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# Offspring resulting from transfer of cryopreserved embryos in camel (Camelus dromedarius)☆

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**Abstract**

The dromedary embryos, collected at hatched blastocyst stage, survived freezing and thawing in the presence of a high concentration of ethylene glycol (7.0 mol/L) with sucrose (0.5 mol/L) and direct plunging in liquid nitrogen. The rate of survival, as judged by the morphological appearance of the embryos after thawing, was high (92%). The transfer of frozen-thawed embryos into the recipients during the breeding ($n = 20$) and non-breeding season ($n = 25$) resulted in two and one pregnancy, respectively. One of the two pregnant recipients, with embryos transferred during the breeding season, delivered a normal healthy male calf at term. To our knowledge, this offspring is the first camelid produced following transfer of a frozen-thawed embryo.


*Keywords:* Cryopreservation; Camel; Dromedary; Vitrification; Rapid-freezing; Embryo

## 1. Introduction

Dromedary camels have an important social, economic and cultural role in the Arabic peninsula. Rearing of these animals is a highly important activity for thousands of native families living in the deserts. In United Arab Emirates camel racing is a traditional sport comparable with horse racing in the western world. Camelids are seasonal breeders [1–4]

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and induced ovulators [5]. Programs exist for genetic improvement of these animals as meat and fiber producers and production of highly valued racing animals through embryo transfer [6]. However, scanty information is available on application of other reproduction technologies like in vitro maturation, in vitro fertilization, intracytoplasmic sperm injection, cryopreservation of gametes, and other associated technologies in this species [6,7]. Cryopreservation and other related modern reproductive technologies offer many advantages to commercial animal production [8]. Among the benefits of cryopreservation is the simplified exchange of genetic material between geographically separated groups. This exchange allows for a greater genetic diversity within a group and for control of genetic traits. Embryo cryopreservation provides an avenue for a long-term storage and retrieval of genetic strains over a long period of time. The management of any embryo transfer programs can be effectively handled by use of this technology. Without the use of embryo freezing, synchronization of donor females and recipient females is critical. Donor females are hormonally stimulated to superovulate, or to create an above normal number of ova. Under normal circumstances, this creates the need for a large ratio of recipients to donor females. By cryopreserving fertilized ova, the problem of synchronization is minimized, since ova can be thawed and made available whenever the recipients are available. Since the first successful cryopreservation of mammalian zygotes and embryos resulting in live births of mice [9], the adaptations of the technique have been used to preserve embryos of a number of mammalian species successfully [10]. There have been attempts at cryopreservation of camelid embryos [11–16], however, to our knowledge, there are no reports of birth of an offspring in this species following transfer of frozen-thawed embryos. More than a decade ago, a single pregnancy following transfer of five frozen-thawed dromedary embryos was reported [11]. These authors, however, neither described the freezing and thawing procedure they adopted, nor the number of embryos they transferred per recipient. Again, a pregnancy following transfer of three conventionally frozen-thawed dromedary embryos into a single recipient was reported in 1999 [12]. There have been also few attempts at cryopreservation of Ilama (Lama glama) embryos [13–16] and two pregnancies following transfer of eight vitrified embryos into four recipients were reported [16].

Conventionally embryos and oocytes are frozen with a slow cooling procedure. During this procedure the gametes are exposed to a low concentration of cryoprotectants like glycerol, ethylene glycol, dimethyle sulfoxide and others for 5–15 min and slowly cooled (generally at the rate of 0.3–0.5 °C/min) to an intermediate temperature of about 35 °C and then plunged into liquid nitrogen for further storage. The basic embryo cryopreservation techniques are being constantly improved. As an alternative to conventional freezing procedures, a number of rapid-freezing protocols including vitrification protocols have been developed and adopted during the last two decades [10]. Rapid-freezing, the plunging of embryos and oocytes into liquid nitrogen was first attempted with mouse embryos [17], after similar methods proved successful for freezing fibroblasts [18]. The freezing protocol, which was applied in this experiment, was developed and optimized for mouse oocytes and embryos [19–23]. During non-conventional freezing procedures (vitrification/rapid-freezing), embryos or oocytes are exposed to a high concentration of a single or a combination of different permeable and non-permeable cryoprotectants for a very short period (20 s–2 min) and plunged into

liquid nitrogen before final storage. These freezing procedures have an advantage over the slow, conventional method in that these are simple, less time consuming and do not require costly freezing units. The freezing protocol used in this experiment has been found to be either equally effective or superior to conventional freezing procedure [19,23]. There has not been any attempt to freeze dromedary embryos using any of the rapid-freezing or vitrification procedures and the conventional freezing procedure has not resulted in the birth of an offspring. Therefore, in this experiment, the morphological survival of dromedary embryos, frozen by a simple vitrification procedure [23] and their development after transfer to recipients, during the breeding and non-breeding season, was investigated.

## 2. Materials and methods

### 2.1. Animals and their management

Dromedary camels aged 6–13 years were used in this experiment. The experimental animals were kept in fenced pens partly covered for shade. All camels were in good physical condition and were fed a diet of mixed concentrates and hay once a day and water was available ad libitum. All the procedures were performed either on standing camels, restrained in stocks, or in a sitting position. Animals were sedated with Rompun (1.2–2.0 mL i.v., Bayer, Leverkusen, Germany) 10 min before the start of the procedure. Ultrasonography was used to determine that donors were not pregnant and had no follicular cysts or intrauterine accumulation of fluid, and also to monitor follicular development and diagnose ovulation. For transrectal ultrasonography, a Pei Medical (Model 240 Parus, The Netherlands) realtime scanner with a 7.5 MHz array transducer was used. Embryos were flushed from donors during the physiological breeding season for the camels in Arabic peninsula, November to March and transferred into the recipients during the breeding as well as non-breeding season, April to October.

### 2.2. Treatment of donors

All donors were treated daily with progesterone-in-oil (125 mg Jurox, im, Rutherford, Australia) for 10–14 days. On the last day of progesterone therapy, camels were treated with 400 mg pFSH (Follitropin-V, Veterpharm, Ontario, Canada) in declining doses of $2 \times 80$, $2 \times 60$, $2 \times 40$ and $2 \times 20$ mg over 4 days with or without a single injection of 2000 IU of eCG (Folligon, Intervet, Boxmeer, The Netherlands) with first pFSH injection [24]. The ovaries of all the donor camels were scanned on Day 5 after the start of treatment, and thereafter at intervals of 1 or 2 days to determine which camels had responded and to assess the optimal time of breeding. When the majority of follicles had reached the size of 1.0–1.7 cm, donors were mated twice to one of the two mature male camels, 12 h apart. Donors were also treated with 3000 IU of hCG (Chorulon, Intervet) at the time of first mating as a further stimulus for ovulation.

### 2.3. Embryo recovery

The day of approximate ovulation (36 h after hCG) was considered Day 0 and embryos were recovered on Day 6 or 7. Embryos were flushed non-surgically using a flushing catheter (Gibbon, Rüsch, Armagh. Northern Ireland) and for flushing phosphate buffered saline medium (PBS) with 2% FCS (Gibco BRL, Life Technologies, Eggenstein, Germany) was used. The catheter was passed through the cervix and the balloon inflated with 30–50 mL of air to seal the internal os. The uterus was filled to moderate capacity with embryo flushing medium by injecting the fluid in 50 mL fractions through the catheter into the uterus with the operator's hand in the rectum to monitor filling. The medium was then recovered by gravity flow into sterile embryo filter (EmCon, Immuno System Inc., Wisconsin, USA) and the residual medium searched for embryos using a stereomicroscope. Embryos were transferred to fresh drops of PBS with 10% FCS and washed three times. Embryos were assessed morphologically and graded 1–4 (IETS-grading system). Embryos ($n = 55$) used for cryopreservation in this experiment were collected from the dromedaries ($n = 15$) during the breeding season. All the embryos except for one morula were hatched blastocysts.

### 2.4. Freezing and thawing procedure

Embryos were frozen with a freezing protocol described earlier [23]. In short, embryos were pre-equilibrated at room temperature in 1.5 mol/L ethylene glycol (EG, Sigma, Deisenhofen, Germany) with 0.25 mol/L sucrose (Sigma) in PBS supplemented with 10% FCS for 5 min. Embryos were then transferred to 7.0 mol/L EG with 0.5 mol/L sucrose and directly loaded into the middle of a 0.25 mL straw (Minitube, Landeshut, Germany) containing the same medium. The other two ends of the straw were filled with 0.5 mol/L sucrose. The straws were capped (for identification) and heat sealed on one side. Within 45 s from transfer of embryos to 7.0 mol/L EG with sucrose, the straws were directly and vertically plunged into liquid nitrogen. Embryos were stored in liquid nitrogen for periods not less than 24 h. The straws with frozen embryos were thawed by removing them from liquid nitrogen and immersing in a water bath at 25 °C for 10 s. The dilution of the cryoprotectant was achieved by transfer of embryos to 0.5 mol/L sucrose for 5 min and washing three times in PBS with 10% FCS. Embryos were cultured for 1–2 h in tissue culture medium (TCM 199, Sigma) supplemented with 0.25 mg/mL sodium pyruvate, 0.6 mg/mL calcium lactate, 0.1 mg/mL L-glutamine, 0.8 mg/mL sodium bicarbonate, 1.4 mg/mL HEPES and 50 μg/mL gentamicin (all chemicals from Sigma) and 10% estrus camel serum. Embryos were assessed morphologically and those intact, re-expanding and showing no signs of degeneration were classified as transferable. The transferable embryos were then transferred to PBS with 10% FCS, loaded into straws and transported to the site of embryo transfer (30 km away) in a transport incubator (Minitube, Germany) maintained at 37 °C.

### 2.5. Recipients

Recipients were selected from the pool of adult animals, which were healthy and in good condition. The ovaries were scanned to monitor follicle development and those

having large follicles (1.0–1.7 cm) were treated with 20 μg of the GnRH analogue, buserline (Receptal, Hoechst Animal Health, Milton Keynes, UK) and examined for the ovulation 36 h later. If ovulation was confirmed by ultrasound examination, the animal was used 6 or 7 days later (−24 h asynchronous) as an embryo recipient.

### 2.6. Embryo transfer

For embryo transfer, the straw containing a single embryo was loaded in an embryo transfer gun (IMV Technologies, L'aigle, France). The transfer gun was advanced through the vagina within the palm of the operator's sterile gloved hand and, after the index finger had penetrated the cervix, the tip of the instrument was guided to the middle of the annular cervical rings. The sanitary sleeve was then retracted and the operator's hand was placed in the rectum to direct the instrument tip into the left horn. The embryo was deposited with minimum amount of medium. Pregnancy in the recipient was diagnosed by ultrasound scanning of the uterus on Day 20 after ovulation followed by confirmation of a viable conceptus at monthly intervals.

### 2.7. Genetic identity of the calf

To confirm the genetic identity of the calf and embryo donor, we analyzed DNA extracted from blood leukocytes from the calf, the embryo donor and recipient dams and four other dams from the herd. Blood samples were collected into preservative-free sodium heparin tubes from the animals. DNA was extracted from a 5 mL aliquot of whole blood using commercially available kit (Nucleon, Tepnel Life Science PLC, Manchester, UK). Subsequently, microsatellite genotypes for each animal were obtained by performing PCR on the extracted DNA, using 12 pairs of primers from llama and dromedary microsatellite markers [25–30]. Fragment analysis was carried on an ABI Prism 310 Genetic Analyzer and the data analyzed using Genotyper V3.7.

## 3. Results

Embryos ($n = 49$) were thawed and 92% ($n = 45$) were assessed as of transferable quality. Two of the four non-transferable embryos recovered after thawing were in fractions (similar to split embryos). One embryo was in two and the other in three fractions. In vitro culture of these five embryo fractions resulted in the reorganization and re-expansion of four fractions within 4–6 h and had an appearance of a normal expanded blastocyst 24 h later. These embryos were not transferred as some of these blastocysts may only consist of trophoblasts. The transferable embryos ($n = 20$ and 25) were transferred to recipients during breeding and non-breeding season, respectively. Two of the recipients with embryos transferred during the breeding season and one during non-breeding season were diagnosed pregnant on Day 20. In one of the two pregnant animals, with embryos transferred during the breeding season, fetal resorption was noticed between 2nd and 3rd month of pregnancy. The other recipient delivered one healthy and normal male calf after the completion of term. The parturition was normal and minimal help was required at the time of parturition.

The recipient expelled the placenta within 2 h of parturition. The calf weighed 30 kg at the time of birth, which is normal for the species. The other recipient with an embryo transferred during the non-breeding season aborted a dead and partly autolysed fetus on Day 215. The cause of the abortion could not be ascertained, however, it may not be associated with embryo freezing and thawing procedure.

The genetic identity of the live born calf was confirmed by comparison of 12 dromedary and llama microsatellite loci. The results of the genotyping analysis are presented in Table 1. The microsatellite genotype of the calf was identical to that of the embryo donor.

## 4. Discussion

This is the first report of birth of an offspring following transfer of frozen-thawed embryos in camelids. The results indicate that embryos from the dromedary can survive vitrification procedure. The freezing protocol adopted for freezing of embryos in this experiment is based on our previous experience and proven effectiveness of this protocol with mouse embryos and oocytes [19–21,31]. A high rate of survival of intact as well as biopsied mouse [22] and bovine embryos [32] has been achieved with this protocol. This freezing protocol is simple, less time consuming and does not require any freezing machine. We used a combination of a permeable cryoprotectant (EG) at a high concentration (7.0 mol/L) in combination with a non-permeable cryoprotectant (sucrose) at 0.5 mol/L concentration. Using this protocol, the solutes vitrify when straws are plunged into liquid nitrogen, however, during devitrification some innocuous ice crystals may be formed. This combination of the cryoprotectants, their concentration and the time of exposure were optimized using mouse embryos and oocytes as a model [19–21]. The results of the present experiment prove that this protocol is also effective for cryopreservation of the dromedary embryos. The survival of embryos after thawing is high (92%). This survival rate is even superior compared with embryos frozen-thawed with conventional freezing procedure in this species [12]. These authors cryopreserved 34 embryos conventionally using eight different cryoprotectant solutions to find out a suitable cryoprotectant for freezing the dromedary embryos. However, the number of embryos used in some experimental groups was low (e.g. in one group there were only four embryos). Using one of their cryoprotectant solutions, three embryos survived the freezing procedure and the transfer of these three embryos to a single recipient resulted in a pregnancy. There has been another attempt at freezing the dromedary embryos [11]. These authors reported a single pregnancy after transfer of five embryos, however, the details of freezing and thawing procedure, survival results, and the number of embryos transferred per recipient was not mentioned in the report. Although, we did not attempt to freeze the dromedary embryos with conventional freezing procedure but if the previous results of the above authors are any indication, then conventional freezing procedure may not be suitable for this species. Conventional freezing procedure has resulted in pregnancy rates similar to those obtained with nonfrozen embryos in some domestic animal species like cattle [10], goats [33] and in laboratory animals like mice [22]. However, it has to be taken into account that following thawing approximately 5–10% of the embryos degenerate and are excluded from the transfer. There is a large variation in the freezing sensitivity of embryos and

Table 1
Genotyping results for the calf born from a cryopreserved embryo, the embryo donor and recipient dams and four other dams in the herd

| Marker | Calf | Embryo donor dam | Embryo recipient dam | Dam 1 | Dam 2 | Dam 3 | Dam 4 |
|---|---|---|---|---|---|---|---|
| YWLL38 | 186.5, 186.5 | 186.5, 191 | 189, 191 | 181.1, 191.7 | 191.8, 191.8 | 185, 191 | – |
| VOLP03 | 148.7,170.8 | 148.8, 170 | 148.5, 172.9 | 148.7, 180 | 148.2, 171.9 | 148.8, 169.7 | 148.8, 169.7 |
| YWLL08 | 147.5, 147.5 | 147.6, 166.7 | 147.6, 169.4 | 151.9, 164 | 133.1, 170.9 | 133.8, 149.5 | 133.8, 147.7 |
| CVRL05 | 160.5, 173.6 | 159, 159 | 171.2, 173.7 | 158, 160 | 170.6, 172.9 | 160.7, 181.8 | 160.4, 160.4 |
| CVRL01 | 217, 237.6 | 233.6, 237.6 | 215.1, 231.4 | 217.3, 237.6 | 221.1, 237.3 | 216.9, 216.9 | – |
| LCA33 | 133.1, 146 | 141.8, 145.5 | 141.6, 147.6 | 141.6, 150.4 | 141.5, 161.5 | 141.3, 141,3 | 143.9, 147.7 |
| YWLL44 | 104, 108 | 104.3, 104.3 | 104.3, 104.3 | 103.8, 103.8 | 104.1, 108.3 | 103.3, 107.3 | 104.1, 108.2 |
| CVRL04 | 135.4, 146.1 | 135.3, 135.3 | 135, 146.4 | 134.8, 134.8 | 135.2, 146.5 | 134.7, 145.9 | 146.5, 151.2 |
| LCA18 | 227.3, 227.3 | 227.2, 227.2 | 228.4, 228.4 | 228.5, 232.5 | 226.1, 231.4 | 227.4, 227.4 | 243, 243 |
| LCA66 | 236, 243.8 | 241.9, 243.7 | 236, 239.9 | 238, 240 | 238, 249.1 | 236.1, 243.9 | 231,241 |
| CVRL07 | 281.3, 281.3 | 281, 289.3 | 277, 277 | 285.5, 285.5 | 289.4, 289.4 | 304, 304 | 280, 280 |
| VOLP67 | 179.1, 179.1 | 148.5, 178 | 155.3, 157.5 | 148.7, 157.4 | 149, 175.5 | 185.1, 185.1 | 168, 168 |

oocytes from different animal species [34]. Embryos and oocytes from some animal species like bovine, ovine and caprine seem to be less sensitive compared to e.g. porcine embryos and oocytes [34]. There has not been any detailed investigation to assess the freezing sensitivity of different stages of the dromedary embryos with different freezing protocols. The dromedary embryos, when flushed on Days 6.5–7 after ovulation, are generally at hatched blastocyst stage. The freezability and survival of this embryo stage, with conventional freezing procedure, has been found to be superior to zona intact morulae and blastocysts in goats [35]. Similar observations have been reported for pig embryos when vitrification procedure was used for cryopreservation [36]. However, bovine hatched blastocysts may not be suitable for cryopreservation with conventional freezing procedures [37]. A higher survival of blastocysts compared with morulae in goats [38], sheep [39] and in bovine [see 10,40 for reviews] has been reported.

In this experiment, all embryos except for a single morula were at hatched blastocyst-stage at the time of freezing. The results indicate that this stage is suitable for freezing with this freezing protocol. Of the four embryos, which were not of transferable quality after thawing, one was frozen at morula stage, probably its quality was compromised at that time and the other two were recovered in fractions of two and three following freezing and thawing procedure. We have not observed such embryo fractioning in mouse or cattle when the same combination of cryoprotectants and freezing and thawing protocol was adopted [19–23,31,32]. Four of the 5 fractions reorganized and re-expanded after culture in vitro, similar to the development of embryos split mechanically [33,41].

The rate of survival and development of frozen-thawed embryos after transfer to recipients is very low. Only two of the 45 recipients carried the fetuses beyond first term of pregnancy, one delivered a live calf and the other aborted a dead and partly autolysed fetus on Day 215. A number of previous attempts, at freezing of embryos in camelids, did not result in the birth of an offspring [11–16]. The Pregnancy rates achieved with frozen-thawed embryos, is lower (about 5%) compared to our own results with nonfrozen embryos (about 25%, unpublished observations). Except for one report [42] generally low pregnancy rates in the dromedaries compared with other domestic animals have been reported following embryo transfer [6,11,43]. Different factors, which have been considered to influence the pregnancy rates following embryo transfer in dromedaries, are season, age and parity of recipient, quality of embryos and degree of donor and recipient synchronization [11,42,43]. However, these assumptions are not supported by enough data. Further investigations are, therefore, required to study the reason of low pregnancy and survival of dromedary embryos following transfer of frozen-thawed as well as non-frozen embryos.

It may be concluded that the dromedary embryos at hatched blastocyst stage can be frozen successfully with a simple vitrification procedure. This is the first report demonstrating successful transfer of frozen-thawed embryo resulting in the birth of an offspring in camelids. Further investigations are required to optimize the rate of development after transfer of frozen-thawed embryos to recipients in this species.

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