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ORIGINAL ARTICLE

Coronary corium, a new source of equine mesenchymal stromal cells

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Abstract

Mesenchymal stromal cells (MSCs) have attracted great attention for therapeutic applications. Since cells derived from different tissues have different properties, using the right tissue source may impact their efficiency in regenerative medicine. This study describes for the first time the isolation and characterization of MSCs derived from the equine coronary corium, which may be useful for treating diseases such as laminitis. Seven coronary corium samples were used for isolation of cells (ccMSCs). Adherent cells were characterized for morphology, immunophenotype, proliferation and differentiation potential, in vitro migration and colony-forming capacity. The cells displayed the characteristic fibroblastoid morphology, with population doubling time increasing until passage 7 and reaching a plateau in passage 10. Cells were negative for CD14 and CD45, and positive for CD73 and CD90. ccMSCs showed chondrogenic and osteogenic, but not adipogenic differentiation, and migrated with nearly total closing of the empty area in 48 h, in the scratch assay. The clonogenic potential was in average 18% to 23%. This study describes for the first time the establishment of mesenchymal stromal cell cultures from the equine coronary corium. The results are similar to MSCs isolated from many other equine tissues, except for restricted differentiation potential. As coronary corium stem cell regulation may contribute to the pathogenesis of equine chronic laminitis, the use of ccMSCs in cell therapy for this significantly debilitating disease should be further investigated.

Keywords Mesenchymal stromal cells · Horse · Coronary corium · Laminitis

Introduction

Mesenchymal stem or stromal cells (MSCs) are multipotent progenitor cells that have attracted great attention in recent years for therapeutic applications. Initially described in the 1970s (Friedenstein et al. 1970), the cells received their current name 20 years later, in reference to adherent, culture-expanded bone marrow cells (Caplan 1991). MSCs have been extensively investigated for their biological properties and regenerative potential. Due to their perivascular niche, they are present in virtually all tissues (da Silva Meirelles et al. 2006), where they have an important role in organ homeostasis, tissue regeneration and ageing (da Silva Meirelles et al. 2008). As recently reviewed (Fan et al. 2020), the therapeutic potential of MSCs is attributed to multiple activities including the differentiation into various cell types and mainly paracrine effecs, with mitochondrial transfer and production of extracellular vesicles mediating cell survival and regeneration. The immunomodulatory properties of MSCs, exerted through interactions with all types of immune cells, are particularly important and result in regulation of the inflammatory microenvironment (Weiss and Dahlke 2019).

A great number of preclinical and clinical studies have explored the potential of MSCs to treat a variety of diseases (reviewed by Gurusamy et al. 2018). However, the translation of beneficial preclinical results observed in animal models into the clinical setting has been limited, due to factors such as heterogeneity of cell types and protocols used. This has been seen more often in cardiovascular diseases (Epstein 2019).

Luiza Lopes da Silva and Maiele Dornelles Silveira contributed equally to this work.

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These observations emphasise the need for a better understanding of the biology of MSCs and their physiological behaviour. Since MSCs derived from different tissues also have different properties (da Silva Meirelles et al. 2006), using the right source of cells may impact their therapeutic effects in regenerative medicine.

MSCs have been described in laboratory, domestic and wild animal species (reviewed by Gugjoo et al. 2019a). Equine MSCs, derived from tissues such as bone marrow (Fortier et al. 1998), adipose tissue (Vidal et al. 2007; Shojaee et al. 2019), endometrium (Corradetti et al. 2014; Rink et al. 2017; Cabezas et al. 2018), hair follicles (Michler et al. 2018), synovial fluid and membrane (Prado et al. 2015), amniotic membrane (Corradetti et al. 2014), umbilical cord wall and blood (Koch et al. 2007; Cremonesi et al. 2008; Alizadeh et al. 2018; Merlo et al. 2019), have also been investigated. As reviewed by Gugjoo et al. (2019b), cultures derived from equine tissues exhibit the general morphological, immunophenotypic and functional aspects of mesenchymal stromal cells (De Schauwer et al. 2011).

The therapeutic use of equine MSCs, generally isolated from bone marrow, adipose or foetal tissues, has also been investigated. Although initially considered mainly for tendon, joint and bone disorders, more recently their potential to treat many other conditions, including inflammatory, immuno-mediated, reproductive and neurological diseases has been explored (reviewed by Gugjoo et al. 2019b). This therapeutic approach has also been tested in horses with laminitis, a common and debilitating musculoskeletal disease that results in morphological and functional changes within the hoof. In this condition, which is the most serious disease of the equine foot, converging metabolic and inflammatory mechanisms result in lamellar attachment failure through loss of epithelial cell adhesion (reviewed by van Eps and Burns 2019). Curative treatment strategies in more advanced cases are still unknown, and a few studies describe the use of mesenchymal stromal cells (Morrison et al. 2014; Angelone et al. 2017). As a whole, the results do not allow a definite conclusion on the effect of MSCs, mainly due to the variation in cell sources, numbers and protocols, as well as the small number of patients and lack of appropriate control groups. There is a clear need for a more complete understanding of the biology of MSCs from different tissues, so that the best sources and conditions can be established in each case.

According to the current understanding of the physiological role of native MSCs, their presence in a perivascular niche in virtually all tissues allows them to recognise soluble signals produced by injured regions, become activated and contribute to tissue repair by secreting cytokines and chemokines (da Silva Meirelles et al. 2016). In this context, it is possible that cells isolated from the injured region are better prepared to recognise and respond to repair signals, as has been shown for instance for the osteogenic potential of bone marrow- and adipose tissue-derived MSCs (Mohamed-Ahmed et al. 2018). Therefore, knowing and testing the therapeutic potential of cells derived from different tissues may contribute significantly to a better use of cell therapy protocols. The present study describes for the first time the isolation and characterization of MSCs derived from the equine coronary corium, which allows not only broadening of the knowledge on the biological characteristics of MSCs, but would also provide cells for therapeutic applications.

Material and methods

Reagents and culture media

All reagents used in this study were from Sigma Chemical Co. (Sigma-Aldrich Brasil Ltda, SP, Brazil), unless otherwise stated. Normal culture medium (NCM) was composed of Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies do Brasil, SP, Brazil) with HEPES (free acid, 2.5–3.7 g/l) and 10% foetal bovine serum (Cultilab, SP, Brazil). Ca2 + − and Mg2 + −free Hank's balanced salt solution (HBSS) was used to wash the tissues and cells. Plasticware used was supplied by Greiner Bio-One Brasil (SP, Brazil).

Animals and samples

Seven coronary corium samples were collected from healthy adult horses of both sexes slaughtered at the Foresta equine slaughterhouse (São Gabriel, RS, Brazil). The samples were collected until 1 h after the death of the animals. Five samples (cc1 to cc5) were used for all experiments described below, except for immunophenotyping which was performed in cells from two samples (cc6 and cc7).

The study was approved by the Animal Research Ethics Committee of Universidade Federal de Pelotas (n° CEEA 2322–2016).

Isolation and cultivation of coronary corium mesenchymal stromal cells (ccMSCs)

Prior to collection, a surgical preparation was made in the coronary region by shaving and antisepsis with 10% iodopolividone and iodised alcohol. With the assistance of sterile surgical instruments, the dorsal coronary tissue was accessed through an incision approximately five centimetres long by one centimetre wide in the band of horny tissue that forms the upper back of the hoof wall, thus allowing visualisation and subsequent resection. The collection time respected the maximum limit of 1 h after the slaughter of the animals.

The coronary corium samples were cut into small pieces, washed with HBSS and digested with collagenase type I

(250 U/mL in DMEM/10 mM HEPES) for 30 min at 37 °C. After centrifugation at 400 x g for 10 min, at room temperature, the pelleted cells and tissue fragments were resuspended in NCM supplemented with 1% antibiotic-antimycotic solution (Gibco) and plated in 175 cm^2 culture flasks. After 3 days incubation at 37 °C in a humidified atmosphere of 5% CO_2 in air, half the medium was changed. When a confluence of 70% to 90% was reached, all the medium was discarded and the cell monolayer was incubated with 0.25% trypsin in 0.01% EDTA for 5 min at 37 °C, collected and washed in HBSS. The cultures were passaged twice weekly. Fresh cells in passages 3 to 5 were used in all experiments, except for determination of population doubling times when older cultures were also analysed. Experiments were performed in triplicates.

Morphological analyses and photographs

Adherent cell cultures were routinely observed under an inverted phase-contrast microscope (Axiovert 25; Carl Zeiss, Hallbergmoos, Germany). Photomicrographs were taken with a digital camera (AxioCam MRc, Carl Zeiss), using AxioVision 3.1 software (Carl Zeiss).

Proliferation potential of ccMSCs

From passages three to 10, the adherent monolayer was grown to 80–85% confluence, detached by trypsin/EDTA treatment, washed and viable cells were counted using a Neubauer chamber, after trypan blue staining. The population doubling time (PDT) of the cultures was calculated by the formula: log (final cell number) - log (initial cell number) = K x T, where K is the generation constant (0.008963) and T is time in days (Roth 2006). Experiments were performed in triplicates.

Differentiation potential of ccMSCs

Cultures cc1 to cc5 were analysed for trilineage potential. The cells were plated into 6-well culture plates, in NCM and at a concentration of 10^4 cells/cm^2. After 48 h, the inducing media were added and the plates were incubated for 28 days. The osteogenic medium was NCM supplemented with 10^{-8} M dexamethasone, 5 μg/mL ascorbic acid 2-phosphate and 10 mM β-glycerophosphate. For chondrogenic differentiation, NCM was supplemented with 6.25 μg/mL insulin, 10 ng/mL TGF-β1 and 50 nM ascorbic acid 2- phosphate. For adipogenic differentiation, cultures cc1 to cc7 were maintained in NCM supplemented with 10^{-8} M dexamethasone, 2.5 μg/mL insulin, 100 μM indomethacin, and 3.5 μM rosiglitazone (GlaxoSmithKline, Middlesex, UK). For analysis of osteogenic, chondrogenic and adipogenic differentiation, the cultures were washed, fixed with 4% paraformaldehyde and stained with Alizarin Red S, Alcian Blue and Oil Red O, respectively. Undifferentiated controls were cultured for the same period of time and stained.

Immunophenotyping

The immunophenotype of cultures cc7 and cc8 was determined by flow cytometry. The cells were trypsinized, washed and incubated for 30 min at 4 °C with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies specific for human CD14, CD45, CD73 and CD90, and isotype controls were used (Becton Dickinson, San Jose, CA). Flow cytometry was performed with a BD FACSCanto II (Becton Dickinson). At least 10,000 events were collected.

In vitro migration assay

The capacity of ccMSCs to migrate was analysed using the in vitro scratch assay. Adherent cells were plated in 6-well culture plates and grown to 70–80% confluence, and a pipette tip was used to scratch the cells and create an artificial wound. Photographs taken at 0, 24 and 48 h using an inverted phase-contrast microscope (Axiovert 25, Carl Zeiss) were analysed with the ImageJ software (National Institutes of Health version 1.48v). Cell migration was analysed by the correlation between time and closure of the space in the scratch area.

Analysis of clonogenic potential

The clonogenic potential of ccMSCs was analysed by limiting dilution, in triplicate samples. The cells were centrifuged, resuspended in HDMEM and 200 cells/well were seeded in 6-well plates. After culture for 10 days at 37 °C with 5% CO2 in air, with medium change every 3 days, the cells were washed, fixed with 70% ethanol for 5 min at room temperature and stained with Giemsa. Colonies were considered as cell clusters with more than 50 cells, and the result was expressed as % colony-forming cells.

Statistical analysis

Results are expressed as mean ± standard deviation. Data were tested for normality and analysed by one-way analysis of variance followed by Tukey's post hoc test, with significance set at $p < 0.05$. Prism 5 software (GraphPad Software Inc., San Diego, CA, USA) was used for analysis of the data and graph drawing.

Results

Healthy MSC cultures were derived from the five coronary corium samples processed. The first passage was performed around five or six days after plating of pelleted cells and

fragmented tissues in culture flasks. The cells displayed characteristic fibroblastoid morphology during all culture time (Fig. 1a). The PDT of the cultures increased until passage 7, and after that it was decreased until seemingly reaching a plateau in passage 10 (Fig. 1b). Immunophenotyping showed that the cells were negative for CD14 and CD45, weakly positive for CD73 and strongly positive for CD90 (Fig. 1c). Chondrogenic and osteogenic differentiations were observed in the five cultures analysed, as represented in Fig. 2. Staining with Alizarin Red S and Alcian Blue, respectively, revealed the matrices secreted by the differentiated cells. However, no adipogenic differentiation was observed, even when additional samples were analysed (not shown).

To evaluate the capacity of ccMSCs to migrate in vitro, a scratch assay was performed with cultures derived from the five coronary corium samples. The closure of the scratch area was recorded at 0, 24 and 48 h (Fig. 3). Although for some of the cultures the closure was not complete in 48 h, the process was effective in all of them and the differences were not significant.

The clonogenic potential of ccMSCs was analysed by plating the cells at low concentration and counting the colonies formed after 10 days of culture. The results, expressed as te percentage of plated cells capable of forming colonies, showed that cultures derived from the five samples had, on average, around 18% to 23% cells with clonogenic capacity (Fig. 4).

Discussion

This study describes for the first time the establishment of mesenchymal stromal cell cultures from the equine coronary corium. The results are supported by determination of the morphology, immunophenotype and proliferative capacity, as well as in vitro migration and clonogenic potential of the cells.

The horse hoof is formed by keratinisation of epithelial layers, which are associated with a dermal region or corium. The corium is formed by a dense matrix of highly vascular tough connective tissue. It has the capacity for self-renewal, cell proliferation and differentiation, and is therefore responsible for epithelial regeneration and growth (Daradka and Pollitt 2004). When laminitis develops, the attachment mechanism between the hoof lamellae and the distal phalanx fails and the corium becomes compressed, presenting limited

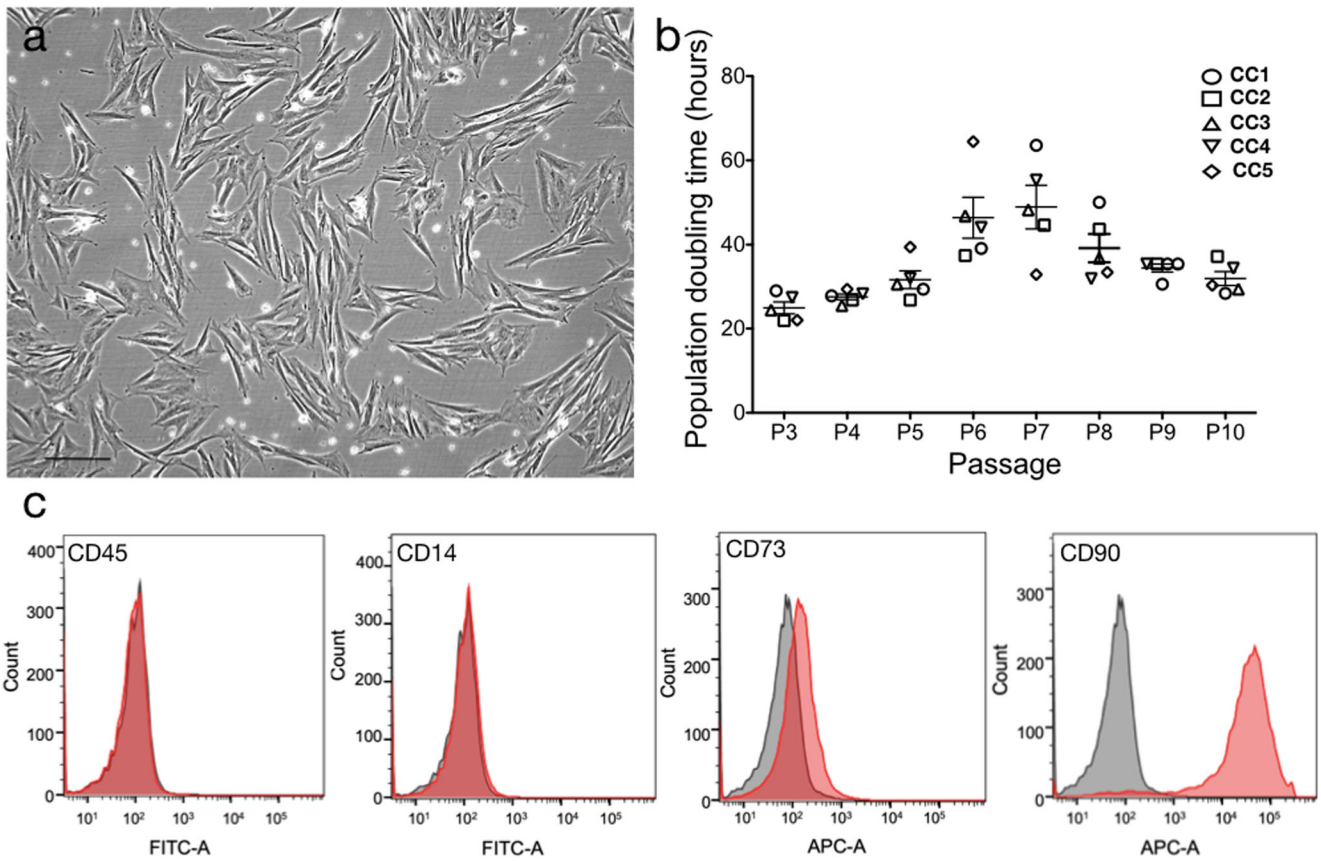

Fig. 1 **a** Representative phase-contrast photomicrograph of ccMSCs. Bar = 100 μm. **b** Population doubling time of cultures derived from five samples of coronary corium (cc1 to cc5), showing mean ± standard deviation. **c** Immunophenotype of ccMSCs (cc6 and cc7). Representative results show that cultures were negative for CD14 and CD45, weakly positive for CD73 and strongly positive for CD90

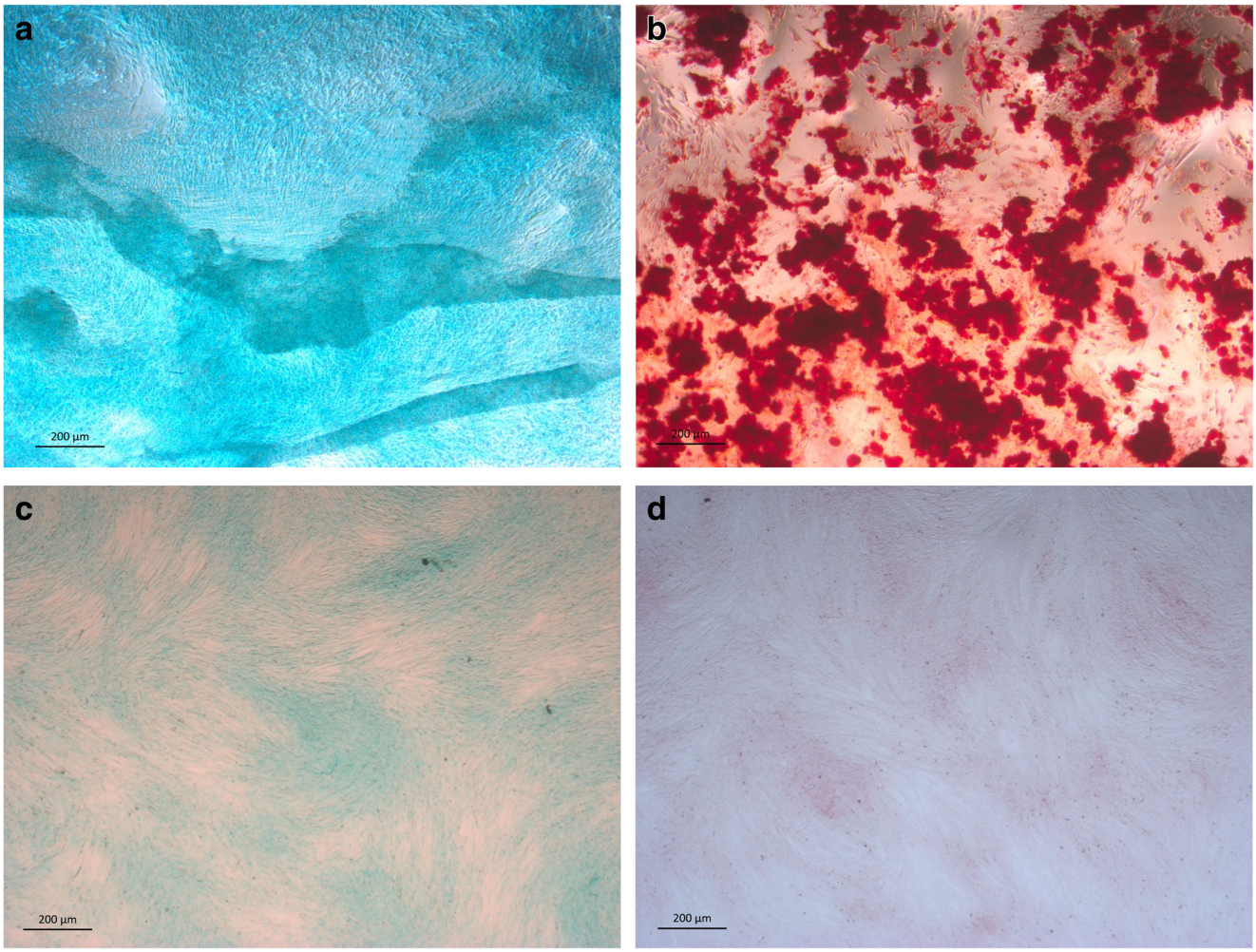

Fig. 2 Representative photomicrographs of the chondrogenic (**a**) and osteogenic (**b**) differentiation processes observed in cultures derived from coronary corium samples cc1 to cc5. Control cultures (**c** and **d**) were not stained. Bars = 200 µm

proliferative potential with a shift towards differentiation. This may reflect reduced activity of epidermal stem cells in laminitic hoof (Carter et al. 2011).

Healthy MSC cultures were established from the seven coronary corium samples used in the present study. The collection of tissues from deceased horses did not influence the results, as already described for bone marrow-derived MSCs when samples obtained from euthanised or live horses were compared (Schröck et al. 2017). The maximum time frame after death has still to be determined. In the above-mentioned study, bone marrow was collected 30 min after euthanasia, whereas an interval of 1 h was allowed in the present work.

There is a clear advantage in using cells isolated from cadaveric donors, if allogeneic MSCs can be used in cell therapy with no adverse reaction. This question is still under debate. While some studies show that allogeneic MSCs can induce allorecognition responses, which include the expansion of immune cell subpopulations and detectable levels of allo-specific antibodies (Schnabel et al. 2014; Wang et al. 2019), a recent review shows that, although MSCs are not as immune-privileged as previously though, evidence from clinical trials suggest that allogeneic MSCs transplantation is safe and can be used as an "off-the-shelf" medical product (Kot et al. 2019).

A high number of progenitor cells is expected from this type of tissue, due to its high vascular network (da Silva Meirelles et al. 2006). The characteristics of ccMSCs were similar to cells isolated from other tissues. The cells presented the typical spindle-shaped morphology which has been described for equine MSCs derived from bone marrow (Colleoni et al. 2009), endometrium (Rink et al. 2017), hair follicles (Michler et al. 2018), periosteum and muscle (Nino-Fong et al. 2013; Radtke et al. 2013). This morphology is maintained when cells are isolated or cultivated under different conditions. Adipose tissue-derived cells, for instance, keep the same morphology even when cultures are initiated with $CD146^{+}$ (a pericyte marker)-sorted cells (Esteves et al. 2017). The fibroblastoid morphology is therefore considered one of the important characteristic features of MSCs, across species or tissue sources.

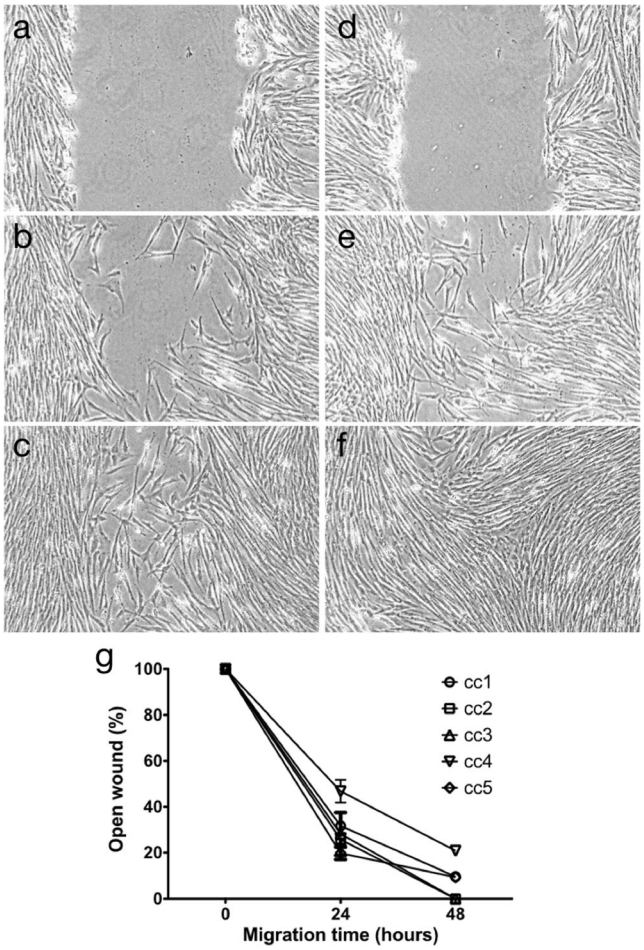

Fig. 3 In vitro scratch assay. Closure a scratch produced on the five ccMSC cultures (cc1 to cc5) was evaluated by images recorded at 0, 24 and 48 h, and the percent of closed area (or "healed wound") was determined with the ImageJ software. For some cultures (**a**, **b** and **c**, cc1) the area was not completely closed in 48 h as it was for others (**d**, **e** and **f**, cc2), but the difference was not significant. Values are mean ± standard deviation of three independent experiments

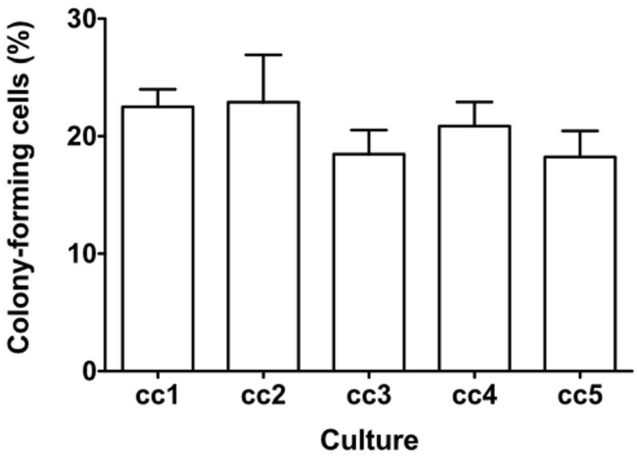

Fig. 4 Clonogenic ability of MSC cultures (cc1 to cc5), expressed as mean percentage of plated cells able to originate colonies ± standard deviation

The rapid increase in cell number is one of the main advantages of mesenchymal stromal cells, when considering their application in regenerative medicine. We observed an increase in PDT of ccMSCs up to passage 7, after which it decreased and seemed to reach a plateau around passage 10. This initial increase in growth rates has been reported in other studies on equine MSCs. Esteves et al. (2017) observed adipose-derived cells until passage 6, with a continuous increase in PDT. Similar results were reported for cord blood-derived MSCs, studied up to passage 10 (Alizadeh et al. 2018). Other studies have shown maintenance of growth rates during the first ten passages, for MSCs isolated from bone marrow (Vidal et al. 2006) or from cord blood and cord tissue (Lepage et al. 2019). A simultaneous comparison of equine MSCs from different tissues showed variable growth patterns until passage 8, with higher replication rates for cells isolated from adipose tissue and tendon than umbilical cord blood and bone marrow (Burk et al. 2013).

ccMSCs readily differentiated into osteogenic and chondrogenic lineages, when maintained in inducing media. Trilineage differentiation is another hallmark of mesenchymal stromal cells, as defined by the International Society of Cell Biology (Dominici et al. 2006; Viswanathan et al. 2019), and has already been reported for equine MSCs isolated from many tissues (reviewed by Gugjoo et al. 2019b). Special attention has been given to the chondrogenic potential, due to interest in therapeutic applications of these cells (Ortved and Nixon 2016).

ccMSCs however showed absence of adipogenic differentiation when cultured in inducing medium. The result was confirmed by submitting two additional cultures to the adipogenic medium. Although trilineage differentiation is one of the minimal criteria to define MSCs (Dominici et al. 2006), little or no adipogenic differentiation potential has already been described for cells isolated from different tissues, such as murine (Nadri and Soleimani 2007) and human amniotic fluid (Chen et al. 2019) or human umbilical chord (Kern et al. 2006; Shetty et al. 2010). In these cases, lack of adipogenic differentiation was suggested to be related to the ontogenetic age of the cells. However, a similar situation was described for MSC-like cells isolated from the equipe superficial digital flexor tendon (Williamson et al. 2015), explained by the authors as a possible selection, for unknown reasons, of a subpopulation of cells with restricted differentiation potential. This possibility is supported by the report of a biophysical sorting of human bone marrow MSCs resulting in large- and small-MSCs, with lack of adipogenic differentiation of the first population (Poon et al. 2015).

We have previously used the differentiation protocol described in the present study to induce adipogenesis in MSCs isolated from different tissues and species, such as virtually all murine organs and tissues (da Silva Meirelles et al. 2006), canine and rat bone marrow (Marx et al. 2014 and Corsetti

et al. 2017, respectively), and adipose tissue from humans (Markarian et al. 2014), and from a wild rodent species, *Ctenomys minutus* (Pereira et al. 2018). The reasons for restricted differentiation potential of ccMSCs observed in the present study are not clear, and further studies would be necessary to identify a biological reason for that, or the existence of subpopulations with different adipogenic potential.

The immunophenotype of equine ccMSCs was determined with anti-human antibodies, which have also been used in other reports (Pascucci et al. 2011; Barberini et al. 2014; Esteves et al. 2017; Schröck et al. 2017). Our results have also been similar to the above-mentioned studies, and agree with the surface marker phenotype proposed by the International Society for Cell Therapy for MSCs (Dominici et al. 2006).

Two frequently investigated functional characteristics of MSCs are the capacity to migrate in vitro and to form colonies from single cells. In vitro migration capacity, which simulates a stress situation to which the cells must respond, can be analysed with several methods. We used the scratch assay, in which an artificial "wound" is created in the cell monolayer and the speed of closure of the scratch is measured (Jonkman et al. 2014). The cultures isolated from the coronary corium closed the scratch area in a period close to 48 h, showing adequate migration capacity.

Using this assay, Cabezas et al. (2018) observed that equine MSCs isolated from endometrium closed the scratch faster than adipose tissue-derived cells, but for both populations the scratch was completely refilled in 24 h, which was faster than our results for ccMSCs. In another study using the scratch assay, but with results observed at 24 h only, the average percentage of migration of MSCs derived from equine adipose tissue, bone marrow, umbilical cord blood and Wharton's jelly ranged between 24.5 ± 21.4% and 43.0 ± 7.7% (Merlo et al. 2019), similar to our results.

The clonogenic or colony-forming assay is used to evaluate the percentage of more primitive cells in the population, as a measure of self-renewal capacity. Our results, showing a range between 18% to 23% cells with clonogenic capacity for the five samples, are lower than those reported for bone marrow-derived cells, 32% and 42% (Bourzac et al. 2010 and Lovati et al. 2011, respectively). A much lower clonogenic capacity of 8.64 ± 4% was reported by Zahedi et al. (2017) for equine bone marrow-derived MSCs, which could be explained by different characteristics of isolated cells or cell isolation method, according to the authors. A still lower clonogenic capacity was described for adipose-derived MSCs (5.5%, Alipour et al. 2015).

In conclusion, this study described for the first time the isolation and characterization of equine MSCs from the coronary corium. As a tissue that acts as an "essential glue" holding the hoof onto the foot (Colville and Bassert 2016), the corium is involved in laminitis. Since the administration of MSCs isolated from umbilical cord blood, adipose tissue and bone marrow (Morrison et al. 2014; Angelone et al. 2017) have shown beneficial effects, it is proposed that ccMSCs should also tested in cell therapy protocols for this significantly debilitating disease.

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Availability of data and material The datasets generated during the current study are available from the corresponding author on reasonable request.

Authors' contributions All authors contributed to the study conception and design. Nance Beyer Nardi and Charles Ferreira Martins conceived the experiments and wrote the paper. Luiza Lopes da Silva, Maiele Dornelles Silveira, Patrícia Bencke Grudzinski and Carla Augusta Sassi da Costa Garcia performed the experiments. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethics approval All procedures performed in studies involving animals were in accordance with the ethical standards and were approved by the Animal Research Ethics Committee of Universidade Federal de Pelotas, Brazil (n° CEEA 2322–2016).

Consent to participate Not applicable.

Consent for publication Not applicable.

Code availability Not applicable.

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