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# Preservation of dromedary camel embryos at 4 °C for up to 5 days: Factors affecting the pregnancy and pregnancy loss rates

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ABSTRACT

The aims of the present study were to evaluate the effect of cooling of the dromedary camel embryos on the pregnancy and pregnancy loss rates, and to investigate the factors which might affect the outcomes of the transfer of cooled embryos. After the donors (n = 56) had been super-ovulated and mated, they were flushed at Day 8 or 9 post-mating. Of 487 collected embryos, 110 were refrigerated at 4°C for up to 5 days in holding medium (HM), flushing medium supplemented with 10% fetal calf serum (FM + FCS) or TCM199 supplemented with 50% FCS and HEPES (TCM + FCS + HEPES). Both fresh (n = 377) and cooled embryos were transferred individually into synchronized recipients. Pregnancy diagnoses were carried out at Days 18–19, 30 and 60 post-mating of the donors. Transferring of fresh embryos into the recipients resulted in significantly higher pregnancy rates at Days 18–19 (53.1% vs. 38.2%, $P < 0.01$), Day 30 (46.4% vs. 31.8%, $P < 0.01$) and Day 60 (42.4% vs. 26.4%, $P < 0.005$) compared with those of cooled embryos, respectively. Pregnancy rates after transferring cooled embryos progressively decreased with the prolongation of the storage period. A significant difference in the pregnancy rate (56% vs. 13%, respectively, $P < 0.05$) was recorded only at Days 18–19 between cooled embryos held for one day and those held for 5 days. The pregnancy rates at Days 18–19, Day 30 and Day 60 were non-significantly higher when TCM + HEPES and FCS medium used for cooling of embryos compared to those of FM + FCS or HM medium. Cooling of spherical embryos resulted in significantly higher pregnancy rates at Days 30 (45.6% vs. 17.0%, respectively, $P < 0.005$) and 60 (42.1 vs. 9.4%, respectively, $P < 0.005$) and a significantly lower pregnancy loss rate (11.1% vs. 66.6%, respectively, $P < 0.005$) compared to those resulting from cooling of folded embryos. Neither the size of embryo nor the day of flushing had a significant effect on the pregnancy and pregnancy loss rates after the transfer of cooled embryos.

In conclusion, pregnancy could be obtained after the transfer of camel embryos refrigerated for up to 5 days. In addition, higher pregnancy rates could be obtained if only spherical embryos are selected for cooling.



## 1. Introduction

Nowadays, camels are not only used for transportation, meat or milking, but considered as one of the most leading sport animals in the Gulf region due to the huge governmental financial support and the financial gain to the owners of winning camels [1]. Camel reproductive efficiency under natural conditions is very low [2]; the average calving interval is more than 2 years [3]. Assisted reproductive technologies (ART) such as embryo transfer (ET) and artificial insemination (AI) have been used to improve the low reproductive efficiency. Currently, ET is one of the most widely used technologies to assist camel reproduction with a successful commercial application [4].

The availability of healthy synchronized recipients at the time of

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embryo transfer is among the main factors for successful outcomes of ET. The highest pregnancy rate is obtained if the embryos are transferred to the recipients that have ovulated one or two days post-mating of the donors [5]. In commercial ET centers, the number of recovered embryos from super-ovulated donors is sometimes higher than the number of synchronized recipients. To overcome this problem, non-ovulated recipients can be used if they are primed by progesterone for at least 48 h before ET [6] or the surplus embryos are preserved by cooling [7,8], freezing [9] or vitrification [10,11]. Using asynchronized (non-ovulated) recipients necessitates daily administration of progesterone for the whole gestation period (13 months) or until a corpus luteum (CL) is induced which is costly and not practical [12]. Whilst freezing or vitrification can preserve embryos for a very long time, these technologies require relatively more time, equipment, chemicals, and expertise. Additionally, only a few trials reported positive pregnancies after the transfer of vitrified embryos in camelids [11,13,14].

Cooling of embryos is a simple and fast technique requiring fewer tools and less time compared with freezing and vitrification. Cooled embryos are usually kept at 4 °C, in a metabolically inhibited but still viable state. In cattle, the pregnancy rate ranged from 42.8% to 59% and from 15.4% to 44% after the transfer of embryos refrigerated for 48 and 72 h, respectively [15–17]. Increasing embryo refrigeration period more than three days has been done with some modifications of the component of storage medium, its pH and carriers. A pregnancy rate of 75% was obtained from the transfer of embryos held at 4 °C for 7 days in a modified cooling medium [7]. In camelids, there are only two studies that evaluated the pregnancy rate after transferring of a small number of embryos cooled for 24 h. The pregnancy rate was 62.5% (20/32) in the dromedary [5] and 21.4% (3/14) in llama [8]. To the best of our knowledge, this is the first study to evaluate the effect of cooling of dromedary camel embryos for more than 24 h on the pregnancy and pregnancy loss rates. In addition, the factors which might affect the outcome of the transfer of cooled embryos were investigated.

## 2. Materials and methods

### 2.1. Animals and their management

This study was carried out at Tharb Camel Hospital, state of Qatar, in two breeding seasons (December to April 2017 to 2019). The donors (n = 56) and recipients (n = 294) used in the present study aged 5–14 years and were free from brucellosis, trypanosomiasis, and reproductive problems. The dromedaries were fed on Rhodes, wheat bran and dried alfa alfa and they had free access to water and mineral blocks. They were maintained in groups of 40–50 she-camels in fenced pens.

### 2.2. Superovulation and breeding of the donors

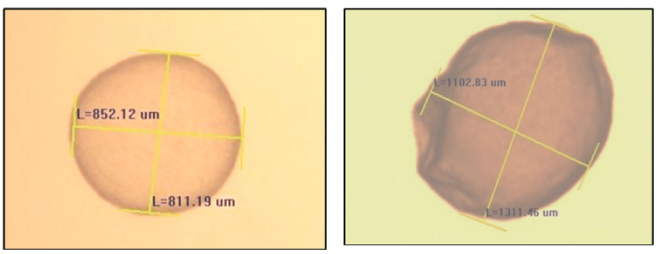
Transrectal ultrasonography was carried out in both donors and recipients by using Ibex Evo® scanner (E.I. Medical Imaging, Colorado, USA) supplied with a 6–8 MHz linear rectal transducer. The donors were superovulated according to Skidmore et al. [5]. Briefly, the donors having a follicle(s) with a diameter between 12 and 16 mm were administered GnRH (Buserelina®, Zoovet, Argentina) to induce ovulation. After 4 days, ovulation was confirmed by transrectal ultrasonography and superovulation protocol was applied using a combination of single administration of 2500 IU eCG (Folligon®, MSD Animal Health, Canada) and 400–440 mg porcine FSH (Folltropin V®, Bioniche Animal Health, Canada) twice daily in decreasing doses for 4–6 days. The donors were administered 500 μg Cloprostenol (Estrumate®, MSD Animal Health) at the same time as the last dose of FSH. Thereafter, the follicular growth was monitored by transrectal ultrasonography and when the majority of follicles reached 12–16 mm in diameter, the donors were mated twice at a 12-h interval and received 3000 IU hCG (Chorulon®, MSD Animal Health) immediately after the first mating. Day of mating was considered as Day 0 for the estimation of the day of embryo flushing and days of pregnancy in the recipients after embryo transfer.

### 2.3. Embryo collection and evaluation

The donors were flushed non-surgically in a standing position at Day 8 (n = 20) or 9 (n = 36) post-mating as described by Tibary and Anouassi [3]. Briefly, the vulva and surrounding perineal area were cleaned with sterilized dry gauze and then were disinfected with isopropyl alcohol 70%. Thereafter, an 18 to 22-gauge Foley catheter (Bioniche, Canada) was introduced into the base of the uterine horn by the recto-vaginal technique. The cuff of the Foley catheter was inflated with 30–40 ml air and the uterine horn was flushed repeatedly with 30–70 mL commercial flushing medium (Euroflush®, IMV Technologies, France). A total of 1000 ml flushing medium was used per donor. The flushing medium was recovered by gravity through Y tubing into a sterile embryo filter (IMV Technologies). The residual filtrate was examined for the presence of embryo(s) in a gridded searching plate under a stereomicroscope with a 3.2-megapixel digital camera (Wesco WS7, California, USA). The hatched embryos were transferred into 5-well plates containing holding medium (IMV Technologies) and washed four times. The collected embryos from each donor were counted and two diameters of each embryo were measured using Wescometrics software (Wesco) and the average diameter was recorded (Fig. 1). In addition, they were classified according to their diameter into small and medium embryos (300–750 μm in diameter) and large (751–1200 μm in diameter) embryos and into spherical and folded embryos according to their shape.

### 2.4. Cooling procedures

Except for flushing and holding media, all chemicals added or used for the preparation of chilling media are from Sigma–Aldrich, USA. The excess embryos (over the number of synchronized recipients) had been preserved by cooling for up to 5 days until the synchronized recipients (any recipient which had been administered GnRH 7–8 days before the day of ET and had confirmed ovulation) were available. The surplus embryos (n = 19) in season 2017–2018 were cooled using only the holding medium, each in a well of 5-well plates, while in season 2018–2019, they were randomly cooled in one of the following three cooling media:




(A) (B)

**Fig. 1.** Large spherical (A) and folded (B) dromedary camel embryos flushed at Day 9 after mating.

a) Holding medium (HM) (n = 43): each embryo was transferred individually into a well of 5-well plate containing 1.5 mL (n = 11) of holding medium or a 1.5-mL Eppendorf tube (n = 32) containing 0.5 ml of holding medium.
b) Flushing medium plus fetal calf serum (FM + FCS) (n = 23): These procedures were modified from Skidmore et al. [5]. Embryos were washed in 4 successive drops of fresh flushing medium before each being transferred into a well of 5-well plate containing 1.5 mL of flushing medium supplemented with 10% v/v, FCS.
c) Tissue culture medium (TCM 199) plus 50% FCS plus HEPES (TCM199 + FCS + HEPES) [7] (n = 25). The embryos were washed in 4 successive wells containing TCM199, 50% v/v FCS and 25 mM HEPES. The following mixtures were loaded in a succession into a 0.5-ml straw: the medium, an air bubble, the medium containing one embryo, an air bubble and more medium [7].

Five-well plates and Eppendorf tubes containing the embryos were wrapped in aluminum foil and the loaded straws were kept in visotubes held in a goblet (Cryobiosystem, IMV Technologies, France) and then placed in a refrigerator set at 4 °C till transferred into the synchronized recipients. The storage period of embryos in each group ranged from 1 to 5 days.

### 2.5. Treatment of recipients and embryo transfer

The recipients were examined by transrectal ultrasonography and those having a 12–16 mm follicle in diameter were administered 20 μg buserelin acetate at 24–48 h post-mating of the donors. Ovulation was confirmed in the recipients if progesterone (P4) level was ≥1 ng/mL a day before ET and/or by detection of CL by transrectal ultrasonography at the time of ET.

Fresh embryos were individually aspirated with the holding medium into a 0.25- ml straw, while the cooled embryo was washed in fresh holding medium (at room temperature) before being loaded into the straw. The sheathed embryo transfer gun was loaded with the straw and then introduced into the cervix and guided through the rectum to the anterior tip of the left uterine horn where the embryo was deposited.

### 2.6. Blood sampling and progesterone assay

Blood samples were collected from the jugular vein of the recipients a day before ET for confirmation of ovulation and at Day 10 after ET for pregnancy diagnosis. The collected samples were centrifuged at 1500 *g* for 15 min for separation of sera. Progesterone concentrations in the fresh sera of the donors and recipients were measured in a commercially available assay kit (Elecsys Progesterone III for Cobas e 601, Roche Diagnostics GmbH, D-68305 Mannheim) by electro chemiluminescence immunoassay (ECLIA) according to Ayad and Ouada [18]. The minimum detection limit of the assay was 0.05 ng/mL. The inter- and intra-assay coefficients of variation for high (7.84 ng/mL) and low (2 ng/mL) P4 concentrations were 8.1% and 0.9% and 3.72% and 4.77%, respectively.

### 2.7. Diagnosis of pregnancy

The females were considered pregnant at Days 18–19 post-mating of the donors if blood P4 level was ≥2 ng/ml and they showed tail cocking (erected and coiled tail) when approached by a camel bull. This cut-off value (≥2 ng/ml) was chosen because, at this level the highest sensitivity and specificity of the P4 test were obtained in the previous observations in our lab. The pregnant she-camels were re-examined by transrectal ultrasonography at Days 30 and 60 of pregnancy. Pregnancy losses were diagnosed when the embryo with the surrounding fetal fluids was not detected or the embryo was detected without the heartbeat.

### 2.8. Statistical analyses

All data of holding medium group were presented together as no difference was observed between Eppendorf and 5-well plates in terms of pregnancy rates at Days 18–19 (11/32 *vs.* 10/30), Day 30 (9/32 *vs.* 8/30) and Day 60 (8/32 *vs.* 7/30), respectively and pregnancy loss rate (3/11 *vs.* 3/10, respectively). Pregnancy rates were calculated by dividing the number of diagnosed pregnant recipients at Days 18–19, 30 and 60 by the total number of the transfers × 100. The pregnancy loss rate was calculated by dividing the number of the lost pregnancies between Days 18 and 60 post-mating by the total number of pregnancies at Days 18–19 post-mating × 100. The effect of the type (fresh and cooled) and shape (spherical and folded) of the embryos, duration of the storage period (between Day 1 and Day 5), type of cooling medium and day of embryo flushing on the pregnancy and pregnancy loss rates was evaluated by using Fisher's exact test. When there were multiple comparisons, Bonferroni's corrections were applied by multiplying the *P*-value obtained from Fisher's exact test by the number of multiple comparisons. Student t-test was used to compare the means of diameter of cooled embryos flushed at Day 8 and those flushed at Day 9. Also, the test was used to compare the means of diameter of spherical-and folded-shaped embryos [19]. The significance level was set at $P < 0.05$.

## 3. Results

Three hundred seventy-seven fresh embryos and 110 cooled embryos were transferred into synchronized recipients during the study period. None of the cooled embryos showed marked change in their shape and transparency during the storage period. Out of the 487 transferred embryos, 242 resulted in pregnancies (fresh: n = 200/377; cooled: n = 42/110) at Days 18–19. A total of 53 pregnancies were lost (fresh: n = 40/200; cooled: n = 13/42) between Days 18 and 60 (Table 1). In season 2017–2018, transferring of 19 cooled embryos resulted in 6 confirmed pregnancies (31.6%) at Days 18–19 and Day 30. Two pregnancies were lost between Days

**Table 1**
Effect of the type of transferred embryo on the pregnancy and pregnancy loss rates in the dromedary camels.

| Evaluating criteria | Type of the transferred embryo | |
|---|---|---|
| | Fresh embryo (n = 377) | Cooled embryo (n = 110) |
| Pregnancy rate at Days 18–19 | 53.1 %[a] (200/377) | 38.2 %[b] (42/110) |
| Pregnancy rate at Day 30 | 46.4 %[a] (175/377) | 31.8 %[b] (35/110) |
| Pregnancy rate at Day 60 | 42.4 %[c] (160/377) | 26.4 %[d] (29/110) |
| Pregnancy loss rate | 20.0% (40/200) | 31.0% (13/42) |

[a,b] $P < 0.01$.
[c,d] $P < 0.005$.

31 and 60 and eventually, four alive calves were born and weaned.

Transferring of fresh embryos into recipients resulted in significantly higher pregnancy rates at Days 18–19 ($P < 0.01$), Day 30 ($P < 0.01$) and Day 60 ($P < 0.005$) compared with those of cooled embryos. On the other hand, there was a non-significant difference in the pregnancy loss rate between fresh (20%) and cooled (31%) embryos (Table 1).

Pregnancy rates after transferring of cooled embryos progressively decreased with the prolongation of the storage period. A significant difference in the pregnancy rate ($P < 0.05$) was found only at Days 18–19 between cooled embryos held for one day and those held for 5 days (Table 2).

Cooling of embryos in TCM + HEPES plus FCS medium resulted in non-significantly higher pregnancy rates at Days 18–19, Day 30 and Day 60 compared with those of embryos cooled in FM + FCS or HM medium (Table 3).

Regarding the effect of embryo shape, cooling of spherical embryos resulted in significantly higher pregnancy rates at Days 30 ($P < 0.005$) and 60 ($P < 0.005$) and a significantly lower pregnancy loss rate ($P < 0.005$) compared with those resulting from cooling of folded embryos (Table 4). The diameter of the spherical embryos was significantly smaller than that of the folded embryos (Mean ± SD: 677.6 ± 218.5 vs. 808.8 ± 276.5 μm, $P < 0.01$, respectively; Table 4). However, transferring of cooled spherical embryos having small/medium size (300–750 μm in diameter) resulted in significantly higher pregnancy rate at Day 60 ($P < 0.05$) and lower pregnancy loss rate ($P < 0.05$) compared with those of folded embryos having the same size range. Also, significant differences were recorded in the pregnancy rate at Days 30 ($P < 0.05$) and 60 ($P < 0.01$) and pregnancy loss rate ($P < 0.05$) between the spherical and folded embryos having a large diameter (751–1200 μm) (Table 5). Within the group of the same embryo shape (spherical or folded), there were non-significant differences in the pregnancy rates at Days 18–60 and pregnancy loss rate between small to medium-sized embryos and large-sized embryos (Table 5).

Regarding the effect of the day of embryo flushing, there was a non-significant difference in the pregnancy and pregnancy loss rates between cooled embryos flushed at Day 8 and those flushed at Day 9. Furthermore, there was a non-significant difference in the diameter of cooled embryos flushed at Day 8 and those flushed at Day 9 post-mating (682 ± 214.4 vs. 769.4 ± 270.2 μm, respectively, Table 6).

## 4. Discussion

The present field study is the first in camelids to demonstrate that pregnancies could be obtained after the transfer of embryos kept at 4 C for up to 5 days. A relatively large number of cooled embryos (n = 110) were transferred in the present study compared with that transferred in the only two published studies in dromedary (n = 32) [5] and llama (n = 14) [8]. However, in the current study, the number of embryos in some groups was relatively small because several variables (storage period, storage medium, shape and size of embryo and day of embryo flushing) which might affect the outcomes of transferring cooled embryos were investigated. Additionally, these variables were evaluated using different volumes of storage medium (0.5 mL vs. 1.5 mL) and different storage devices (Eppendorf, 5-well plates and 0.5 ml straw) which might

**Table 2**
Effect of the cooling storage period of the embryos on the pregnancy and pregnancy loss rates in the dromedary camels.

| Evaluating criteria | Storage period (d) | | | | |
|---|---|---|---|---|---|
| | 1 (n = 27) | 2 (n = 31) | 3 (n = 25) | 4 (n = 11) | 5 (n = 16) |
| Pregnancy rate at D 18-19 | 56.0%[a] (15/27) | 45.2% [ab] (14/31) | 36% [ab] (9/25) | 18.2% [ab] (2/11) | 13.0%[b] (2/16) |
| Pregnancy rate at D 30 | 41.0% (11/27) | 45.2% (14/31) | 28.0% (7/25) | 9.1% (1/11) | 13.0% (2/16) |
| Pregnancy rate at D 60 | 37.0% (10/27) | 32.3% (10/31) | 24% (6/25) | 9.1% (1/11) | 13% (2/16) |
| Pregnancy loss rate | 33.3% (5/15) | 28.6% (4/14) | 33.3% (3/9) | 50.0% (1/2) | 0.0% (0/2) |

[a,b]$P < 0.05$.

**Table 3**
Effect of the type of cooling medium of the embryo on the pregnancy and pregnancy loss rates in the dromedary camel.

| Evaluating criteria | Medium of cooling | | |
|---|---|---|---|
| | HM (n = 62) | FM + FCS (n = 23) | TCM 199 + HEPES + FCS (n = 25) |
| Pregnancy rate at D 18-19 | 33.8% (21/62) | 39.1% (9/23) | 48.0% (12/25) |
| Pregnancy rate at D 30 | 27.4% (17/62) | 30.4% (7/23) | 44.0% (11/25) |
| Pregnancy rate at D 60 | 24.2% (15/62) | 17.4% (4/23) | 40.0% (10/25) |
| Pregnancy loss rate | 28.6% (6/21) | 55.5% (5/9) | 16.7% (2/12) |

HM: holding medium; FM: flushing medium; FCS: fetal calf serum; TCM: Tissue culture medium.

**Table 4**
Effect of the shape of cooled embryos on the pregnancy and pregnancy loss rates in the dromedary camel.

| Evaluating criteria | Shape of the embryo | |
|---|---|---|
| | Spherical (n = 57) | Folded (n = 53) |
| Diameter of the embryo (Mean ± SD μm) | 677.6 ± 218.5[a] | 808.8 ± 276.5[b] |
| Pregnancy rate (%) at D 18-19 | 47.4 (27/57) | 28.3 (15/53) |
| Pregnancy rate (%) at D 30 | 45.6[c] (26/57) | 17.0[d] (9/53) |
| Pregnancy rate (%) at D 60 | 42.1[c] (24/57) | 9.4[d] (5/53) |
| Pregnancy loss rate (%) | 11.1[c] (3/27) | 66.6[d] (10/15) |

[a,b] $P < 0.01$.
[c,d] $P < 0.005$.

**Table 5**
Effect of the shape and diameter of cooled embryos on the pregnancy and pregnancy loss rates in the dromedary camel.

| Evaluating criteria | Diameter of the embryo (μm) | | | |
|---|---|---|---|---|
| | 300-750 (n = 56) | | 751-1200 (n = 54) | |
| | Shape of the embryo | | | |
| | Spherical (n = 36) | Folded (n = 20) | Spherical (n = 21) | Folded (n = 33) |
| Pregnancy rate at D 18-19 | 50.0% (18/36) | 35.0% (7/20) | 42.8% (9/21) | 24.2% (8/33) |
| Pregnancy rate at D 30 | 47.2% (17/36) | 25.0% (5/20) | 42.8%[a] (9/21) | 12.1%[b] (4/33) |
| Pregnancy rate at D 60 | 44.4%[a] (16/36) | 15.0%[b] (3/20) | 38.1%[c] (8/21) | 6.1%[d] (2/33) |
| Pregnancy loss rate | 11.1%[a] (2/18) | 57.1%[b] (4/7) | 11.1 %[a] (1/9) | 75.0%[b] (6/8) |

[a,b] $P < 0.05$.
[c,d] $P < 0.01$.

**Table 6**
Effect of the day of flushing of cooled embryos on the pregnancy and pregnancy loss rates in the dromedary camel.

| Evaluating criteria | Day of embryo flushing | |
|---|---|---|
| | Day 8 (n = 36) | Day 9 (n = 74) |
| Diameter of embryo (Mean ± SD μm) | 682.1 ± 214.4 | 769.4 ± 270.2 |
| Pregnancy rate at D 18-19 | 33.3% (12/36) | 40.5% (30/74) |
| Pregnancy rate at D 30 | 27.7% (10/36) | 33.7% (25/74) |
| Pregnancy rate at D 60 | 25.0% (9/36) | 27.0% (20/74) |
| Pregnancy loss rate | 25.0% (3/12) | 33.3% (10/30) |

have affected the results. However, in the current study neither the volume nor the storage device (Eppendorf vs. 5-well plates) in holding medium group had any effect on the outcomes of transferring cooled embryos.

Pregnancy rates at Days 18–19 (53.1%) and Day 60 (42.4%) obtained in the present study after transfer of fresh embryos were within the range reported at the same days of pregnancy (50%–85% and 21.5%–44.4%, respectively) by a commercial camel embryo transfer center [4]. In addition, pregnancy loss rate between Days 18 and 60 (20%) was similar to that (22.6%) obtained by Tinson et al. [20], while it was much lower than that (40.5%) reported in another study [21]. The difference in pregnancy loss rates between the two studies might be attributed to the difference in the age of donors and recipients, quality of transferred embryos and the year [4,20,21].

Since the embryos were cooled for only 24 h in the two published studies in dromedary camels [5] and llama [8], our data (not shown) used for comparison included subgroups in which the embryos were cooled for 24 h and kept in the same basic medium used in each of these two studies. The pregnancy rate at Day 60 (50%, n = 6) obtained in the present study is comparable with that (63%) reported by Skidmore et al. [5] in the dromedary camels, when embryos were cooled for 24 h in flushing medium plus 10% FCS in both studies. However, in the study of Skidmore et al. [5], embryos were flushed only at Day 8 post-mating and cooled in 5-ml bijou tubes. Additionally, the shape and size of embryos were not mentioned in that study. On the other hand, a higher pregnancy rate at Day 60 (75%, n = 4) was obtained in the present study when compared with that (21.5%) reported at Day 45 in llama [8] after transferring similar-sized embryos which had been cooled for 24 h in 0.5-ml straws containing TCM 199 plus HEPES plus FCS in both studies. The difference in the pregnancy rate between the present study and that of llama [8] might be attributed to the difference in concentration of FCS (50% vs. 10%, respectively), the technique of embryo transfer and the day of embryo flushing (D 8 to 9 vs. D 7, respectively). The viability of cattle embryos increased from 54% to 73% by increasing the concentration of FCS from 12.5% to 50% in the cooling medium [7]. Regarding the technique of embryo transfer, embryos were transferred by the recto-vaginal technique in the present study, while in llama, the trans-cervical technique was used which might have prevented the technician from depositing the embryos into the tip of the left uterine horn of the recipient. Bon-Durant et al. [15] recommended the tip of the horn as the preferred site for deposition of cattle embryos especially for those weakened by refrigeration. Regarding the difference in the day of flushing between the two studies, there was a non-significant effect of the day of flushing on pregnancy and pregnancy loss rate in the current study.

In the current work, the overall pregnancy rate at Days 18–19 (38.2%) for cooled embryos was the same as that (38.2%) reported in cattle when embryos cooled for the same period as in our study (5 days) [16]. On the other hand, a much higher pregnancy rate (75% n = 32) was reported in cattle after embryos were refrigerated for 7 days [7] when compared with the pregnancy rate (48%) obtained in our study in the group of embryos cooled in the same cooling medium (TCM199 + FCS 50% HEPES, n = 25) and carrier (0.5 ml straw). This higher pregnancy rate might be attributed to the difference in the sensitivity of camel and cattle embryos to cooling. In addition, only high-quality embryos were transferred in the study of Ideta et al. [7]. Camel embryos might be more sensitive to cooling than cattle embryos due to the higher cytoplasmic lipid content in camelids embryos [22]. In pig, removal of lipid content (delipation) from the embryo by centrifugation increased its tolerance to chilling [23].

Prolongation of embryo storage period up to 4 days non-significantly decreased pregnancy rates at Days 18–19, 30 and 60. A significant effect was observed only at day 5 of storage in the present study. These results were inconsistent with that reported in cattle, where storing embryos at 4 °C for 5 days [17] or 7 days [7] had little effect on the pregnancy rate. Cooling of cattle embryos for 3 days led to death of more embryonic cells, especially in the inner cell mass, compared with the normal range [16]. Since camel embryos are more sensitive to cooling compared with cattle embryos, more embryonic cells (either in the inner cell mass or trophoblast) might have died by cooling at Day 5 of storage, which led to a significant decrease in the pregnancy rate in the present study.

Cooling of embryos in TCM199 supplemented with 50% FCS and HEPES resulted in non-significantly higher pregnancy rates and lower pregnancy loss rate when compared with those of embryos cooled in holding medium or flushing medium supplemented with 10% FCS. If the number of transferred embryos in the present study was higher, the difference in the pregnancy and pregnancy loss rates among groups might have reached the significance level. This difference in the pregnancy and pregnancy loss rates among the groups might be attributed to the difference in the composition of media and storage devices. Since the composition of both commercial flushing and holding media used in the present study are not known, it is difficult to find out the exact component of

TCM199 + FCS 50% + HEPES medium responsible for the better outcomes obtained in the group. However, the higher percentage of FCS (50%) used to supplement TCM 199 compared with that (10%) used to supplement the flushing medium might be among the reasons for the higher pregnancy rate. This explanation is reinforced by the results in cattle [7] as mentioned above in the discussion. Serum is important in the cooling medium as it contains a wide variety of substances, including energy substrates, growth factors, cytokines and hormones [24]. It also contains amino acids that play important roles as osmolytes and pH buffers [25]. Also, keeping the embryos in 0.5 -mL straws in TCM199 + FCS 50% + HEPES group might be among the reasons for the higher pregnancy rate in this group. The larger surface area of the straw compared with those of a well in 5-well plates and Eppendorf tubes used for keeping embryos in holding and flushing media groups might have affected the cooling rate of the embryos. The effect of the surface area of the carrier used for storing cold camel embryos is worth a further study.

Irrespective of the size of the embryo, significantly higher pregnancy rates, and lower pregnancy loss rate were obtained in the present study when the spherical-shaped embryos were cooled compared with those of folded ones. Similar findings were observed in dromedary fresh embryos [21]. Folded embryos might be more fragile and might have not withstood the required manipulation before and during their transfer into recipients.

In the current study, there were non-significant differences in the pregnancy rates between cooled embryos flushed at Day 8 and those flushed at Day 9. A possible explanation for these results might be that cooled embryos flushed at Day 8 and Day 9 were in the same developmental stage This explanation was confirmed by our findings where there was a non-significant difference in the diameter of embryos flushed at Day 8 and those flushed at Day 9 post-mating. Also, Herrid et al. [26] demonstrated that the day of flushing (Days 7.5, 8 and 8.5 post-mating) had no effect on camel embryo viability after vitrification. In contrast, the day of flushing (Day 7 vs. Day 8 post-mating) markedly affected the outcome of vitrification and freezing in another study [10].

In conclusion, pregnancies could be obtained after the transfer of camel embryos refrigerated for up to 5 days. In addition, higher pregnancy rates could be obtained if only spherical embryos are selected for cooling. Storing viability of camel embryos for 5 days enables embryo transfer centers to transport the embryos from one geographical region to another in a simple, cheap and safe way and allow recipients to be in more synchrony with the donors.

## Authors' contribution

**Ahmed Abd-Elfattah**: conceptualization, methodology, investigation, writing original draft, data curation. **Mohsen Agag**: conceptualization, writing- review and editing, supervision. **Mostafa Nasef**: conceptualization, methodology, writing original draft. **Shanmugavelu Muthukumaran**: methodology. **Mohamed El-Raey**: conceptualization, methodology, writing-review and editing. **Ahmed El-Khawaga**: conceptualization, writing-review and editing, supervision. **Aly Karen**: conceptualization, writing-review and editing, supervision, formal analysis, data curation, visualization.

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