Relatório Final de Estágio

Mestrado Integrado em Medicina Veterinária

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ABSTRACT

Cell-based therapies have been exponentially growing over the last few years and stand out in Regenerative Medicine. Canine Umbilical Cord Mesenchymal Stem Cells (cUC-MSCs) and their products' characteristics have given them popularity in this field, leading to the development of more studies and clinical trials on its therapeutic potential.

Stem cells' therapeutic effect is essentially attributed to their secretome and studies have been conducted to understand if there are differences between applying cells themselves or only their bioproducts, specially the secretome (known as Conditioned Media – CM – when produced in the laboratory).

When preparing therapeutic cell dosages, the commonly used cells' culture medium requires supplementation with Fetal Bovine Serum (FBS), which raises ethical, health and animal welfare concerns. However, CM is a xeno-free product and the preference for its use may represent a safer and more ethic therapeutic choice.

Therefore, since stem cells are exposed to their own biofactors, it's crucial to understand how secretome interacts with them. In this respect, PrestoBlue™ Cell Viability Assay was performed to compare cUC-MSCs viability when cultured with CM at two different concentrations, 1x and 5x, and only with standard culture medium, at different time points (24, 72, 120 and 168 hours). The results support that CM might be able to increase cell viability since it provided a statistically significant higher viability at later time points, when compared to the standard medium. Also, CM concentration was not a decisive factor in cells' viability, allowing great viability values to be obtained using CM plain, saving both time and CM. More studies are needed to prove the effectiveness of CM in replacing FBS-supplementation and even as an alternative to cell-based therapies. However, the results encourage the use of CM as a mean for treatment optimization.

KEYWORDS: Conditioned Media; Canine Umbilical Cord Mesenchymal Stem Cells; Secretome.

RESUMO

O uso das terapias celulares como opção terapêutica, na área da Medicina Regenerativa, tem vindo a crescer exponencialmente ao longo dos últimos anos. As características das Células Estaminais Mesenquimatosas do Cordão Umbilical Canino (cUC-MSCs) e dos seus bioprodutos favoreceram o desenvolvimento de estudos e de ensaios clínicos direcionados à avaliação do seu potencial terapêutico.

O efeito terapêutico destas células é essencialmente atribuído ao secretoma e vários estudos foram já realizados para determinar se existem diferenças entre aplicar as células ou apenas os seus bioprodutos, especialmente o secretoma (conhecido como Meio Condicionado - CM - quando produzido em laboratório).

Ao preparar dosagens terapêuticas de células, o meio de cultura normalmente utilizado é suplementado com Soro Fetal Bovino (FBS), o que levanta preocupações éticas, de saúde e de bem-estar animal. Contudo, o CM não é xenogénico e a preferência pela sua utilização pode representar uma escolha terapêutica mais segura e em termos éticos, mais consensual.

Assim sendo, é crucial compreender a interação entre estas células e o seu secretoma. Para isso, o ensaio de viabilidade celular PrestoBlue™ foi realizado com o objetivo de comparar a viabilidade das cUC-MSCs quando cultivadas com CM em duas concentrações diferentes, 1x e 5x, e apenas com o meio de cultura *standard*, em tempos diferentes (24, 72, 120 e 168 horas). Os resultados parecem indicar que o CM poderá ser capaz de aumentar a viabilidade celular, uma vez que forneceu uma viabilidade significativamente maior em medições mais tardias comparativamente ao meio *standard*. Além disso, a concentração de CM não demonstrou ser decisiva na viabilidade celular, permitindo obter valores adequados poupando tempo e CM. São necessários mais estudos para provar a eficácia do CM na substituição do FBS e mesmo como alternativa às terapias celulares. Contudo, os resultados encorajam a utilização deste produto como forma de otimização terapêutica.

PALAVRAS-CHAVE: Meio Condicionado; Células Estaminais Mesenquimatosas do Cordão Umbilical Canino; Secretoma**.**

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LIST OF ABBREVIATIONS

AD	Adipose tissue
AMP	Amphotericin B
BM	Bone marrow
CD	Clusters of Differentiation
CM	Conditioned Media
CR^{2}AL	Centro de Reabilitação e Regeneração Animal de Lisboa
CRAA	Centro de Reabilitação Animal da Arrábida
cSM-MSCs	Canine synovial membrane-derived mesenchymal stem cells
cUC-MSCs	Canine umbilical cord mesenchymal stem cells
DMEM HG	Dulbecco's Modified Eagle Medium High Glucose
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
EDTA	Ethylenediamine tetraacetic acid
FBS	Fetal bovine serum
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBSS	Hank's Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA-DR	Human leukocyte antigen DR
HSCs	Hematopoietic stem cells
IFN-γ	interferon-gama
IL-	Interleukin (IL-2, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18)
IP-10	Interferon gama-induced protein 10
ISCT	International Society for Cellular Therapy
KC-like	Keratinocyte chemotactic-like cytokine
MCP-1	Monocyte chemotactic protein-1 chemokine

MHC-II	Major histocompatibility complex II
MSCs	Mesenchymal stem cells
P3/4/6/7	Passage 3/4/6/7
PBS	Phosphate-buffered saline solution
PCR	Polymerase chain reaction
PS	Penicillin-streptomycin
Scs	Stem cells
TNF-α	Tumor necrosis factor alpha
UC	Umbilical cord
UCB	Umbilical cord blood

1. INTRODUCTION

Regenerative Medicine is a medical field that has been exponentially growing over the last few years, both in Human and Veterinary Medicine. This field focuses on biological regeneration of damaged tissues, cells, or organs, based on the use of cell-therapies (e.g., stem cells), secretome, growth factors and other tissue engineering techniques (Greenwood et al., 2006).

Cell-based therapies currently stand out in Regenerative Medicine research. Stem cells (Scs) are classified according to their source as embryonic, adult (hematopoietic stem cells (HSCs) and non-hematopoietic/mesenchymal stem/stromal cells (MSCs)) or induced pluripotent Scs (Evans & Kaufman, 1981; Takahashi & Yamanaka, 2006), and considering their stage of development and differentiation as totipotent, pluripotent or multipotent cells (Wagers & Weissman, 2004).

MSCs can be defined as adult multipotent cells with mesodermal origin capable to replicate as undifferentiated cells, differentiate into various cell lineages (related to fat, tendon, muscle, cartilage, bone, skin and connective tissue) and self-renew (Barry & Murphy, 2004; Bianco et al., 2008; Dominici et al., 2006; Mendicino et al., 2014; Pittenger et al., 1999). These characteristics, the ease and success of harvesting along with the few ethical restrains and the potential therapeutic application of these stem cells, have given them wide popularity in many clinical and laboratory investigation areas.

Despite this, stem cells' therapeutic potential and interest lies, mainly, in their interaction with the host's immune system. MSCs can modulate or suppress immune system cells' activity through the secretion of cytokines, growth factors and chemokines, with paracrine effects, which together are called secretome. These biological molecules can prevent apoptosis (Tögel et al., 2007) and fibrosis (Suga et al., 2009), can support wound healing, proliferation and differentiation of cells *in situ* and angiogenesis (Blobe et al., 2009), can reduce inflammatory response and, consequently, enhance MSCs' maintenance and the regeneration environment (Kalinski, 2012). Considering that inflammation is commonly present at the lesion site, where stem cells are injected into or attracted to, *in vitro* MSCs preconditioning, using pro-inflammatory cytokines such as interferon-gama (IFN-γ) and tumor necrosis factor alpha (TNF-α) ("priming"), is frequently performed. Priming approaches may increase MSCs' therapeutic potential by allowing them to be pre-activated and predisposed to act in an immunomodulatory way.

As previously mentioned, stem cells' therapeutic effect is nowadays, essentially attributed to their secretome. Studies have been conducted in order to understand if it is crucial or not to apply stem cells in injured tissues, once their secretome can lead to similar

regenerative outcomes (Carvalho et al., 2011; Pereira et al., 2014). MSCs' culture supernatant, rich in specific bioactive factors secreted to the extracellular space (cytokines and other growth factors), is known as Conditioned Media (CM). CM's composition fluctuates according to cells' stimulation, stem cells type and tissue (Vizoso et al., 2017). Using CM, instead of stem cells in regenerative therapies, the risk of applying cells with malignant characteristics as well as the risk of rejection diminishes, cell dosage and storage requirements may be reduced, and the response capacity in emergency/acute situations increased – off-the-shelf secretome therapies – (Volarevic et al., 2018).

The culture medium commonly used to culture and expand MSCs require the supplementation with Fetal Bovine Serum (FBS). The production and use of this serum raises disturbing ethical and animal welfare concerns, as it requires the sacrifice of young animals (Swamynathan et al., 2014). Furthermore, FBS may possibly work as a vector of virus, zoonotic agents and even mycoplasma (Swamynathan et al., 2014). CM is xeno-free, i.e., does not have components from different species that may lead to immune reactions, does not imply young animals' sacrifice to be obtained, and the preference for its use may represent a safer therapeutic alternative.

In Veterinary Medicine, several sources of MSCs have already been identified and recognized in cats, dogs and horses, namely the umbilical cord stroma and blood (Carrade et al., 2011), dental pulp and periodontal ligament (Mensing et al., 2011), bone marrow (Webb et al., 2012), adipose tissue (Webb et al., 2012), synovial membrane and fluid (Prado et al., 2015) and peripheral blood (Sato et al., 2016). Interestingly, MSCs were isolated for the first time from mice bone-marrow (Friedenstein et al., 1966) and the first application of stem cells-based therapies in Veterinary Medicine history was performed in a horse with suspensory ligament desmitis (Herthel DJ, 2002).

Although MSCs can be isolated from virtually any body tissue/organ (da Silva Meirelles et al., 2006), bone marrow (BM-) and adipose tissue (AD-) are the most common sources of MSCs for therapeutic purposes. This is mostly because, when compared to other sources, they enable for a greater number of cells to be obtained/isolated. On the contrary, the harvesting process of bone marrow mesenchymal stem cells (BM-MSCs) is painful, invasive and it has already been described negative correlation between donor aging and cells' therapeutic potential in both BM- and AD-MSCs (Wu et al., 2014).

Considering this, umbilical cord (UC-) represents a great alternative source of MSCs and it is gaining popularity between regenerative therapies. The UC can be collected immediately after a cesarean section through a noninvasive and easy process that does not raise any ethical concerns, unlike collecting bone marrow. This tissue was traditionally abandoned since the UC had no further applications, supporting the fact that its use has no

consequences on the mother or the newborn. Furthermore, the umbilical cord tissue is an excellent reservoir of young stem cells (less mature when compared to other MSCs' sources), with high proliferative, growth and differentiation potential (Kim et al., 2018; Richardson et al., 2016). Umbilical cord mesenchymal stem cells (UC-MSCs) are also known for long-term viability at higher passages and for having low immunogenicity (Kim et al., 2018). As a result, UC-MSCs are well tolerated in allogeneic cell-therapies (use of stem cells obtained from a donor who is different from the recipient, but both from the same specie) (Fong et al., 2012). Another great advantage of UC-MSCs is that they are non-tumorigenic (Gauthaman et al., 2012).

On the other hand, umbilical cord tissue/matrix can be hard to obtain in Veterinary Medicine. Cesarean sections are rarely performed, only in medically justified circumstances, such as dystocia and, nowadays, most pet owners look forward to sterilizing their animals (Sultana et al., 2018). Furthermore, in natural births, the mothers' maternal instinct leads to the ingestion of birth's material, including the umbilical cord and in other species, such as horses, it's not feasible to collect this tissue aseptically. Nonetheless, stem cells' banking options (publics and privates) throughout the world have grown over time (Arutyunyan et al., 2018), making it easier to get canine umbilical cord mesenchymal stem cells (cUC-MSCs) and enabling a quicker response to therapeutic needs.

To date, in Veterinary Medicine, MSCs have been studied and applied mostly in horses and dogs, especially to treat orthopedic disorders (Fortier & Travis, 2011). Nevertheless, encouraging results prompted the pursuit of new studies about MSCs' therapeutic potential application in other diseases (liver, renal, dermal, neuronal, olfactory, respiratory, reproductive, and digestive diseases) (Voga et al., 2020).

The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) established three minimal criteria to standardize human MSCs characterization. Briefly, human MSCs can be defined by their capacity to adhere to plastic surfaces and their fibroblast-like morphology when cultured in flasks; their ability to differentiate into at least three different cell lineages (chondrogenic, osteogenic and adipogenic) when cultured in appropriate differentiation culture conditions; and expression of clusters of differentiation (CD) like CD105, CD73, and CD90 and reduced/non-expression of hematopoietic markers [CD45, CD34, CD14 or CD11b, CD79α or CD19, and major histocompatibility complex- (MHC-) II/human leukocyte antigen- (HLA-) DR] (Dominici et al., 2006). Unfortunately, thus far, no standards were approved to characterize veterinary MSCs and their products (antibodies that are routinely applied are human-specific), making the human guidelines the only ones available. Even though, human MSCs-based therapies are deeply studied, and uniformed factors were established to consider them as effective and

safe (de Girolamo et al., 2013), the diversity of veterinary species, their biological differences and MSCs' heterogeneous phenotypes and behavior *in vitro*, makes it difficult to describe specific patterns for MSCs therapeutic use and their safety. Currently, research focus on finding new critical surface markers, such as CD271 and STRO-1, with the aim of establishing standardized and reliable guidelines for veterinary medicine MSCs' characterization (Álvarez-Viejo et al., 2015; Fitter et al., 2017).

Nonetheless, protocols based on cells' biological behavior in culture and their expansion features, their karyotype, their potential to differentiate into multilineages, immune-cytochemical and -phenotypic traits, and PCR (Polymerase chain reaction) can be performed to biologically characterize MSCs (Alvites et al., 2020; Seo et al., 2009; Zhang et al., 2018; Zucconi et al., 2010).

As aforementioned, cells' morphology and behavior, when cultured, is a minimal criteria to characterize them as MSCs. Also, these cells are expected to maintain their morphology and ability to proliferate even at higher passages. Here, karyotype analysis is especially important to exclude chromosomal abnormalities, that are typically identified in higher passages.

Once MSCs can differentiate into at least three different cell lineages, several osteogenic, adipogenic and chondrogenic differentiation protocols are currently available. Likewise, specific surface markers' expression can be analyzed by flow cytometry and immunocytochemical assays.

Finally, when specific genes that define cells as adipocytes, chondrocytes, or osteocytes, are present, PCR can be used to detect them, validating the true capacity of MSCs to follow an adipogenic, chondrogenic, or osteogenic differentiation through methods' results.

When compared to BM- and AD- stem cells, or even umbilical cord blood (UCB) ones, cUC-MSCs are a prospective therapeutic option in Veterinary Regenerative Medicine that has received minimal research to date. Despite this and as previously mentioned, MSCs' secretome seems to represent the key to their therapeutic action. Considering that CM could be a safer therapeutic option to stem cells, developing studies to understand CM's full potential is gaining particular interest. Since stem cells are exposed to their own biofactors when cultured and after transplantation, it's crucial to understand how the secretome interacts with/affects them. In this respect, the main purpose of this study was to assess a possible xeno-free alternative to replace both the standard culture medium and even the one used in cell-based therapies, in order to optimize treatment procedures and their outcomes.

The laboratory procedures from cUC-MSCs' isolation to CM' production are described in detail too, as one of the major purposes of this internship was to understand the principles of cell culture and therapies, learning more about Regenerative Medicine and its relevance in the currently therapeutic available options. Likewise, as part of the internship's clinical component at Centro de Reabilitação Animal da Arrábida (CRAA) and Centro de Reabilitação e Regeneração Animal de Lisboa (CR^2AL), therapeutic stem-cell dosage preparation protocols for clinical use are also discussed.

2. MATERIALS AND METHODS

2.1. cUC-MSCs isolation

For the purpose of this work, cUC-MSCs were used. Although no umbilical cords were received to isolate cUC-MSCs during the internship period, to perform this study it was crucial to understand how cell isolation from a specific tissue is performed, and the precautions that must be taken. Also, isolating MSCs is time-consuming, and previously isolated and banked cells were employed instead. Nonetheless, all the procedures needed to isolate cells from the umbilical cord were retrospectively debated and are herein briefly detailed. Samples are collected and soaked in transport media, containing Dulbecco's phosphate-buffered saline (DPBS, 14190169, Gibco®) supplemented with 3% penicillin-streptomycin (PS, 15140122, Gibco®), 3% amphotericin B (AMP, 15290026, Gibco®), the umbilical cord is rinsed a few times with a phosphate-buffered saline solution (PBS) to prevent dehydration and clean the tissue. After cleansing, the tissue is cut into small fragments (with 5mm maximum) allowing the selection of segments with more Wharton's jelly and less clots.

Even though, the UC is usually collected in cesarean sections (Sultana et al., 2018), contamination can occur during its collection, manipulation, or transport. As such, before cell-isolation, it's crucial to eliminate all possible contaminants from the tissue, with a decontaminant solution (3% PS, 3% AMP, Hank's Balanced Salt Solution (HBSS) 1:10 and distilled H_2O) in a water-bath (37ºC with agitation).

Subsequently, the tissue is submitted to an enzymatic digestion. Enzymatic digestion detaches cUC-MSCs from extracellular matrix, using DMEM HG medium (Dulbecco's Modified Eagle Medium High Glucose) (D5671, Sigma®), supplemented with 354 U/mL collagenase type I (17100017, Gibco®) and 3 mg/mL dispase II (D4693-1G, Sigma®), 20% fetal bovine serum (FBS, One Shot Fetal Bovine Serum, Gibco®), 1.5% PS and 1.5% AMP. After soaking the tissue in this solution for 60-80 minutes in a water-bath (37ºC with agitation), it is filtered with a cell strainer and then centrifuged at 3000 rpm for

10 minutes. The supernatant is rejected, and the cell-pellet is resuspended in a standard culture medium (MEM α, GlutaMAX™ Supplement, no nucleosides) (αMEM, 32561–029, Gibco®), supplemented with 20% FBS, 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid – HEPES – (15630122, Gibco®), 1.5% AMP and 1.5% PS). Following, the cells are seeded in T-flasks with the same culture medium and incubated in a controlled humidified atmosphere (80%) with 5-6% CO_2 at 37ºC. T-flasks should be observed daily, under an inverted phase contrast microscope (Axiovert 40 CFL, Zeiss), to evaluate cells adherence to the plastic surface, their confluence, and the estimated consumption of the medium. Overall, medium is refreshed every 2-3 days days or whenever an excess of debris and suspended cells is observed.

2.2. Cell culture

2.2.1. Cryopreservation and thawing

The selection of the most suitable cryopreservation protocol revolves around the choice of the best cryoprotective agent. The correct formulation depends on the specie, the MSCs origin, and the freezing technique itself (Fuller, 2004; Thirumala et al., 2005).

Cryoprotectants considerably minimize the formation of ice crystal and decrease the cooling point of the medium, preventing membrane damage and reducing cell-loss (Fuller, 2004; Thirumala et al., 2010). Furthermore, improving the cryopreservation method allows greatest results when reconstituting viable stem cells from frozen umbilical cord matrix.

When considering UC-MSCs, the most used cryoprotectant is dimethyl sulfoxide (DMSO, D2650, Sigma®) (Raffo et al., 2019).

cUC-MSCs are cryopreserved with culture medium and 10% DMSO (representing usually 200µL of the 2ml total criovial volume). Each cryovial usually contains 1-2x10^6 cells/ml. When the vials are correctly filled and labelled (MSCs origin, passage number, quantity, date, and culture medium) they start a gradual cooling cycle (1°C decrease per minute until −80°C) in a freezing container with Isopropyl alcohol (Nalgene® Mr. Frosty®, ThermoScientific®). Cryovials are transferred to liquid nitrogen containers (−195.79 °C) usually after 5 days.

When thawing cryovials containing MSCs, the process must be accomplished rapidly. Cryovials are partially thawed in a 37ºC water-bath and then transferred to the laminar flow hood. Here, the cells are collected from the vials to a falcon tube, which is filled with standard culture medium. The falcon is centrifuged at 1600 rpm for 10 minutes, the supernatant is then discharged, and the cell-pellet is resuspended in culture medium. Hereafter, cells can be counted, cultured, or preserved in accordance with the assay's objectives.

For the purpose of this work, both the cryopreservation and the thawing techniques were performed with cUC-MSCs, and results are further presented.

2.2.2. Trypsinization

MSCs are usually detached from culture vessels (microplates, T-flasks, i.a.) with trypsin or trypsin/Ethylenediamine tetraacetic acid (EDTA) solutions when the intended confluence is achieved (70-80%). At this point, the medium is rejected and the cultured cUC-MSCs are washed 2-3 times with DPBS to eliminate cellular waste products, medium proteins, dead cells and remaining medium. Cells are detached using a 0.25% Trypsin-EDTA solution (25200-072, Gibco®) and incubated for 3 min under standard conditions as described previously. After incubation, detachment is confirmed by observing suspended round cells under an inverted phase contrast microscope. When cells are detached, standard culture medium is added to the culture vessel as to neutralize the trypsin's enzymatic action and to allow rinsing and collection of the suspended cells to a falcon tube. Rinsing needs to be performed carefully and around 3 times, as to collect as many cells as possible. Following, the falcon tube is centrifuged at 1600 rpm for 10 min. The supernatant is rejected as to eliminate the enzyme, and the cell-pellet is resuspended in fresh medium, ideally equaling 1 ml. From this point Trypan Blue Exclusion Assay can be used to count the cells and evaluate their viability.

2.2.3. Trypan Blue Exclusion Assay

Trypan Blue Exclusion Assay is performed to calculate the concentration of viable cells by differentiating these ones from the dead cells (blue-stained). The quantification is performed mixing 10μL of 0.4% Trypan Blue dye solution (T8154-20ML, Sigma-Aldrich®) with 10μL of the MSCs suspension (1:1 dilution) and then filling the Countess™ II FL slide. The slide is read by the Countess II FL Automated Cell Counter (Thermo-Fisher Scientific) at least three times and total cell number and % of viability is assessed. From this moment on, cells can be further passed, cryopreserved, and used in other *in vitro* experiments or to prepare therapeutic dosages. For the purpose of this work, both the trypsinization and the Trypan Blue Exclusion Assay techniques were performed with cUC-MSCs and results are further presented.

2.3. Conditioned Media production and preparation

cUC-MSCs are seeded with standard culture medium in T-175 culture flasks until confluence reaches 70-80%. Then, the medium is discharged, and cells are cleansed 2-3 times with DPBS. When producing CM, DPBS should be completely removed from the cells

and extracellular matrix, so DMEM (basal medium, without any supplementation) is frequently used to wash the cells (also 2-3 times) after the cleansing with DPBS. Following this step, DMEM is rejected, and 13ml of fresh non-supplemented media (no FBS, PS or AMP), DMEM/F12 GlutaMAX™ (10565018, Gibco®) is added to each T-flasks. Cells are incubated (same atmosphere characteristics as described previously) for the desired time and finally the CM (rich in cells self-produced bioactive factors) collected. By using an unsupplemented media, cells are obligated to produce their own bioactive factors, what will culminate in a CM with variable characteristics that can be further analyzed.

To perform this study, 48h of conditioning was considered, and CM collected at this timepoint, centrifuged for 10 minutes at 1600 rpm and then supernatant was collected. Following, CM is filtered in a 0.2 µm Syringe filter (Filtropur S, PES, Sarstedt®). CM was used plain (CM 1x) and after concentration 5 times (CM 5x). As to do so, Pierce™ Protein Concentrator, 3k MWCO, 5–20 mL tubes (88525, Thermo Scientific®) are manually sterilized (the protein concentrators cannot be autoclaved) following the manufacturer's instructions. Briefly, each concentrator is filled with 70% ethanol (v/v) for 30 minutes and centrifuged at 3000 xg for 10 minutes. The ethanol is rejected and replaced by PBS, which is also centrifuged. Each concentration tube is subjected to two rounds of the described centrifugation protocol. Finally, the concentrators are left to dry in the laminar flow hood for 10 minutes and, after that, the superior compartment is filled with CM 1x. The concentrators are centrifuged until the intended concentration of CM is achieved in the top compartment (5x).

2.4. PrestoBlue™ Cell Viability Assay

PrestoBlue™ Cell Viability Assay is based on cells' membrane permeability to a blue resazurin-based solution to determine viability. Viable cells, using mitochondrial enzymes, reduce the phenoxazine dye (resazurin) into resorufin, which is fluorescent red, leading to a color change in the culture medium that can be quantified using ultraviolet-visible spectrophotometry.

This assay was performed to compare cUC-MSCs viability when cultured with CM at two different concentrations, 1x and 5x, and with standard culture medium, at different time points (24, 72, 120 and 168 hours). Thus, this assay included three groups, shown in **Figure 1**: one group (group A) cultured with standard culture medium (αMEM supplemented with 10% FBS, 1% HEPES, 1% AMP and 1% PS), other group (group B) cultured with αMEM supplemented with 10% FBS, 1% HEPES, 1% AMP, 1% PS and 5% CM concentrated 1x (CM 1x), and finally one group (group C) cultured with αMEM

supplemented with 10% FBS, 1% HEPES, 1% AMP, 1% PS and 5% CM concentrated 5x (CM 5x).

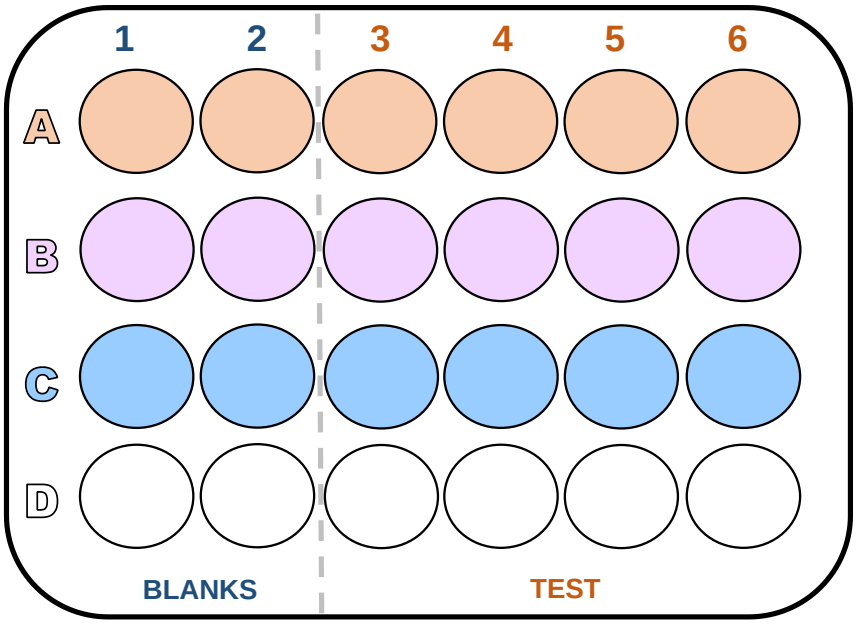


Figure 1. 24 well-plates with the tested groups in PrestoBlue™ Cell Viability Assay: group A – standard culture medium, group B – CM 1x and group C – CM 5x.

Using a 24 well-plate, cUC-MSCs are seeded in quadruplicates at a density of 6.000 cells/cm^2 ("TEST"), and each test group has two blank wells (unseeded – "BLANKS"), as presented in **Figure 1**. After 24h incubation (allowing cell adhesions), and at every time-point (24, 72, 120 and 168 hours) medium is removed from the wells, and fresh testing/standard media is added to the corresponding test/control groups among with 10% (v/v) of 10x Presto Blue™ cell viability reagent (Invitrogen, A13262, Thermo Fisher Scientific, Waltham, MA USA). cUC-MSCs are incubated in a controlled humidified atmosphere (80%) with 5% CO_2 at 37ºC for 1 hour. After the incubation period, 100 µl of the supernatant of each well is transferred to a 96 well-plate and, using a Thermo Scientific Multiskan FC plate reader, the absorbances are read at two wavelengths absorbance (570nm – excitation wavelength and 595 nm – emission wavelength). These readings are normalized and the results from the blank wells are rectified (Caseiro et al., 2019). Following, remaining supernatant is removed from the 24 well plate and DPBS is employed for well rinsing and removal of the dye residues a minimum of two times. Corresponding fresh media is added to each well and the plate is incubated until the following 48 hours for the next reading, until the end of the assay.

2.5. Therapeutic cell dosage

Whenever cells are prepared for therapeutic purposes, they are usually sent inside vials filled with autologous or allogenic canine serum/plasma. Due to stem cells therapeutic potential, cell dosages can be used for rehabilitation in case of musculoskeletal, joint and nerve injuries. cUC-MSCs are great for MSCs-based therapies due to its collection procedure (non-invasive and few ethical controversy), the low immunogenicity and the high rate of proliferation (Arutyunyan et al., 2018). However, other MSCs, such as canine synovial membrane-derived mesenchymal stem cells (cSM-MSCs), could be used in these cases.

A cell dosage was prepared in the Veterinary Cell Therapies Laboratory (ICBAS) and further sent to the CR^2AL to be applied in a clinical case of hip joint osteoarthritis. Information regarding the therapeutic cell dosage and the evolution of this clinical case is described in Annex A.

2.5.1. Canine serum preparation and inactivation

Before cell dosage preparation, it is necessary to collect, prepare and inactivate autologous or allogenic canine serum which will be used as a medium and vector for cUC-MSCs or other cell types, like cSM-MSCs.

Animal's blood is collected to a dry tube and, once in the laboratory, total blood is transferred to a 15ml sterile tube (if needed, more than one). The tube is centrifuged for 10 minutes at 2500-3000 rpm. After centrifugation, the supernatant (serum) is carefully transferred to a new sterile tube and the falcon in heated in a water bath at 56ºC for 30 minutes to inactivate the serum. Following these 30 minutes, the tube is immediately refrigerated on ice. The inactivated and cooled serum is filtrated to a new sterile tube with a 0.22 µm filter and a syringe (the filter may saturate, so more than one filter may be needed). The inactivated serum is ready for use and the container must be identified, sealed with parafilm, and freezed at -20°C for further use.

2.5.2. Cell dosage preparation

Cell vial and the inactivated serum tube require initial heat in a water bath at 37ºC. When only one small ice fragment is observed inside de vial, cells must be quickly collected (with a sterile Pasteur pipette) and transferred to a sterile tube, adding 3ml of inactivated, centrifuged, and sterile serum. DPBS is added to the tube to make 10ml of final volume. The solution is centrifuged for 10 minutes at 1600 rpm, and the supernatant is rejected. The cell-pellet is resuspended in approximately 2 ml of the serum with up-and-down movements

to homogenize the suspension. Cell dosage should be administered immediately, and if it's not possible, they can be refrigerated for a short period of time (up to 24-36 hours).

To administer the therapeutic cell dosage, cells must be aspirated into a needle (approximately 18G)-syringe system, changing the needle before administration.

3. RESULTS

3.1. cUC-MSCs isolation and culture

When cultured and expanded, the cUC-MSCs used in this study presented fibroblastic morphology (**Figure 2**) and capacity to adhere to plastic surfaces as expected.

More detailed characterization of these cells was not carried out since it had already been done (banked cells were used), it's time-consuming, and it was not one of the study's major goals.

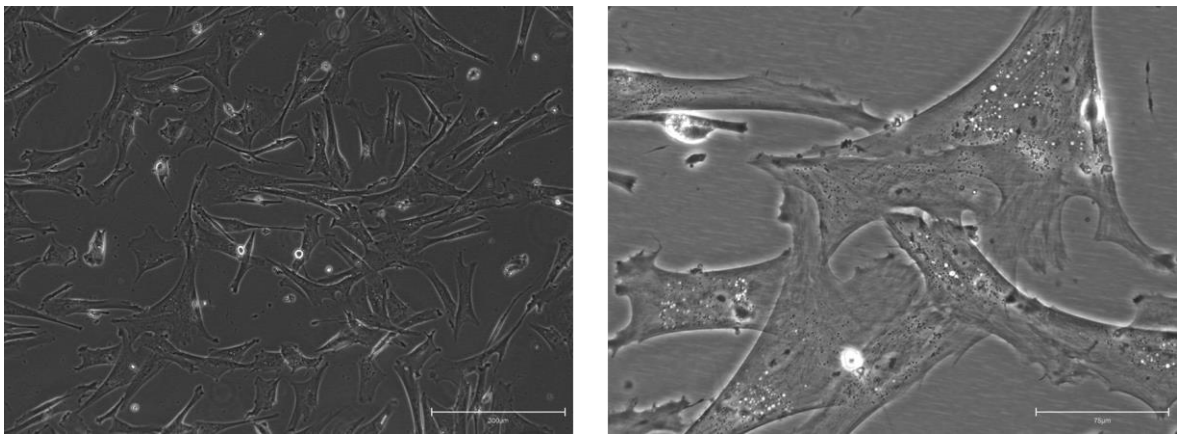


Figure 2. cUC-MSCs with fibroblastic morphology (higher magnification on the right).

3.2. Trypan Blue Exclusion Assay

Two banked cryovials were thawed for this study. The first cryovial to be thawed had been cryopreserved with 1.5 million cUC-MSCs at passage 3 (P3), whereas the second one with 1 million at passage 6 (P6).

For each vial the quantification was performed mixing 10µL of 0.4% Trypan Blue dye solution (T8154-20ML, Sigma-Aldrich®) with 10µL of the MSCs suspension (1:1 dilution) and then filling the Countess™ II FL slide, as previously described. Each slide was read by the Countess II FL Automated Cell Counter (Thermo-Fisher Scientific) three times.

Trypan Blue Exclusion Assay determined a total of 1.43×10^6 viable cells in the first thawed cryovial and 9.6×10^5 viable cells in the second one. In both cryovials, the average cell viability was nearly 98%. These results are shown in **Table 1**.

Table 1. Trypan Blue Exclusion Assay results. One slide was prepared and read three times (first read, second read and third read) for each cryovial using the Countess II FL Automated Cell Counter (Thermo-Fisher Scientific), then the average of the three readings was calculated (average of readings).

	First cryovial	**Second cryovial**
Concentration of cryopreserved cells	$1.5x10^6$	$1x10^6$
Passage	P3	P6
First read	Total concentration: $1.45x10^6$/mL Live: 96% ($1.39x10^6$/mL)	Total concentration: $0.98x10^6$/mL Live: 97% ($0.95x10^6$/mL)
Second read	Total concentration: $1.48x10^6$/mL Live: 98% ($1.45x10^6$/mL)	Total concentration: $0.99x10^6$/mL Live: 99% ($0.98x10^6$/mL)
Third read	Total concentration: $1.47x10^6$/mL Live: 99% ($1.46x10^6$/mL)	Total concentration: $0.95x10^6$/mL Live: 99% ($0.94x10^6$/mL)
Average of readings	Viability average: **98%** **$1.43x10^6$/mL**	Viability average: **98%** **$0.96x10^6$/mL**

3.3. Conditioned Media production and preparation

Two sample of the produced CM were collected to characterize its bioactive factors profile (growth factors, cytokines and chemokines). One sample was retrieved from the CM produced by the first cryovial' cells (Group A) and the other from the second one (Group B). Note that, at this moment, cells were already passed so the following results consider this passage.

Bioactive factors detection and quantification was performed as described by Caseiro et al. (2019). The canine CM panel assessed: includes granulocyte-macrophage colony-stimulating factor (GM-CSF) – a proliferative and anti-apoptotic biofactor –, IFN-γ, several interleukins (IL-2, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18), interferon gama-induced protein 10 (IP-10), keratinocyte chemotactic-like cytokine (KC-like), monocyte chemotactic protein-1 chemokine (MCP-1) and TNFα. The analysis was performed twice for each biomarker and results are presented in **Figure 3**.

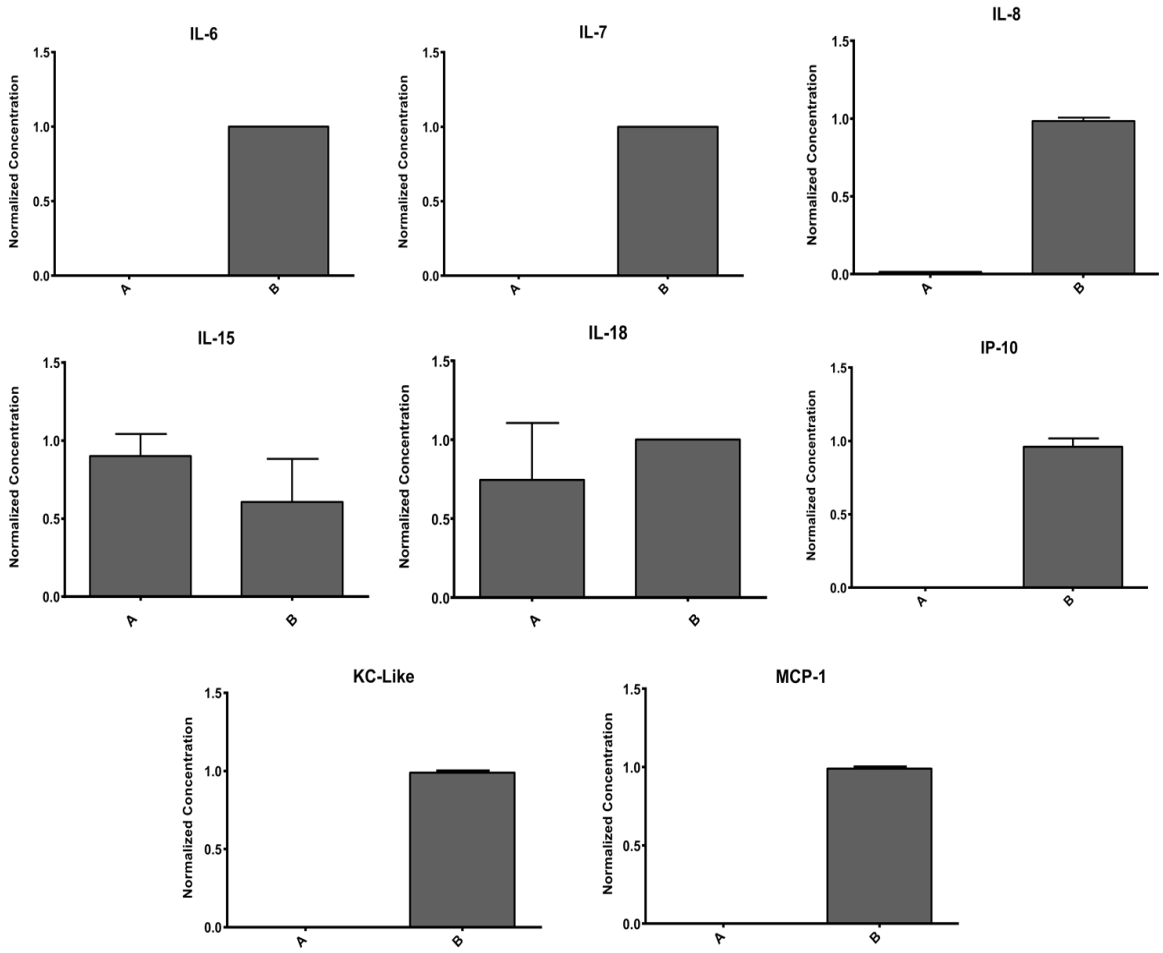


Figure 3. Conditioned Media bioactive factors detection and quantification. Only the detected factors are shown, and data was normalized (mean ± SEM).
A – cUC-MSCs CM obtained from the first cryovial (cells now at P4), B – cUC-MSCs CM obtained from the second cryovial (cells now at P7).

CM characterization revealed the absence of some searched bioactive factors (GM-CSF, IFN-γ, TNFα, IL-2 and IL-10) and others (IL-6, IL-7, IL-8, IP-10, KC-like and MCP-1) were only detected in the CM obtained from cells at a higher passage (group B in **Figure 3**). Interestingly, only two bioactive factors were detected and measured in both CM groups (IL-15 and IL-18), and the results don't appear to follow any pattern. **Table B1** from Annex B shows the mean concentration of each biomarker in the CM under analysis, normalized between 0 and 1 (mean ± SEM) and the absolute value of each measure is shown in **Table B2**.

3.4. PrestoBlue™ Cell Viability Assay

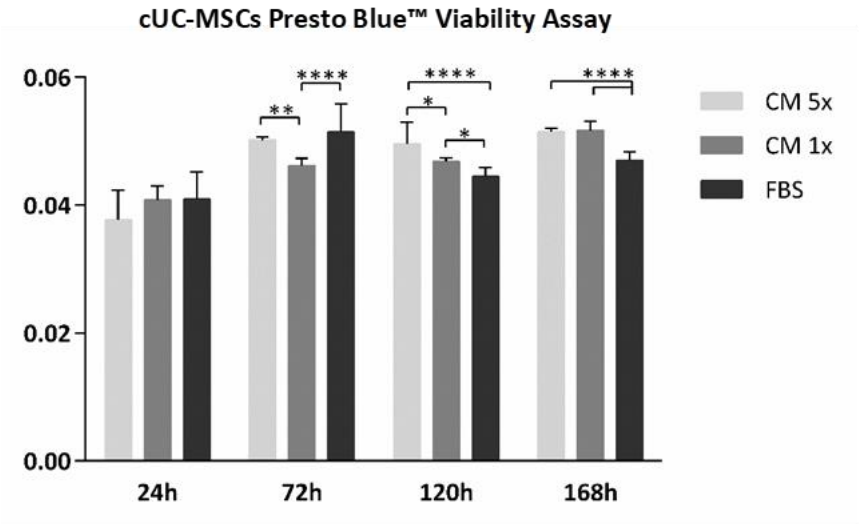


Figure 4. cUC-MSCs (at P4) Presto Blue™ Viability Assay at all the time points (24, 72, 120 and 168h). The results are presented with the * symbol and indicate $0.01<p\leq0.05$, $0.001<p\leq0.01$ and $p\leq0.0001$ with *, ** and **** respectively.

Figure 4 shows cUC-MSCs (P4) viability, assessed by PrestoBlue™ assay, at each time point (24, 72, 120 and 168 hours). At 24h the viability differences between groups were not statistically significant. At 72h cell viability was higher in the control group (FBS medium), followed by CM 5x and CM 1x, with statistically significant differences, as shown in the figure. Cell viability decreased in the control group at 120h while CM 5x presented the greatest viability results. In the images presented in **Figure 5** it is possible to observe cUC-MSCs appearance in each culture medium.

Finally, the last time point (168h) revealed that cell viability was similar between the CM 5x and CM 1x groups, with no statistically significant differences as shown in the figure. Unlike the control group, that had the lower viability results at this time point proven by the significant statistical differences represented in the figure.

Table C1 from Annex C shows the statistical analysis of each group results (FBS, CM1x and CM5x) at each time point (24, 72, 120 and 168 hours).

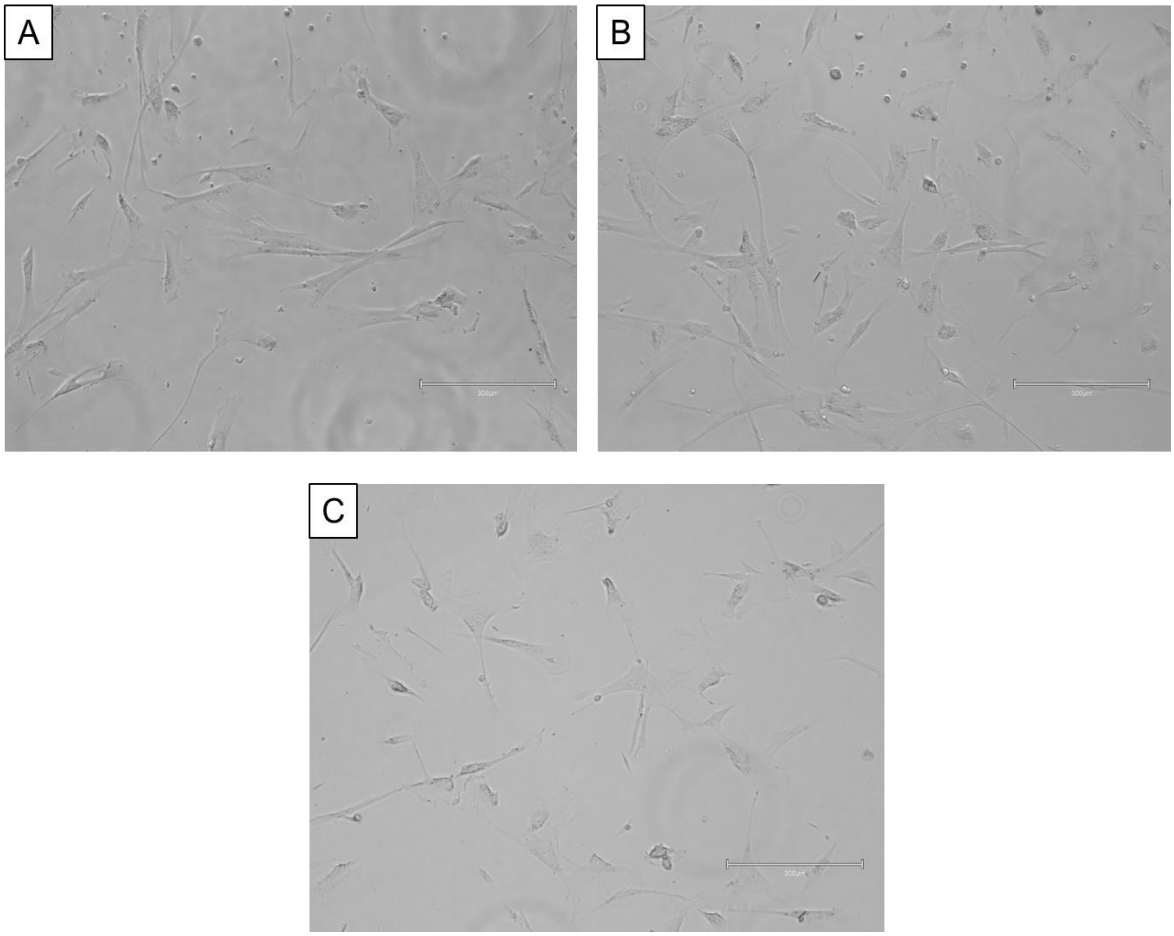


Figure 5. cUC-MSCs (P4) at 120h during Presto Blue™ Viability Assay.
A – Cell culture with CM 1x, B – Cell cultured with CM 5x, C – Cell culture only with standard culture medium.

4. DISCUSSION AND CONCLUSION

Stem cells have revolutionized regenerative medicine' therapeutic procedures. MSCs, in particular, have been applied in several clinical trials (on animals with natural disease) and preclinical trials (using animal models) to treat a wide variety of illnesses [such as skin wounds (Harman, 2013), spinal cord injuries (Lim et al., 2007), diabetes (Zhu et al., 2011), peripheral nerve injuries (Tomita et al., 2013), tendinites (Del Bue et al., 2008), etc.] with promising outcomes. This provides new therapeutic alternatives for both human and veterinary medicine, focusing in the “One Health” concept, especially when choosing the most suitable animal model for each study to obtain conclusions capable of being translated to human medicine (Lopes et al., 2022).

The lack of uniformed factors to prove MSCs effectiveness and safety, despite being widely studied, remains a major concern regarding the use of these cells for therapeutic

purposes. The major risks of MSCs-based therapies are pro-inflammation, fibrosis (once these cells can differentiate into myofibroblasts) and tumorigenicity. MSCs can produce excessive quantities of chemokines, growth factors and cytokines, which interact with cancer cells' surface receptors and enhance tumor growth. Considering this, cUC-MSCs were chosen to perform this study especially for having low immunogenicity (Kim et al., 2018) and the lower carcinogenic potential (Gauthaman et al., 2012).

Banked cells were thawed, cultured and, when calculating the concentration of viable cells using Trypan Blue Exclusion Assay, viability was approximately 98%. These findings lead the group to assume that the cryopreservation and thawing protocols are effective and do not significantly affect cells' viability, which is particularly relevant for cell-based therapies, since it is possible to bank cells for long periods of time and easily thaw cryovials whenever stem-cells or their products are necessary. Furthermore, only high viability results (>95%), even after cryopreservation and thawing, encourage the use of these cells *in vivo*, for regenerative purposes, as it is more likely to achieve therapeutic success. Viable cells are less prone to produce unfavorable treatment reactions (such as tumor growth) and have higher secretory activity, encouraging CM production.

Using CM, instead of stem cells, in regenerative therapies, the risk of applying cells with malignant characteristics, the risk of rejection, cell dosage and storage requirements can be reduced (Volarevic et al., 2018). Also, CM contains great concentration of biofactors with potential pro-regenerative effect, is xeno-free and does not raise concerns related to allogeneic transplants (no biological tissues from genetically different organisms are transplanted). Therefore, PrestoBlue™ was performed to compare cUC-MSCs viability and their behavior when cultured with CM, at different concentrations, and with standard culture medium, at different time points (24, 72, 120 and 168 hours).

As expected, at 24h no statistically significant differences between groups were observed as cells were adjusting to the mediums and adhering to the plastic surfaces. At 72h cell viability increased in all groups. However, cell viability in the control group (only FBS medium) was higher than the others. At this time point CM 5x group' viability was significantly higher than the CM 1x group since it had a higher concentration of biofactors favorable to cell growth, especially at this early stage. Over time, cell viability tends to decrease since these cells are finite. However, at 120h the control group suffered a significant decrease in cells' viability, while the study groups substantially maintained their results. This support the idea that CM might be able to increase cell viability since it provided a statistically significant higher viability at later time points, when compared to the standard medium. Considering this, CM can be a safer and better option to FBS.

By comparing the results of the two CM groups, it is reasonable to deduce that CM concentration was not a decisive factor in cells' viability. Therefore, cUC-MSCs with great viability can be obtained using less concentrated CM (plain), saving both time (manual concentration protocols, such as Pierce™ Protein Concentrator, are time-consuming) and CM.

For clinical purposes, it is possible to produce viable higher quantities of CM using bioreactors, reducing both production time and the number of used cells. Since CM can be produced faster and on a larger scale, treatments can be optimized by initiating them as soon as possible. Also, CM production can be optimized by priming MSCs with cytokines (such as IFN-γ and TNF-α) or other molecules that may improve cells' proliferation, differentiation and therapeutic efficiency (Noronha et al., 2019).

Furthermore, the CM is completely customizable, meaning that it is possible to obtain CM enriched in certain biofactors considering its intended use. In laboratory, CM of various origins (choosing cells closest to the ones where CM will be applied), with or without priming (choosing priming approaches), can be produced, and extensively characterized. It's also feasible to use lesion extraction factors to induce stem-cells to produce CM directed to the intended lesion or tissue. This allows the selection of the most appropriate CM, by clinical and laboratory teams together, to treat each clinical case.

CM analysis revealed the absence of some biomarkers (GM-CSF, IFN-γ, TNFα, IL-2 and IL-10) and others (IL-6, IL-7, IL-8, IP-10, KC-like and MCP-1) were only detected in the CM obtained from cells at a higher passage. It's difficult to explain these results with certainty, especially because there are no guidelines for each biomarker produced from these cells with predicted values. Considering that the detected and quantified biomarkers were mainly present only in the CM produced by older/mature cells, the results could suggest that cells at higher passages are more likely to respond positively to conditioning, producing biofactors that are not typically present in lower passages. However, more research is required to establish if there is a pattern.

Nonetheless, the possibility of metabolomic characterization and quantification of CM biofactors content encourages its use as a versatile tool that can be adjusted based on the therapeutic purpose. It's feasible to modify cells' secretome and hence create CM rich in the intended biofactors using specific priming approaches, as previously mentioned.

When choosing the best therapeutic strategy there are also some disadvantages associated to CM' use, such as the absence of panels and uniform values for each biofactor and for each species (Caseiro et al., 2016). CM biofactors content varies significantly depending on cells' type and passage number, species and even the used laboratory

protocol (Caseiro et al., 2016). All these variables make it challenging to standardize the CM produced for therapeutic purposes.

Unfortunately, this curricular internship period did not allow for a more in-depth and thorough investigation of the findings presented in this work. More studies are needed to determine the potential effectiveness of CM in replacing FBS-supplemented medium and even as an alternative to cell-based therapies. Since CM has been shown to positively influence long-term viability of cUC-MSCs in this research, the next step would be to investigate this cells' behavior and viability *in vitro* using only CM (without FBS). Then, although being safer, it would be possible to determine whether the CM is a viable alternative to the standard medium.

Additionally, the suggested therapeutic cell dosage preparation protocols could be adjusted to produce cell dosages using cells with CM and dosages using exclusively CM (with no biological material). Using these two types of dosages and one with cUC-MSCs cultured with standard medium (with FBS) and another one supplemented with CM, in preclinical and clinical trials, it would be possible to ascertain *in vivo* cells' viability, the efficiency of each therapeutic option and the potential short, medium and long-term effects on the patient.

Nonetheless, these findings support the use of CM as a xeno-free therapeutic optimization tool with no associated ethical concerns.

Regenerative Medicine is a rapidly evolving field with an increasing number of possible and safe therapeutic alternatives. More research is still required to better improve and optimize the use cUC-MSCs and their products, particularly CM. Stem cells and their bioproducts are undeniably promising therapeutic options.

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ANNEX A – Therapeutic cell dosage application in a real clinical case

A therapeutic cell dosage of cSM-MSCs was prepared in the Veterinary Cell Therapies Laboratory (ICBAS) and further sent to the CR^2AL to be applied in a clinical case. This cell dosage contained $4x10^6$ cells at P3 with 98% viability (Trypan Blue Exclusion Assay).

This clinical case involves a 9-year-old male dog with hip joints osteoarthritis, worse on the left hip joint, with lameness and pain when palpating the joints and extending the limbs. In 2020, this animal began a rehabilitation protocol at CR^2AL and received an intraarticular therapeutic cell dosage on the left hip joint on November 5th of the same year. On January 27th of 2022 another therapeutic cSM-MSCs dosage was administered. **Figure A1** demonstrates the therapeutic cell dosage application in this case.

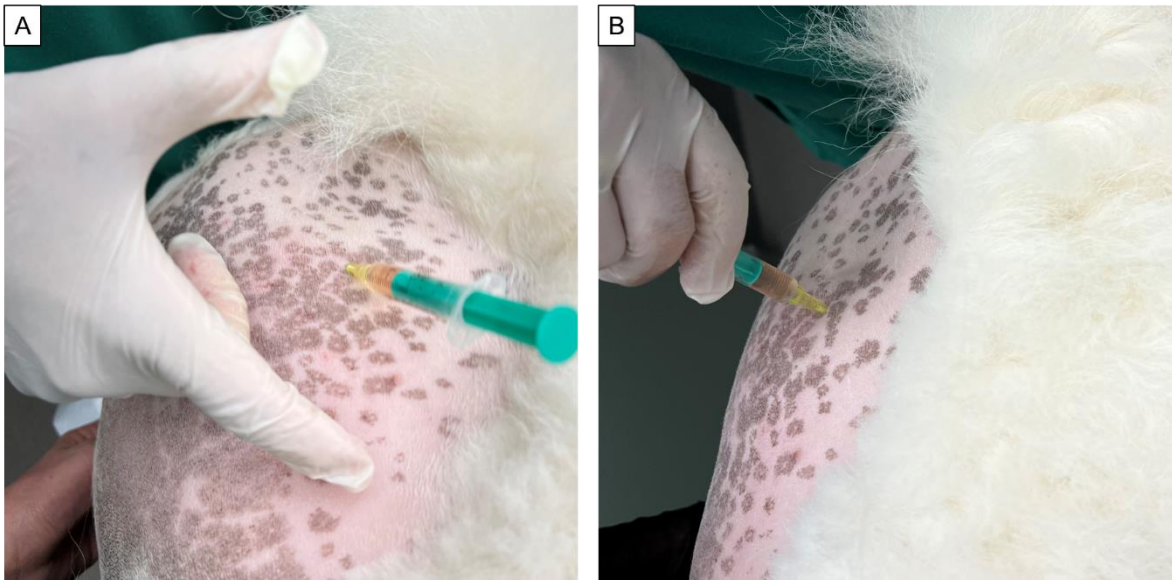


Figure A1. Administration of cSM-MSCs in a dogs' left hip joint with osteoarthritis.
A – left hip joint identification and syringe positioning, B – cells' administration.

Two weeks after stem cells therapy, the animal still presented slight pain when extending the pelvic limbs. One month after the therapeutic cell dosage administration, the dog no longer demonstrated lameness or pain when manipulating the affected joints.

Although no cell dosages were prepared using cUC-MSCs during this intership period, the preparation and application techniques previously described are the same for both stem cell types.

Regenerative medicine and its therapeutic modalities have been showing promising results in both clinical and pre-clinical trials and in real clinical cases. The evolution of this

clinical case demonstrates stem-cells therapies' effectiveness and potential in the prognosis of certain illnesses, such as osteoarthritis.

Furthermore, combining stem-cells therapies with rehabilitation modalities that are tailored to each clinical case has shown to improve the chances of a successful treatment and to accelerate the recovery process.

ANNEX B – cUC-MSCs Conditioned Media Analysis – Secretome

Table B1. Mean normalized concentration of each biomarker in each CM group of cUC-MSCs (mean ± SEM).

Biofactor	Mean normalized concentration – **Group A** **cUC-MSCs CM – P4**	Mean normalized concentration – **Group B** **cUC-MSCs CM – P7**
IL-6	0	1.00 ± 0.00
IL-7	0	1.00 ± 0.00
IL-8	0	0.99 ± 0.01
IL-15	0.90 ± 0.10	0.61 ± 0.20
IL-18	0.74 ± 0.26	0.50 ± 0.50
IP-10	0	0.96 ± 0.04
KC-like	0	0.99 ± 0.01
MCP-1	0	0.99 ± 0.01

Table B2. Absolut concentration of each biofactor in each CM group of cUC-MSCs (pg/ml).

Biofactor	Group A cUC-MSCs CM – P4		Group B cUC-MSCs CM – P7	
	Observed Concentration (pg/mL) Replicate 1	Observed Concentration (pg/mL) Replicate 2	Observed Concentration (pg/mL) Replicate 1	Observed Concentration (pg/mL) Replicate 2
IL-6	0,00	0,00	69,93	69,93
IL-7	0,00	0,00	10,01	10,01
IL-8	0,00	0,00	392,92	381,63
IL-15	15,25	18,26	15,25	9,23
IL-18	5,35	3,49	5,35	1,71
IP-10	0,00	0,00	10,72	11,65
KC-like	0,00	0,00	151,33	147,60
MCP-1	0,00	0,00	2726,31	2775,79

ANNEX C – PrestoBlue™ Cell Viability Assay - Statistical analysis

Table C1. cUC-MSCs Presto Blue™ Viability Assay statistical analysis (mean ± SEM) of each group results (FBS, CM1x and CM5x) at each time point (24, 72, 120 and 168 hours).

	FBS	**CM1x**	**CM5x**
24h (Mean ± SEM)	0,041 ± 0,00123	0,041 ± 0,00065	0,038 ± 0,00136
72h (Mean ± SEM)	0,051 ± 0,00128	0,046 ± 0,00033	0,050 ± 0,00014
120h (Mean ± SEM)	0,045 ± 0,00041	0,047 ± 0,00017	0,050 ± 0,00102
168h (Mean ± SEM)	0,047 ± 0,00038	0,052 ± 0,00042	0,052 ± 0,00015