of diagnosed pregnant recipients at 18 days}}{\text{Number of recipients that received embryos}} \times 100$$

$$\text{Rate of EPL} = \frac{\text{Number of 2 or 3 months-pregnant recipients}}{\text{Number of 18 days-pregnant recipients}} \times 100$$

$$\text{Rate of live birth} = \frac{\text{Number of born calves}}{\text{Number of 3 months-pregnant recipients}} \times 100$$

$$\text{Rate of abortion} = \frac{\text{Number of 3 months-pregnant recipients - number of born calves}}{\text{Number of 3 months-pregnant recipients}} \times 100$$

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regression. The differences were considered significant at a probability level of P<0.05. Hosmer-Lemeshow Test was used to assess the goodness-of-fit of the logistic regression model for our data analyses. Rates of abortions and/or stillbirths and live births were simply presented without further statistical analyses due to low their overall numbers. Statistical analyses were done using SPSS® 22 for Windows® (SPSS Worldwide Headquarters, Chicago, IL, USA).

Results

Data on ovarian super-stimulation, recovered oocytes, and the developed blastocysts are outlined in Table 3. A total of 446 cumulus-oocyte complexes (COCs) were obtained from 614+ follicles sourced from 33 oocyte donors. On average, each donor yielded 18.6 follicles and 14.4 COCs, with a recovery rate of 76.9%. Notably, 87.8% of the oocytes were in the metaphase II stage. Subsequently, 392 metaphase II oocytes were reconstructed with donor cells, with 334 donor cells successfully fusing with the oocyte cytoplasm, resulting in a fusion rate of 84.3%. By Day 2, 247 fused oocytes had undergone at least one division to form a 2-cell embryo, yielding a cleavage rate of 73.2%. By Day 7, a total of 103 blastocysts were obtained, with a development rate of 32.8% (Table 3). Pregnancy rates at 18 days were higher (P < 0.05) with double or 3-4 embryo transfers compared to single embryo transfers (Table 4). At two months of pregnancy, double embryo transfers maintained a higher pregnancy rate than single embryo transfers. At three months of pregnancy, double embryo transfers showed higher pregnancy rates (P < 0.05) than single and 3-4 embryo transfers (Table 4). Notably, no live births were recorded in the 3-4 embryo transfer group, while a total of four live births were reported in the single and double embryo groups (two from each group). At two months of pregnancy, a higher rate of pregnancy loss (P < 0.05) was observed in the single transfer group compared to the double transfer group (Table 4). At three months of pregnancy, the highest rate of pregnancy loss was noted in the 3-4 embryo transfer group compared to the single or double embryo transfer groups (Table 4). Abortion and stillbirth occurred in two out of four recipients confirmed pregnant at three months received a single blastocyst and the recipient received 3-4 blastocysts (Table 4).

Table 3: Ovarian super-stimulation, recovered COCs, oocyte maturation and development of SCNT-derived embryos in dromedary camels.

Parameter	Number/percentage
Oocyte donor	33
Developed follicles per donor	18.6 ± 6.2
Recovered number of COCs/donor	14.4 ± 5.5
Recovery rate of COCs	76.9 ± 10.5
Maturation rate of oocytes	87.8 ± 12.6
Reconstructed oocytes	392
Fused oocytes	334 (84.3 ± 11.1)
Cleavage of fused oocytes	247 (73.2 ±10.6)
Blastocyst development	103 (32.8 ± 10.4)

Percentage data are presented as mean ± SD

At 18 days post-embryo transfer, the pregnancy rate was higher (P < 0.05) in the recipient group that received hatched (HD) blastocysts compared to those that received unhatched (UH) blastocysts (Table 5). However, there was no difference in pregnancy rates among all tested stages of cloned blastocysts at two months (Table 5). At three months of pregnancy, none of the recipients that received HD blastocysts were pregnant. Only four healthy calves were delivered: one from the group that received UH blastocysts and three from the group that received hatching (HG) blastocysts (Table 5). Early pregnancy losses at two months of gestation were lower (P < 0.05) in recipients that received UH blastocysts compared to those that received HD blastocysts (Table 5). By three months of pregnancy, the lowest pregnancy loss rate (P < 0.05) was observed in the group that received UH blastocysts compared to those that received HG and HD

Table 4: Effect of the number of transferred cloned embryos on pregnancy rate, pregnancy loss, and live birth in dromedary camels.

Number of the transferred embryos	Pregnancy number/recipient (%)			Live birth (%)	Pregnancy loss number/ recipient (%)		Abortion and or stillbirth (%)
	18 days	2 months	3 months		2 months	3 months	
Single	9/39 (23.1) [a]	4/39 (10.3) [a]	4/39 (10.3) [a]	2/4 (50.0)	5/9 (55.6) [a]	5/9 (55.6) [a]	2/4 (50.0)
Double	5/11 (45.5) [b]	4/11 (36.4) [b]	2/11 (18.2) [b]	2/2 (100.0)	1/5 (20.0) [b]	3/5(60.0) [a]	0/2 (0.0)
Three - 4	5/12 (41.7) [b]	3/12 (25.0) [a, b]	1/12 (8.3) [a]	0/1(0.0)	2/5 (40.0) [a, b]	4/5 (80.0) [b]	1/1 (100.0)
Total	19/62 (30.6)	11/62 (17.7)	7/62 (11.3)	4/7 (57.1)	8/19 (42.1)	12/19 (63.2)	3/7 (42.9)

Values within a column with different superscripts are significantly different (*P < 0.05*)

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blastocysts (Table 5). Abortion and stillbirth occurred in the pregnant recipient that received UH blastocyst and in two out of five pregnant females that received HG blastocysts (Table 5). Microsatellite analysis of 17 camel-specific loci revealed a perfect match between the four cloned calves and their somatic cell donor (Table 2). The cloned calves and the donor-cell deceased female camel are presented in Figure 2.

Table 5: Effect of different stages of the cloned blastocyst on pregnancy rate, pregnancy loss, and live birth in dromedary camels.

Blastocyst stage	Pregnancy number/recipient (%)			Live birth (%)	Pregnancy loss number/ recipient (%)		Abortion and or stillbirth (%)
	18 days	2 months	3 months		2 months	3 months	
Unhatched (UH)	3/13 (23.1) [a]	2/13 (15.4) [a]	2/13 (15.4) [a]	1/2 (50.0)	1/3 (33.3) [a]	1/3 (33.3) [a]	(1/2) 50.0
Hatching (HG)	14/44 (31.8) [a, b]	8/44 (18.2) [a]	5/44 (11.4) [a]	3/5 (60.0)	6/14 (42.9) [a, b]	9/14 (64.3) [b]	(2/5) 40.0
Hatched (HD)	2/5 (40.0) [b]	1/5 (20.0) [a]	0/5 (0.0) [b]	0	1/2 (50.0) [b]	2/2 (100.0) [c]	0
Total	19/62 (30.6)	11/62 (17.7)	7/62 (11.3)	4/7 (57.1)	8/19 (42.1)	12/19 (63.2)	(3/7) 42.9

Values within a column with different superscripts are significantly different ($P < 0.05$)

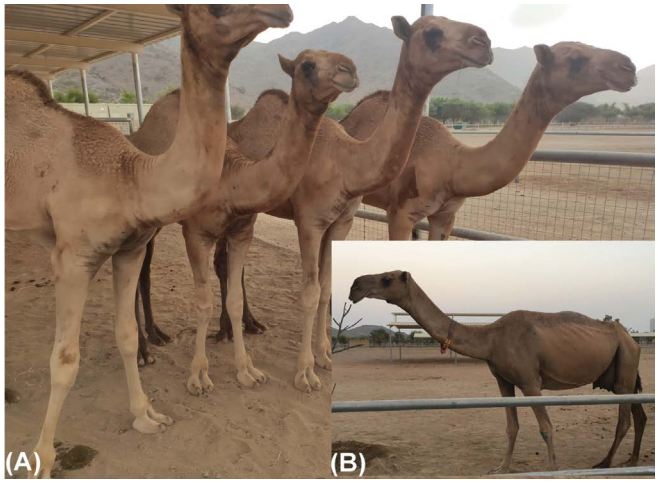

Figure 2: Representing the four cloned offsprings (A) with the donor-cell deceased high-performance milking female (B).

Discussion

To the best of our knowledge, this study is the first successful demonstration of SCNT resulting in the birth of healthy live calves from a deceased and buried female dromedary camel. The camel had been buried in sand for nearly two days under natural hot summer conditions before sampling. Previous studies have shown that, under dry environmental conditions, whether cool or hot with low humidity, a carcass may desiccate rather than decompose in the typical manner [12]. In this case, the camel's burial in sand at temperatures exceeding 42°C desiccated the skin, effectively preventing its deterioration. Previously in our laboratory, somatic cells were collected shortly after death, from the carcasses of various deceased animals, including camels, [2], preserved at 4°C for up to one month postmortem, and used for SCNT. Additionally, viable cells were successfully recovered from bovine skin tissues stored at 25°C for up to approximately two weeks postmortem [13]. Preserving the genetic diversity of high milk-producing females is valuable, and the milk production of her offspring can be further enhanced in subsequent generations by utilizing new assisted reproductive technologies such as ET and in vitro embryo production [3, 14]. The data regarding donor response to hormonal super-stimulation, the quantity of recovered COCs, oocyte maturation, rates of fusion and cleavage of reconstructed oocytes, and blastocyst formation are consistent with our prior studies [1, 5, 8].

The overall pregnancy rate of the cloned embryos, assessed 10 days post-embryo transfer (ET), reached 30.6%, slightly surpassing our previous study's rate of 21.4% [5], as well as another study's rate of 26.4% [15]. Generally, the pregnancy rates of SCNT embryos tend to be lower compared to their in vivo counterparts, which exhibit a pregnancy rate up to 76.8 % [15, 16]. Generally, low pregnancy rates from in vitro-produced embryos in dromedaries is likely stemming from a weak signal during maternal recognition of pregnancy, possibly attributed to the lower cell counts in these embryos in contrast to in vivo-produced embryos [17]. In our current study, the pregnancy rate at 10 days post-ET was higher when two or more embryos were transferred per recipient compared to the transfer of a single embryo per recipient. However, at 2 and 3 months of pregnancy, the highest pregnancy rate was achieved when two embryos were transferred per recipient. Moreover, pregnancy loss was higher at 3 months when more than two embryos were transferred per recipient. These findings are consistent with previous observations that transferring more than two cloned blastocysts per recipient was associated with higher rate of early pregnancy loss [5]. In contrast, transferring one or two in vivo-produced embryos per recipient results in similar pregnancy rates at both 19 days post-embryo transfer (ET) and two months of gestation [6]. This discrepancy may be due to the lower quality of cloned embryos compared to *in vivo* or traditional in vitro-produced embryos, which often necessitates transferring more than one embryo to achieve

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a pregnancy. In the current study, the number of abortions and stillbirths among the confirmed pregnancies at 3 months was 3 out of 7. A similar range was previously recorded with cloned dromedary camels (12 cases out of 31 pregnancies, 38.7%) [1]. This rate of abortion and stillbirth among females receiving cloned embryos is notably higher than the normal range observed in farmed dromedary camels using either natural mating or ET of in vivo embryo production (5.05%) [18]. The increased incidence of pregnancy loss and abortion may be attributed to the transfer of two or more embryos per recipient. In dromedaries, twin pregnancies often lead to an increased incidence of early pregnancy loss and abortion [18]. However, in our study, no instances of twin abortion or stillbirth were recorded.

However, in our study, recipients who received HD blastocysts exhibited a higher pregnancy rate at 10 days post ET (40%) compared to those who received UH blastocysts, both groups experienced pregnancy loss at 3 months. UH and HG blastocysts are fully and partially, respectively, covered by zona pellucida which act as a protective shield to blastomere. While, HD blastocysts, that lacks the protective zona pellucida, is more vulnerable to damage during its handling or the embryo transfer (ET) process. This increased fragility can lead to a higher incidence of early pregnancy loss [5, 19]. In contrast to findings in a previous study [5], we observed that UH cloned blastocysts in our study were associated with a higher pregnancy rate at 10 days post ET compared to transferring HD blastocysts, and they also demonstrated lower rates of early pregnancy loss at 3 months gestation compared to both HG and HD blastocysts. The reason for this discrepancy is unknown, and it is possible that the relatively low number of UH blastocysts tested in our study (three) may have influenced the general outcome.

Conclusion

Transferring double-cloned blastocysts at the unhatched or hatching stage per recipient increases pregnancy rates and reduces pregnancy losses in dromedary camels.

Declaration of Competing Interest

The authors declare no conflict of interest.

Authors' Contributions

MSH, NM, and YBS: Methodology, investigations, conceptualization, statistical analysis, writing, review, and editing. YIJ, YWJ, MK, HK, YB, HSK, KIK, BHE, JYN, KBP, WH, FL, MN, and TMA, helped in the practical ET, investigation and recording of the data. WSH was involved in the conceptualization, supervision, review, and editing of the article.

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