Open Veterinary Journal, (2018), Vol. 8(3): 325-329
ISSN: 2226-4485 (Print)
ISSN: 2218-6050 (Online)
Short Communication
DOI: http://dx.doi.org/10.4314/ovj.v8i3.12

Submitted: 23/09/2017 Accepted: 25/08/2018 Published: 10/09/2018

Isolation and preservation of multipotent mesenchymal stem cells from bone marrow of Arabian leopard (*Panthera pradus nimr*)

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Abstract
The Arabian leopard (*Panthera pardus nimr*) is critically endangered by the International Union for Conservation of Nature, with an effective population of 150-250 across its entire range in the Arabian Peninsula. Isolation and preservation of multipotent mesenchymal stem cells is beneficial both for medical and research purposes. The optimal protocol for collection, handling, culturing and preserving the Arabian leopard mesenchymal stem cells acquired from bone marrow was established. Anesthesia with combination of medetomidine and tiletamine-zozalepam is the safest option even for old animals with concurrent disease including chronic kidney disease.
Keywords: Anesthesia, Arabian leopard, Bone marrow, Mesenchymal stem cells, *Panthera pardus nimr*.

Introduction

The Arabian leopard (*Panthera pardus nimr*) is considered critically endangered by the International Union for Conservation of Nature (IUCN), with an effective population of 150-250 across its entire range in the Arabian Peninsula. Around 80 animals live in captivity in breeding centers and private collections in Arabian Peninsula (Spalton *et al*., 2006; Islam *et al*., 2017). The population has become extremely small, fragmented and susceptible to demographic and genetic stochasticity (Budd and Leus, 2011).

Mesenchymal stem cells (MSCs) have been evaluated as therapies in the veterinary regenerative medicine for years. In feline medicine, MSCs were exploited as therapies for a number of inflammatory, degenerative and immune-mediated diseases including feline chronic gingivostomatitis, acute and chronic kidney disease, enteropathies and asthma (Quimby and Borjesson, 2018). MSCs could also be explored for feline osteoarthritis and cardiomyopathy, among other diseases (Taghavi *et al*., 2015).

Preservation of genetic material such as frozen repositories could provide a high level of insurance against further losses of the diversity or the entire species (Wiidt, 1992). Stem cell cryopreservation is an efficient and safe strategy for maintaining animal genetic resources, but also promising to provide scientific values in other fields of research. Many universities, government organizations and zoos are developing cells banks. Cellular reprogramming for generating induced pluripotent stem cells (iPSCs) was recently adapted to reprogram diverse rare animal species, in an effort toward using this technology for preservation of the genetic material of endangered species. Successful trials resulting in generation of iPSCs from cryopreserved cell lines derived from populations of endangered Mandrillus leucophaeus, northern white rhinoceros, snow leopard were preformed (Leon-Quinto *et al*., 2009; Ben-Nun *et al*., 2011; Verma *et al*., 2012; Mestre-Citrinovitz *et al*., 2016). Somatic cells were reportedly used to clone a wild cat (Gómez *et al*., 2004). Different type of cells are used for storage, including samples of somatic cells, gonads or MSC. MSCs were chosen for storage in the gene bank run by Royal Court Affairs and University of Nizwa.

There is little research information about Arabian leopard and no reports of anesthesia protocol and bone marrow (BM) collection, isolation, incubation and cryopreservation of BM-derived stem cells. Published data suggest that in cats MSCs isolated from adipose tissue, proliferated significantly faster than those delivered from bone marrow and are similar phenotypically (Webb *et al*., 2012).

In the authors' opinion and experience, collection of adipose tissue by excision subcutaneous fat as a solo procedure is contraindicated in leopards when they are exposed to flies.

Authors observed that even a small incision may bring the risk of wound dehiscence. A small lipoma was removed from subcutaneous tissue from the left dorsal thoracic region by a 2-cm skin incision and blunt dissection. The wound was sutured double layer, including subcutaneous tissue, intradermal suture secured with 3 simple interrupted suture with absorbable monofilament (Monosyn 2-0). Five days after surgery the wound opened, infected with signs of myiasis, which necessitated the repetition of the

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anesthesia and the surgical interference to repair the wound. After that, the animal was kept indoors for close observation for three weeks. Such complications can be life-threatening for such a valuable animal and have to be avoided.
The collection of bone marrow by a needle aspiration from iliac wing is not associated with such side effects, especially when the procedure is run concurrently to other treatments and the animal is already under general anesthesia.
The objective of this pilot study was to:
- Harvest MSCs from bone marrow.
- Check resistance of cells for handling.
- Establish a method for culturing and expanding cells.
- Evaluate the cell viability and function after cryopreservation.
- Use the cells for banking and research purposes.

Materials and Methods

Two animals were used for this study; thefirst was an 18 years old male leopard, weighing 31 kg, while the second was a 17 years old female weighing 29 kg from the Omani Wildlife Animal Breeding Centre.Both leopards were anaesthetized for the emergency surgical treatment of the infected wounds with myiasis.
Both leopards had clinical signs of infection including apathy, loss of appetite andvisible wounds with discharge.
The anesthetic protocol was modified from published results of reverse anesthesia of snow leopards (Johansson *et al*., 2013) and involved sedation and anesthesia with a combination of medetomidine (Domitor) at a dose of 0.02 mg/kg and tiletamine-zozalepam (Zoletil) at a dose of 1 mg/kg administered intramuscularly by a blowpipe. Doses of anesthetic agents were reduced due to age-related concurrent disease including chronic kidney disease. Animals were darted in a handling enclosure. Satisfactory induction of anesthesia was observed 10-15 min after injection,.
Each leopard was double-checked for reactions, then approached and moved from enclosure to the surgical facility.
The leopard was intubated and given 100% oxygen at a rate of 3-5 L/min. A 16G catheter was placed in the right cephalic vein, blood was collected for hematology and biochemistry tests then Ringer lactate fluid was administered at a rate of 10 mL/kg/h (Hughes, 2008). Due to the infected wound, an antibiotic was administered as prophylaxis. A single dose of 30 mg/kg Ceftriaxone (Rocephin) iv and a single dose of 8 mg/kg Cefovectin (Convenia) im were administered. Moreover, a single dose of Meloxicam (Metacam inj.) at 1 mg/kg im was given for pain control. Initially surgical debridement and suturing of the wounds were performed. At the second stage the bone marrow was collected from the iliac wing by a transilial approach, a technique adapted from small animal patients (Byers, 2017).
The site was aseptically prepared and infiltrated with 2 ml of a local anesthetic (Mepivacaine). A 1-mm stab incision was made through the skin and a bone marrow aspiration needle was inserted. The bone marrow was collected by a T-handle 15 G x 3" T-handle Jamshid bone marrow biopsy aspiration needle (BD USA) to a syringe with heparin (100 IU/ml of aspirate). A total volume of 5-7 ml of the bone marrow was collected (Fig. 1).
All procedures including wound debridement and collection of bone marrow took 45-50 min and no additional anesthetic agents, except local anesthesia, were given. Leopards were transported to closed hospital enclosure and atipamezole was administered intramuscular 0.3 mg/kg. Recovery from anesthesia took a further 15-20 minutes, and after 2 hours animals behave normally. Observation over the following days did not reveal any adverse reactions from sampling site. Animals were found to be moving normally, without limping or pain reaction.
The bone marrow (5 ml sample) was mixed with 15 ml of cell culture medium (x-vivo, R&D) just after aspiration and was kept for 20-26 hours at 4ºC in 10 ml plastic serum tube (BD 366430) and then transported in an icebox to the cell culture facility at the University of Nizwa.

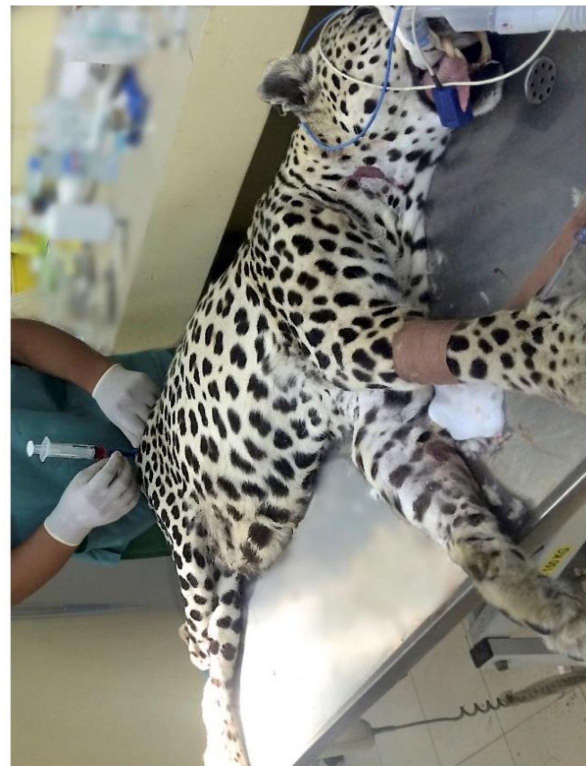

Fig. 1. An 18 years old leopard under anesthesia at the operating room for treatment and aspiration of bone marrow.

Inside a biological safety cabinet, the BM was mixed well and gently laid over a density gradient medium in 50 ml conical tubes to separate the white blood cells from other part of blood. The tube was then centrifuged at 2000 RPM for 20 minutes. The cells buffy coat was collected and transferred to another 15 ml tubes and the cells washed using a cell culture medium (x-vivo , R&D) with 10% FCS, 1% Pen/Strep and 1% antimycotic, at 1000 RPM for 10 minutes. The cell pellet was resuspended using the same medium composition. The total cells counted and then seeded to 75 cm^2 flasks and kept in a CO_2 incubator (5% CO_2) at 37ºC. Two days later, the old medium was removed and fresh medium was added. The medium was changed every three days till the cells reached ≥ 80% confluence. At this stage, the cells were washed with PBS and 3 ml of Trypsin-EDTA (0.5%) was added to the flask for 5 minutes or till the cells detached. A total of 5 ml of the medium was added to the flask to neutralize Trypsin. The cell suspension was centrifuged at 1000 RPM for 10 minutes. The cells were washed again with culture medium, counted and re-centrifuged again at the same speed. This process was repeated for two passages. After the second passage, the cell pellet was resuspended in 10% DMSO 90% FCS at a concentration of about $5x10^6$/ml and stored in liquid nitrogen. To assess the freezing and thawing process, the cells were thawed after around eight months of freezing and re-cultured again using the same conditions. The cells showed good growth with similar reported MSC morphology (Fig. 2).

Results and Discussion

The objective of this pilot study was to establish the best method of collecting, handling, culturing and preserving MSC from Arabian leopard. Anesthesia with a combination of medetomidine (Domitor) at a dose of 0.02mg/kg and tiletamine-zozalepam (Zoletil) at a dose of 1 mg/kg provided good and safe anesthesia even for older animals with concurrent diseases, and lasted for 45-50 minutes.

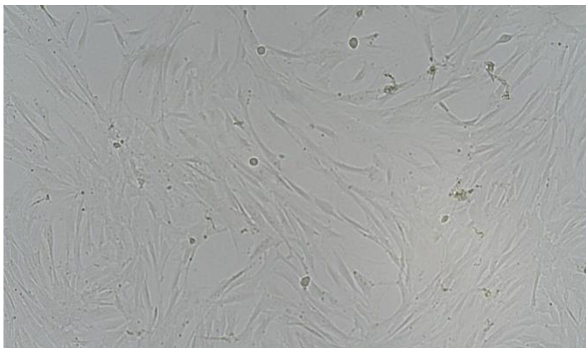

Fig. 2. Leopard BM cells collected on 22nd May 2015. The picture shows cell growth at passage 3, after freeze-thaw procedure in MEM medium. The picture was taken on the 3rd Feb 2016 at 100x magnification.

Oxygen therapy and fluids at a rate of 10 ml/kg/hr prevented post anesthetic complications. Bone marrow aspiration from iliac wing is very convenient and provides sufficient sample for cell culture and preservation without any visible adverse reaction from leopard. Sample storage up to 26 hours at 4ºC in transport medium still provided vital cells for cell culture.

The isolation, expansion and characterization of mesenchymal cells from bone marrow were carried out according to previous protocols (Gnecchi and Melo, 2009; Penfornis and Pochampally, 2011; Gottipamula *et al.*, 2014; Secunda *et al.*, 2015) with some optimization in the procedure. The optimization involved centrifugation speed and timing, periods of expansion and splitting. Also, different culture media were trialled and StemXVivo Mesenchymal Stem Cell Expansion Media (R&D systems) was found to be the most suitable for culturing these cells from leopard. Cells in 10% DMSO and 90% FCS at a concentration of about $5x10^6$/ml survived banking in liquid nitrogen and expressed a good healthy growth.

In the present study, safe and efficient technique of collection, sample storage, expanding and preserving BM-MSC in critically endangered Arabian leopard was demonstrated.

Anesthesia of geriatric patient with concurrent diseases always increase the risk ofcomplications often seen in the older patient; these include hypotension, bradycardia, hypoxemia, and prolonged recovery (Baetge and Matthews, 2012).

Previously published reports described different anesthetic protocols for leopards including:

- dexmedetomidine (0.015 mg/kg), ketamine (3 mg/kg), and midazolam (0.1 mg/kg) in combination with isoflurane (Malmlov *et al.*, 2014).
- medetomidine (0.049 mg/kg IM) and ketamine (3.8 mg/kg IM) injected 20 min later (Leclerc *et al.*, 2017).
- medetomidine 0.04–0.05 mg/kg and ketamine 3–5 mg/kg (Najera *et al.*, 2017).
- tiletamine-zozalepam 6.5–10 mg/kg (Najera *et al.*, 2017).
- ketamine 5mg/kg with xylazine 1.5 mg/kg (Deka *et al.*, 2012).
- medetomidine (0.02mg/kg) with tiletamine-zozalepam (2 mg/kg) (Johansson *et al.*, 2013).

Authors previously experienced prolonged induction time (around 15-20 min) and recovery after reversal with atipamezole (up to 3 hrs for full recovery) after anesthesia with medetomidine and ketamine with other healthy leopard. Since that time, a mixture of medetomidine and tiletamine-zozalepam was used. It provided rapid and smooth induction of anesthesia, allowed quick introduction of monitoring and supportive therapy including fluids and oxygen, which

is very important in geriatric patients. Reducing the dose of tiletamine and zozalepam from the recommended 2 mg/kg to 1 mg/kg produced excellent anesthesia in geriatric leopards. Anesthesia was reversed with atipamezole 0.3 mg/kg. After 15 min, the leopard was in sternal recumbency and after another 2 hours was completely recovered.

In cats, MSCs were first isolated and characterized from bone marrow (bMSCs) in 2002 (Martin *et al*., 2002) and then later readily expanded from fat, fetal fluids and amniotic membranes (Quimby and Borjesson, 2018).

Currently, adipose-derived MSCs (aMSCs) are most commonly used in clinical applications due to ease of attainment and their superior proliferative ability (Webb *et al*., 2012). Adipose tissue is usually collected from ventral abdomen just caudal to the umbilicus by 8 mm punch biopsy or peritoneal fat is collected during spay procedure (Kim *et al*., 2017).

Authors previous experience showed that even a small surgical wound might bring a risk of severe complications. Removing a skin lipoma from the left dorsal thoracic region by 2 cm skin incision and blunt dissection, despite proper surgical wound closure, resulted in myiasis.

Larvae of Old World screwworm fly (*Chrysomya bezziana*), which feed on the living tissue, were found in wounds (Hall *et al*., 2009). As the larvae feed, the wound gradually enlarges and deepens. Infested wounds have a serosanguineous discharge and a distinctive odor. Untreated animals may die in 7 to 14 days from toxicity or secondary infections. Treatment consists of aggressive tissue debridement, wound closure, application of organophosphates insecticides as well antibiotic and pain control therapy (Han *et al*., 2018).

This complication necessitated the repetition of the anesthesia and the surgical repair of the wound. After that, the animal was kept indoors for close observation for three weeks. Such life-threatening complications in critically endangered animals must be avoided.

Bone marrow can be aspirated from many sites including proximal humerus, proximal femur, sternum or iliac crest (Byers, 2017). Old age and concomitant diseases, like infection and chronic kidney disease do not affect quality of the bone marrow (BM)-delivered MSCs and do not contribute to the post-collection complications.

Mixing bone marrow sample with cell culture medium (x-vivo , R&D) in 10 ml plastic serum tube (BD 366430) after aspiration and transport for 20-26 hours at 4ºC provided sufficient sample for isolation and culturing BM-MSC. Field sample storage is important when immediate transport from remote areas might be difficult.

The isolation and expansion of Arabian leopard mesenchymal cells from bone marrow do not differ from previously established protocols (Gnecchi and Melo, 2009; Penfornis and Pochampally, 2011; Gottipamula *et al*., 2014; Secunda *et al*., 2015).

Preservation of tissues, gametes, embryos and stem cells from endangered animals is evolving under auspices of The International Union for Conservation of Nature (IUCN) with cooperation of zoos and universities.

Currently, some large projects are running like Frozen Zoo in San Diego the USA, Frozen Ark Project in the UK, Animal Gene Storage and Resource Centre of Australia and many other projects across the world.

The main goal is to collect genetic materials and live cells for further use in restoring or increasing genetic diversity through advanced reproductive technologies.

The gene bank run by the Royal Court Affairs and University of Nizwa is collecting samples of MSCs among other tissues from endangered wild animals, such as the Arabian tahr (*Arabitragus jayakari*) and the Arabian wolf (*Canis lupus arabs*) for future research and treatment purposes.

Conflict of interest

The Authors declare that there is no conflict of interest.

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