Cryobiology 78 (2017) 95–100

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Collection, processing and freezing of equine bone marrow cells

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ARTICLE INFO

Article history:
Received 24 April 2017
Received in revised form
13 June 2017
Accepted 17 June 2017
Available online 20 June 2017

Keywords:
Cell therapy
Cryopreservation
Heparin
Horse
Bone marrow mononuclear cells

ABSTRACT

There is no consensus on aspects of equine bone marrow collection and processing. The study aimed to describe the collection of large volumes of bone marrow from horses of advanced age, with emphasis on bone marrow mononuclear cells (BMMCs) recovery and viability after cryopreservation. Fourteen horses, aged 3–24 years, were divided into three experiments. E1 studied the feasibility of collecting 200 mL from the sternums of horses of advanced age; E2 examined the number of cells obtained from the first and last syringe of each puncture; and E3 investigated the influence of heparin concentration on the prevention of cell aggregation, and cell viability after freezing in liquid nitrogen. Bone marrow aspirations were done with syringes pre-filled with Iscove's modified Dulbecco's medium and different concentrations of sodium heparin. BMMCs were counted, cell viability was determined, and samples were frozen. Bone marrow collection from the sternum is safe, even at large volumes and from horses of advanced age, and the number of cells recovered decreases with successive aspirations ($p < 0.0001$). Heparin concentration influenced cell aggregation, and recovered cells continued to be commercially viable after 150 days in frozen storage.

1. Introduction

Cell therapy is becoming a common practice in equine clinical and surgical settings, and is more directed to musculoskeletal disorders such as tendonitis, osteoarthritis, and laminitis [20]. Its use has also been demonstrated for the treatment of respiratory diseases in horses [7]. Bone marrow mononuclear cells (BMMCs) have been widely used due to their anti-inflammatory, anti-fibrotic, and microbicidal effects [1]. When compared to other cell types — such as mesenchymal stromal cells (MSCs) — commonly used in cell therapy studies, BMMCs have lower associated costs, lower risk of immunological reactions due to their autologous administration [15], and shorter processing time, with no need to be cultured. This enables their use on the same day of bone marrow collection; alternatively, using cryogenic storage methods, the cells can be used whenever necessary for further injections [2].

In veterinary medicine, there is still no consensus on the best type — or optimal amount — of cells to be used for each treatment [17], which makes uncertain the amount of bone marrow that should be collected. The quantity of cells required for treatment varies according to the species and diseases studied. It ranges from 3×10^6 BMMCs to treat Wistar rats with myocardial infarction [22], to 1×10^7 MSCs to treat horses with tendon lesions [14], to 2×10^6 BMMCs to treat mice with experimentally induced asthma [2].

Recently, we investigated the effects of using BMMCs to treat horses with recurrent airway obstruction (RAO) or severe asthma; these animals received a minimum of 500×10^6 cells, which were obtained from 200 mL of bone marrow collected from the sternum [7]. To date, no studies in horses have shown the quantity of cells that can be obtained from large-volume bone marrow samples, nor have studies identified the minimum number of punctures required to obtain 200 mL of bone marrow. Furthermore, some aspects of collecting bone marrow from these animals remain scarcely discussed, such as the ideal puncture location (sternum or

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http://dx.doi.org/10.1016/j.cryobiol.2017.06.006
0011-2240/

ilium), the relationship between volume collected and the number of cells obtained, and the risks involved in the procedure [3,13].

Therefore, the present study aimed to determine the feasibility of extracting large volumes of bone marrow from the sternum, with additional attention being paid to 1) the quantities of BMMCs that could be recovered, and 2) the extent to which these cells could be cryopreserved without losing viability.

2. Material and methods

2.1. Animals

This study was approved by the Committee on Animal Experimentation of the Pontifícia Universidade Católica do Paraná (PUCPR), Curitiba, Brazil, and is registered as numbers 788 and 988.

Fourteen mixed-breed horses, consisting of males and females, aged 3–24 years and weighing 340–520 kg, were studied. The animals were in good health, as verified by clinical and hematological analyses. Three months before the experiments, the animals were dewormed and vaccinated against tetanus, equine influenza, rabies, encephalitis, and herpes virus.

2.2. Experimental design

The animals were divided into three experiments (Fig. 1). To investigate the feasibility of collecting large volumes of bone marrow from horses of advanced age (which is necessary when using BMMCs to treat horses with severe asthma) [7], eight horses aged 11–24 years were enrolled in Experiment 1 (E1), and had 200 mL of bone marrow collected from their sternums. We also sought to determine whether the number of recovered BMMCs decreased during the course of a series of syringe aspirations taken from a single sternum puncture. Therefore, in Experiment 2 (E2), we focused on the bone marrow samples collected from four animals of the E1, and analyzed the first and the last syringe of each puncture to compare the number of cells recovered. After collection events occasionally resulted in cell aggregate formations and low cell recovery rates, we designed Experiment 3 (E3). In E3, six horses, aged 3–18 years, each had a 20 mL volume of bone marrow collected from their sternums. Three syringes were used for each horse, and each syringe contained one concentration (5%, 10%, and 20%) of heparin; this was done to determine whether cell aggregation would decline, and BMMCs recovery rates would improve, if the heparin concentration was changed. The E3 samples that were collected with the 10% heparin syringes were subsequently aliquoted and frozen in cryotubes, to determine cell viability after frozen storage in liquid nitrogen for periods of 30, 60, 90, 120, and 150 days. This was investigated because an important question in equestrian veterinary medicine has been whether aspirated cells from a healthy animal will remain viable if those cells are frozen for later use.

2.3. Bone marrow collection

Horses were sedated with intramuscular administration of 0.05 mg/kg bwt of 1% acepromazine (Acepran®, Univet Laboratory, São Paulo, Brazil). The collection site between the fourth and sixth sternebrae was the same for all experiments (Fig. 2) [16], and was trichotomized, aseptically prepared, and locally anesthetized with 2% lidocaine (Lidovet®, Bravet Laboratory, Rio de Janeiro, Brazil). Sedation was complemented with intravenous administration of 0.5 mg/kg bwt of 10% xylazine (Sedomin®, König, Buenos Aires, Argentina) with 50 mg of pethidine hydrochloride (Dolosal®, Cristália Laboratory, Itapira, Brazil). The aspiration puncture was

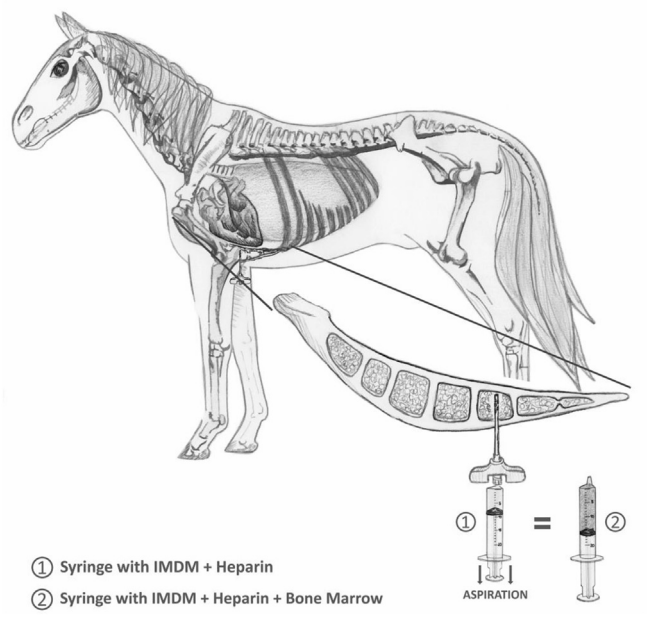

Fig. 2. Bone marrow collection from the sternums of horses, using 20 mL syringes prefilled with 7 mL of Iscove's modified Dulbecco's medium (IMDM) and sodium heparin, to collect 7 mL of bone marrow (resulting in an IMDM-heparin/bone marrow solution with a volumetric ratio of 1:1).

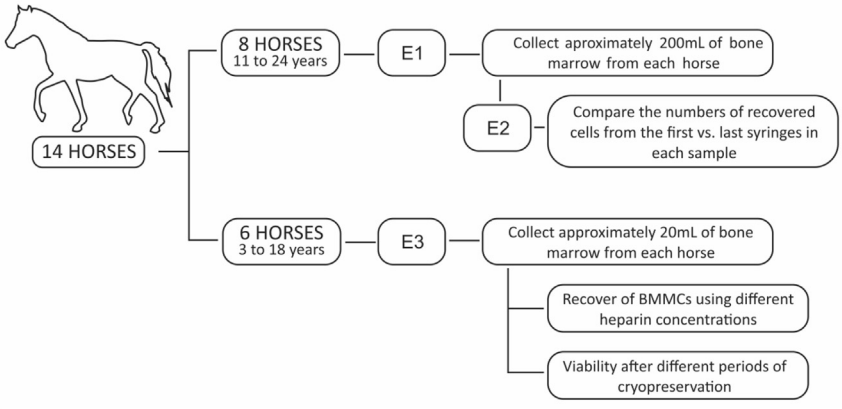

Fig. 1. Schematic diagram of the study: 14 animals, divided into three experiments.