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Allogeneic mesenchymal stem cell-based ready-to-use product for canine osteoarthritis

MARTA MONTOLÍO TORRELL

Allogeneic mesenchymal stem cell-based ready-to-use product for canine osteoarthritis

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MARTA MONTOLÍO TORRELL

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BONE REGENERATION RESEARCH GROUP

DEPARTMENT OF ANIMAL MEDICINE AND SURGERY

Director: JORDI FRANCH SERRACANTA, DVM Ph.D.

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CERTIFICAT de conformitat

Jordi Franch Serracanta, professor titular del departament de Medicina i cirurgia Animal de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, per la present

Certifico que:

El treball de recerca "Allogeneic mesenchymal stem cell-based ready-to-use product for canine osteoarthritis" realitzat per la graduada en Veterinària Marta Montolío Torrell I dirigida per mi, s'ha desenvolupat seguint rigorosament tots els requisits científics i formals necessaris per a poder procedir a la seva defensa com a Tesis Doctoral.

I per a que consti a efectes oportuns, signo el present certificat a Bellaterra a 6 d' octubre de 2025.

SIGNATURA

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List of acronyms

BMP – Bone Morphogenic Protein

$CaCl_2$ – Calcium Chloride

CFU – Colony Forming Unit

CRP – C-Reactive Protein

DAPI - 4′,6-diamidino-2-phenylindole

DJD – Degenerative Joint Disease

ELISA – Enzyme-linked Immunosorbent Assay

EMA – European Medicines Agency

FBS – Foetal Bovine Serum

GMP – Good Manufacturing Practices

IgG - Immunoglobulin G

MHCII – Membrane Histocompatibility Complex II

MSC – Mesenchymal Stem Cells

cMSC – Canine Mesenchymal Stem Cells

Ad-MSC – Adipose-derived Mesenchymal Stromal/Stem Cells

OA – Osteoarthritis

PBS – Phosphate Buffered Saline

PC – Platelet Concentrates

PDGF-BB – Platelet Derived Growth Factor BB

PL – Platelet Lysate

PRP – Platelet-Rich Plasma

RL – Ringer's Lactate

ROM – Range of Movement/Motion

SD – Standard Deviation

β-TGF – Transforming Growth Factor beta

VEGF-A – Vascular Endothelial Growth Factor A

Thesis abstract

This doctoral thesis comprises a series of studies aimed at developing a product based on cell therapy using allogeneic canine mesenchymal stem cells (MSCs) for the treatment of canine osteoarthritis. This therapeutic approach represents a promising strategy for managing degenerative joint diseases such as osteoarthritis. However, one of the main challenges for the clinical application of allogeneic MSCs in veterinary medicine is the effective preservation of cell viability and functionality during the interval between cell processing and intra-articular administration.

The primary objective of this research is to develop and validate a preservation medium capable of maintaining the viability, phenotype, and biofunctional capacity of MSCs derived from canine adipose tissue during short-term storage (24–72 hours) under two temperature conditions: refrigeration and room temperature. In addition to ensuring cellular stability, the medium is expected to provide bioactive properties that benefit the joint environment—such as anti-inflammatory or lubricating effects—that could enhance cellular integration and post-injection therapeutic efficacy.

The working hypothesis is that a medium enriched with Platelet Lysate (PL) and/or Hyaluronic Acid (HA) can maintain cell viability above 80% during the first 24–48 hours. Moreover, these media contain bioactive molecules that may support MSC functionality, contribute to joint homeostasis, and increase their therapeutic potential.

The study is structured in three phases: (1) optimisation of growth factor-rich platelet lysate for use as a medium component; (2) selection of transport media with various supplemented formulations (Lactated Ringer's (RL), RL with HA, RL with PL, and RL with both HA and PL), followed by *in vitro* evaluation of viability, morphology, and immunophenotypic profile of the preserved MSCs; (3) a pilot preclinical phase in a canine model to assess the clinical safety and initial therapeutic potential of the product.

The results of this research suggest that supplemented media such as RL + PL or RL + HA are suitable for MSC preservation for up to 24 hours at room temperature and up to 48 hours under refrigeration. This contributes to the establishment of a standardised protocol for the transport and intra-articular use of MSCs in veterinary practice, supporting their safety in clinical applications and laying the groundwork for future clinical or commercial development.

Resum de la tesi

Aquesta tesi doctoral comprèn una sèrie d'estudis orientats al desenvolupament d'un producte basat en teràpia cel·lular amb cèl·lules mare mesenquimals canines (MSCs) al·logèniques per al tractament de l'artrosi canina. Aquest enfocament terapèutic representa una estratègia prometedora per al maneig de malalties articulars degeneratives com l'artrosi. Tanmateix, un dels principals reptes per a l'aplicació clínica de les MSCs al·logèniques en medicina veterinària és la preservació eficaç de la viabilitat i funcionalitat cel·lular durant l'interval entre el processament cel·lular i l'administració intraarticular.

L'objectiu principal d'aquesta recerca és desenvolupar i validar un medi de conservació capaç de mantenir la viabilitat, el fenotip i la capacitat biofuncional de les MSCs derivades de teixit adipós caní durant l'emmagatzematge a curt termini (24–72 hores), en dues condicions de temperatura: refrigeració i temperatura ambient. A més de garantir l'estabilitat cel·lular, es pretén que aquest medi presenti propietats bioactives beneficioses per a l'entorn articular —com ara efectes antiinflamatoris o lubricants— que afavoreixin la integració cel·lular i l'eficàcia terapèutica posterior a la infiltració.

La hipòtesi de treball és que un medi enriquit amb Lisat Plaquetari (PL) i/o Àcid Hialurònic (HA) pot mantenir una viabilitat cel·lular superior al 80 % durant les primeres 24–48 hores. A més, aquests medis contenen molècules bioactives que poden afavorir la funcionalitat cel·lular, contribuir a la homeòstasi articular i augmentar el seu potencial terapèutic.

El treball es divideix en tres fases: (1) optimització del lisat plaquetari ric en factors de creixement per a l'ús com a component del medi; (2) selecció de medis de transport amb diferents formulacions suplementades (Ringer Lactat (RL), RL amb HA, RL amb PL i RL amb HA + PL), i avaluació in vitro de la viabilitat, morfologia i perfil immunofenotípic de les MSCs conservades; (3) una fase preclínica pilot en un model caní per validar la innocuïtat del producte i el seu potencial terapèutic inicial.

Els resultats d'aquesta recerca suggereixen que els medis testats, com el RL suplementat, són adequats per a la conservació de MSCs durant 24 hores a temperatura ambient i fins a 48 hores en refrigeració. Això contribueix a establir un protocol estandarditzat per al transport i ús intraarticular de MSCs en la pràctica veterinària, facilitant-ne l'aplicació clínica segura i eficaç, i posant les bases per a un possible desenvolupament clínic o comercial futur.

Resumen de la tesis

Esta tesis es un trabajo que incluye diversos estudios orientados a desarrollar un producto basado en terapia celular con células madre mesenquimales caninas para el tratamiento de la osteoartritis canina. Esta terapia representa una estrategia prometedora en el tratamiento de enfermedades articulares degenerativas como la osteoartritis. Sin embargo, uno de los principales desafíos para su aplicación clínica en medicina veterinaria es la preservación eficaz de la viabilidad y funcionalidad celular durante el intervalo comprendido entre su procesamiento y la administración intraarticular.

El objetivo principal de esta tesis doctoral es el desarrollo y validación de un medio de conservación que mantenga la viabilidad, el fenotipo y la capacidad biofuncional de células madre mesenquimales (MSCs) derivadas de tejido adiposo canino durante su almacenamiento a corto plazo (24–72 h) en dos rangos de temperatura: refrigeración y temperatura ambiente. Además de garantizar la estabilidad celular, se busca que dicho medio posea propiedades bioactivas beneficiosas para el entorno articular, como efectos antiinflamatorios o lubricantes, que favorezcan la integración y eficacia terapéutica tras la infiltración.

La hipótesis de este estudio es que un medio enriquecido con lisado plaquetario y/o ácido hialurónico puede mantener una viabilidad celular superior al 80 % durante las primeras 24–48 horas. Asimismo, estos medios contienen componentes bioactivos que podrían favorecer la funcionalidad celular, contribuyendo a mantener la homeostasis articular y aumentando su potencial terapéutico.

El trabajo se estructura en tres fases: (1) optimización del lisado plaquetario rico en factores de crecimiento para su uso como parte del medio; (2) selección de medios de transporte con distintas formulaciones suplementadas (Ringer Lactato (RL), RL suplementado con ácido hialurónico (HA), RL con lisado plaquetario (PL) y RL con HA + PL), y evaluación in vitro de la viabilidad, morfología y perfil inmunofenotípico de las MSCs conservadas; (3) realización de una fase preclínica piloto en modelo canino para validar la inocuidad del producto y su potencial terapéutico inicial.

Los resultados de esta investigación sugieren que los medios testados, como el RL suplementado, son adecuados para la conservación celular durante 24 horas a temperatura ambiente y hasta 48 horas bajo refrigeración. Esto contribuye a establecer un protocolo estandarizado para el transporte y uso intraarticular de MSCs en la práctica veterinaria, facilitando su aplicación segura y eficaz en tratamientos celulares, y sentando las bases para su posible escalado clínico o comercial.

Foreword and Justification

Medicine is found in the intrinsic nature of the organisms.

With the evolution of organisms and the increasing complexity of their biological systems, part of the regenerative and reparative potential observed in certain species has been necessarily sacrificed. Lizards are able to regenerate their tails, and octopuses their tentacles; however, this capacity diminishes in more evolutionarily advanced species such as mammals. Although mammals have largely lost their regenerative ability, it has not disappeared entirely but remains limited. For instance, when a nail is broken, it is capable of regrowth; following an injury, fibroblasts act to repair tissue; and certain organs, such as the liver, retain the ability to regenerate. By building upon this inherent cellular capacity, it becomes possible to enhance and accelerate the intrinsic regenerative potential that persists within all living organisms.

Cellular and tissue ageing, which accompanies advancing age, has become increasingly evident as the life expectancy of many species continues to rise. Ageing is associated with cellular senescence, a process that affects older cells and their microenvironment, thereby predisposing tissues to inflammatory conditions, disease, and a diminished capacity for cellular and tissue regeneration.

Current advances in technology and biomedical research now enable the investigation and application of regenerative medicine therapies based on cellular approaches that may slow or mitigate the progression of cellular senescence, while simultaneously promoting tissue regeneration and establishing an anti-inflammatory microenvironment.

Osteoarthritis represents the most prevalent degenerative disease in dogs, its incidence increasing as a consequence of the ageing canine population. At present, no curative treatment exists, and a range of therapeutic interventions of limited efficacy are applied in an effort to improve patients' quality of life. Although treatments involving stem cells and other biological products are occasionally administered intra-articularly to alleviate clinical symptoms, there is currently no standardised product that combines allogeneic stem cells with an additional active compound capable of potentiating their anti-inflammatory effects. This context provides the rationale for the present study.

This thesis is organised into a series of chapters, each addressing a distinct phase in the development of an allogeneic cell therapy product for the intra-articular treatment of canine osteoarthritis. The first chapter establishes the scientific and conceptual framework underpinning the study. The second chapter, through laboratory experimentation, seeks to standardise a platelet lysate rich in growth factors for its

incorporation into the final product formulation. The third chapter investigates the properties of canine mesenchymal stem cells, ensuring their safety and suitability for use in an allogeneic therapeutic context. The fourth chapter examines the variables and environmental conditions influencing in vivo cell viability. Subsequently, the fifth chapter presents a pilot study designed to evaluate the clinical safety and potential efficacy of the product as a therapeutic approach for canine osteoarthritis. Finally, the sixth chapter synthesises the conclusions and final reflections derived from the research.

Chapter 1

Background and significance

1.1. Canine osteoarthritis and its pathophysiology

Osteoarthritis (OA), also known as Degenerative Joint Disease (DJD), is one of the most common degenerative conditions affecting pets, likely due to the increasing lifespan of the canine population (Barbeau-Gregoire *et al*., 2022). A 2017 study in the UK found that 2.5% of dogs develop OA annually, with an estimated 11.4% expected to develop OA during their lifetime (Anderson *et al*., 2018). Additionally, research by Roitner *et al.* indicated that the OA prevalence in dogs older than eight years—based on radiographic evaluations—ranged from 35.9% to 57.4% depending on the joint affected (Roitner *et al*., 2024). Despite these findings, OA in dogs remains undiagnosed and undertreated, especially in younger canines. A 2024 study revealed that 39.8% of young dogs exhibit radiographic signs of OA, with 16.3% to 23.5% also presenting clinical symptoms in addition to radiographic evidence (Enomoto *et al.*, 2024). These statistics highlight the need for early diagnosis and intervention to manage OA effectively and improve long-term joint health in dogs.

The reason more dogs suffer from the disease than are reported, may be that OA is a chronic and progressive condition. Many dogs do not exhibit obvious signs of pain, masking discomfort. Instead, they may show reduced activity, longer rest periods, or protective behaviours—symptoms that are not always recognised as indicators of pain by their owners— (Belshaw *et al.*, 2020).

Moreover, diagnosis of OA in dogs is still primarily based on digital radiography, which, according to some studies, may not be the most accurate diagnostic tool.

Radiographic changes often appear late in the disease progression, meaning that early cartilage damage, synovial inflammation or subtle degenerative changes may go undetected. Advanced imaging techniques such as magnetic resonance imagining (MRI) or computed tomography (CT), combined with physical exploration, can provide more detailed insights into joint health. However, accessibility and cost limit their routine use (de Bakker *et al.,* 2021; Chung *et al*., 2023).

OA is a painful condition that limits dogs' daily movement, significantly impacting their welfare (Pye *et al*., 2022). As quality of life has become an important consideration for both families and veterinarians, research into new tools and treatments for canine osteoarthritis has intensified (Belshaw & Yeates, 2018; Belshaw *et al*., 2020).

This pathology is characterised by the progressive breakdown of cartilage integrity, resulting from an imbalance between cartilage regeneration and degeneration. This deterioration leads to chronic joint inflammation and pain, significantly affecting mobility and quality of life. The disease is also associated with cartilage erosion, synovial inflammation, and periarticular osteophyte formation, where new bone develops around the damaged joint as a cellular repair response (Yunus *et al*., 2020).

This inflammation and destruction of healthy cartilage are mediated by biomolecules such as C reactive protein (CRP), metalloproteases, inflammatory cells including lymphocytes, cytokines, nitric oxide, and proteolytic enzymes (Yunus *et al*., 2020; Li *et al*., 2017).

While OA can occur as a primary disease, evidence suggests it is most often secondary to dysfunctional joint stress or previous joint injuries. Nonetheless, it is widely accepted that OA has multifactorial causes (Yunus *et al*., 2020). For this reason, it is more commonly found in obese, neutered, and senior dogs, as well

as in certain breeds (Anderson *et al.*, 2018; Vaughan-Scott and Taylor, 1997; Roitner *et al.*, 2024). In human medicine, OA has also been linked to gastrointestinal imbalances caused by alterations in gut microbiota, which may contribute to chronic systemic inflammation (Biver *et al.*, 2019).

As a degenerative disease, OA progresses slowly, gradually worsening over time. Pathophysiology of OA has been extensively studied across various species and conditions (Yunus *et al.*, 2020). Mesenchymal Stem/Stromal Cells (MSCs) are present in a wide variety of tissues, and studies have reported the presence of synovial MSCs in OA-affected joints; however, their functional activity may be downregulated (Luyten, 2004).

1.2. Canine osteoarthritis treatments and their scientific evidence

Although various lines of investigation are currently underway, there is still no cure for canine OA. However, management through multimodal therapies—including physiotherapy, balanced nutrition, weight management, acupuncture, and pharmacological interventions—has been shown to alleviate symptoms, slow disease progression, and enhance the quality of life for affected patients by reducing pain (Fox and Mills, 2010; Pye *et al.*, 2022; Marshall, 2010; Mille *et al.*, 2022).

At present, one of the most commonly used treatment approaches, alongside pharmaceuticals such as NSAIDs, COX-2 inhibitors, bendivetmab (anti-nerve growth-factor), and EP4 inhibitors NSAID (Enomoto *et al.*, 2019; Pye *et al.*, 2022), involves the use of nutraceuticals or nutritional supplements. Interest in and research on these products have been increasing, although their effects remain controversial in some cases (Fox and Mills, 2010).

Nutraceuticals are non-drug substances administered orally, designed to supply nutrients essential for maintaining normal physiological functions (Boothe, 1997). These include omega-3 fatty acids, hyaluronic acid, Boswellia serrata, curcumin, collagen, green lip mussel, chondroitin sulphate, glucosamine hydrochloride, cannabidiol… (Fox and Mills, 2010; Pye *et al.*, 2023; Gamble *et al.*, 2018; Loef *et al*., 2018).

A systematic review and meta-analysis conducted in 2022 evaluated the efficacy of various supplements commonly used for OA management. The study found that chondroitin and glucosamine, despite their widespread use, showed limited or negligible effect on symptom relief. In contrast, omega-3 fatty acids demonstrated significant analgesic properties, potentially reducing joint pain and inflammation. Cannabidiol (CBD) also exhibited pain-relieving effects, although to a lesser extent, and collagen supplements showed some potential benefits for pain relief, but with limited supporting evidence. These findings highlight the need for further research to validate the effectiveness of supplements in OA treatment (Barbeau-Grégoire *et al.*, 2022; Gamble *et al.*, 2018).

Severe cases of canine OA that significantly impact dog's quality of life may require more invasive treatments, such as intra-articular injections of Hyaluronic Acid (HA), which help lubricate the joint and restore a healthier joint environment (Lee *et al.*, 2019; Budsberg *et al.*, 2006). In cases where joint damage is potentially extensive and pain is severe, surgical interventions may be necessary. These include procedures that address the primary cause of OA, such as osteotomies for cranial cruciate ligament repair or osteotomies for hip dysplasia, as well as surgeries aimed at alleviating pain by removing damaged joint structures, such as femoral head ostectomy, arthrodesis (joint fusion), or total joint replacement (Hurley *et al.*, 2007; Guevara and Franklin, 2017).

In recent decades, the limitations of nutraceuticals, inconsistent evidence of their efficacy, and the adverse effects associated with long-term NSAID and

bendivetmab use have driven increased focus on regenerative medicine as a promising alternative for OA treatment (Pye *et al.*, 2022; Farrell *et al.*, 2025). These include innovative therapies such as equine umbilical cord MSCs (Punzón *et al.*, 2022; Beerts *et al.*, 2023) or canine MSCs (Black *et al.*, 2007, Li *et al.*, 2021).

Despite the lack of robust, conclusive evidence supporting many treatments, the expanding range of therapeutic options provides veterinarians with greater flexibility to tailor interventions to individual patient needs (Table 1). This has led to improved clinical management of OA symptoms. However, achieving meaningful prevention or halting the progression of cartilage degeneration remains a significant challenge and is currently achievable only to a limited extent (Yunus *et al.*, 2020; Pye *et al.*, 2022).

Canine Osteoarthritis Therapies			
Pharmacological	Nutraceutical	Regenerative Medicine	Other therapies
NSAIDs: meloxicam, carprofen	Omega-3 fatty acids	Platelet Concentrates: PRP	Joint surgeries
NSAIDs COX-2 inhibitors: Firocoxib, Cimicoxib, Enflicoxib, Robenacoxib,…	Chondroitin sulphate and Glucosamine hydrochloride	Autologous Mesenchymal Stem/Stromal Cells	Intra-articular Hyaluronic Acid
EP4 inhibitors NSAIDs	Boswellia Serrata	Equine Mesenchymal Stem/Stromal Cells (DogStem®)	Weight control
Anti-nerve growth-factor: Bendivetmab	Green lip mussel		Physiotherapy
Gabapentin	Collagen		Balanced nutrition
Opioids: tramadol	Curcumin		Acupuncture
Cannabinoids (CBD)			

Table 1. *Summary of* ***currently available therapies for canine osteoarthritis****, categorised into four groups: pharmacological, nutraceutical, regenerative medicine, and*

other therapies. Sources: Pye et al., 2022; Pye et al., 2023; Punzón et al., 2022; Fox and Mills, 2010; Gamble et al., 2018; Enamoto et al., 2019.

1.3. Regenerative medicine application in veterinary orthopaedics

Regenerative medicine, including MSCs and Platelet Concentrates (PCs), has emerged as a promising therapeutic approach for OA. This is primarily due to its minimal adverse reactions, limited side effects, as well as its ability to provide symptom relief in OA patients (Lee *et al.*, 2019; Beerts *et al.*, 2023; Brondeel *et al.*, 2023; Cho *et al.*, 2024). Several studies have demonstrated the safety of administering allogeneic and xenogeneic MSCs intravenously and intraarticularly in canine patients (Beerts *et al.*, 2023; Brondeel *et al.*, 2023; Cho *et al.*, 2024). Furthermore, the immunomodulatory properties of MSCs, which are believed to underpin their therapeutic effect, remain an active area of research (Weiss and Dahlke, 2019).

Additionally, PCs are routinely employed allogeneically in clinical practice for both human and veterinary medicine, particularly in platelet transfusions for critical care patients suffering from bleeding disorders (Callan *et al.*, 2009). In veterinary medicine, Platelet-Rich Plasma (PRP) and other PCs are widely recognised as affordable, accessible, and cost-effective treatments for a range of conditions in fields such as ophthalmology, dermatology and orthopaedics, as well as for serving as MSCs carriers (Sharun *et al.*, 2021).

Although, *in vitro* studies have indicated that PRP may exhibit pro-inflammatory effects in OA culture environments (Gallego *et al.*, 2022), several *in vivo* studies have demonstrated that PRP can be beneficial for canine OA management (Lee *et al.*, 2019; Catarino *et al.*, 2020; Zhang *et al.*, 2016). Additionally, it has shown potential in enhancing bone healing, (López *et al.*, 2019) and treating tendinopathies when combined with MSCs (Canapp *et al.*, 2016).

Furthermore, PCs have also been proposed and utilised as a substitute for Foetal Bovine Serum (FBS) in cell culture media, particularly for xeno-free MSCs cultures (Bernardi *et al.,* 2017; Wang *et al.,* 2019).

However, *in vivo* efficacy of PCs remains a subject of debate, likely due to the absence of standardised protocols and the intrinsic variability in both the composition of the product and therapeutic outcomes (Franklin *et al.*, 2017; Pye *et al*., 2022; Raleigh *et al*., 2017; Sharun *et al.,* 2021).

Canine Mesenchymal Stromal/Stem Cells (cMSCs) have emerged as a promising treatment for canine OA and are widely used, as equine MSCs or autogenic cMSCs (Punzón *et al.,* 2022; Beerts *et al*., 2023; Black *et al.*, 2007). cMSCs can be obtained from a variety of tissue sources. The most commonly used sources include bone marrow, adipose tissue and umbilical cord. However, other tissues, such as synovia, can also serve as sources when specific characteristics are being targeted (Berebichez-Fridman and Montero-Olvera, 2018; Bearden *et al*., 2017).

Their proven regenerative capacity remains limited due to study design constraints. Despite this, research has demonstrated that cMSCs exert significant immunomodulatory effects, which can help reduce inflammation and improve OA symptoms, offering a therapeutic benefit even if cartilage regeneration has not yet been conclusively established (Brondeel *et al.*, 2021).

1.3.1. Adipose-derived Mesenchymal Stromal/Stem Cells (Ad-MSCs)

Stromal Cells encompass a diverse group of cells, including fibroblasts and other structural cells found in various organs. Among them, a subset with stem-like properties is classified as Mesenchymal Stromal/Stem Cells (MSCs), which can be derived from adipose tissue, bone marrow, and other sources. These MSCs

possess multipotent differentiation potential, enabling them to develop into specialised cell types such as osteoblasts or adipocytes. However, unlike pluripotent stem cells, which originate from embryologic tissues and have the ability to differentiate into any cell type, MSCs are limited in their differentiation capacity and are primarily involved in tissue repair, immunomodulation, and regeneration (Tseng *et al.,* 2024; Manetti, 2021; Choudhary & Choudhary, 2021).

cMSCs are multipotent adult-derived stem cells that are relatively easy to expand and proliferate *in vitro*. They exhibit a spindle-shaped, fibroblast-like morphology and possess plastic adherence properties. Key characteristics that distinguish MSCs from other cell types include their immune privilege, long-term viability, multipotent differentiation potential, ability to inhibit apoptosis, anti-inflammatory modulation, and tissue regeneration capabilities. To meet the criteria for MSCs, these cells must demonstrate differentiation into at least three distinct lineages: osteoblasts, chondroblasts and adipocytes. Additionally, they must express specific surface markers, including CD29, CD44, CD90, CD73 and CD105, while lacking expression of markers such as CD14/CD11b, CD34, CD45 and CD79α/CD19 (Carrade and Borjesson, 2013; Vieira *et al*., 2010; Guest *et al*., 2022; Spencer *et al*., 2012) *(Table 2).*

Adipose-derived canine MSCs (Ad-cMSC) are among the most commonly used MSCs today due to their ability to be isolated in high quantities and their safe procurement. These cells can be obtained from various adipose reservoirs, such as subcutaneous, visceral, and inguinal fat depots. The lower morbidity associated with harvesting adipose tissue and the high cell yield make Ad-cMSCs a more advantageous source compared to others (Guercio *et al*., 2012; Arrigoni *et al*., 2009).

To isolate Ad-cMSC under sterile conditions, adipose tissue is minced and washed with phosphate-buffered saline (PBS) containing antibiotics and antimycotics. The tissue is then digested in an equal volume of PBS containing

bovine serum albumin (BSA) and type I collagenase. The resulting mix is centrifuged and the Stromal Vascular Fraction (SVF) pellet is resuspended in a standard stromal medium (DMEM supplemented with 10% FBS). Following this process, cells should undergo characterisation and can be cryopreserved for extended periods, with viability maintained for at least three years (Vieira *et al*., 2010; Herrera *et al.,* 2023; Taguchi *et al*., 2019; Guercio *et al*., 2012; Spencer *et al*., 2012; Kisiel *et al*., 2012; Franch *et al*., 2023).

Canine Mesenchymal Stem/Stromal Cell characteristics		
Characteristic of cMSCs	Technique	Minimum for characterisation
Fibroblast-like morphology	Microscopical observation during *in vitro* cell culture	Yes
Plastic adherence in cell culture	Microscopical observation during *in vitro* cell culture	Yes
Proliferation capacity and long term viability	Counting during *in vitro* cell culture	Yes
Immune privilege	MHCII negative	Yes, when allogeneic use
Multipotential differentiation capacity	Osteoblast, chondroblast and adipocytes differentiation *in vitro*	Yes. Trilineage differentiation at least once with a specific cell linage
Positive cell surface marker	CD29, CD44, CD90, CD73, CD105	Yes, minimum two
Negative cell surface marker	CD14/CD11b, CD34, CD45, CD79α/CD19	Yes, minimum two
Anti-inflammatory modulation	*In vitro* study	No
Inhibition of apoptosis	*In vitro* study	No

***Table 2. Canine mesenchymal stem cell characteristics, techniques** for their **characterisation** and minimum of proves recommended for their characterisation. Adapted from Guest et al. 2022, Vieira et al. 2010 and Carrade and Borjesson 2013.*

Safety and efficacy of allogeneic cMSCs injections for OA treatment have been well-documented (Kiefer *et al.*, 2016; Guercio *et al.*, 2012). But when cMSCs are applied allogeneically, they must be validated according to the minimum criteria previously described, including trilineage differentiation, surface marker profiling, and plastic adherence. Additionally, antigenicity should be assessed by evaluating Membrane Histocompatibility Complex II (MHCII) (Guest *et al.*, 2022). Typically, MHCII expression is absent in MSCs which is advantageous as it ensures their low immunogenicity when applied in an allogeneic patient (Carrade and Borjesson, 2013).

During cultivation, cells are maintained under controlled conditions in growth media supplemented with FBS, which promotes cell proliferation. A commonly used medium is low-glucose DMEM enriched with 5 - 20% FBS. However, in the absence of standardised protocols for MSCs culture, variations in FBS quality and medium preparations can significantly contribute to variability in cell expansion (Spencer *et al.*, 2012; Taguchi *et al.*, 2019; Bernardi *et al.*, 2017; Vieira *et al.*, 2010; Kisiel *et al.*, 2012; Guercio *et al.*, 2012).

Recent investigations into MSCs, have highlighted Extracellular Vesicles (EVs) as a revolutionary approach in regenerative medicine. Those vesicles, which are secreted by MSCs, contain bioactive molecules such as proteins and growth factors (GFs), allowing them to exert immunomodulatory and regenerative effects without the need for direct MSC transplantation. EVs have shown potential in reducing inflammation, promoting tissue repair and modulating immune responses, making them a promising cell-free alternative (Williams and Ehrhart, 2022).

1.3.2. Platelet concentrates (PCs)

PCs, such as Platelet Lysate (PL) and PRP, are blood-derived biomaterial products that contain platelet concentrations above baseline levels. These concentrates serve as an important source of both autologous and allogeneic GFs, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (b-FGF), transforming growth factor (TGF), insulin-like growth factor (IGF), epidermal growth factor, and others. These GFs may play a significant role in promoting tissue regeneration (Qian *et al*., 2017; Stief *et al*., 2011; Perazzi *et al*., 2013).

However, further studies involving larger populations and comparative group analyses are necessary to establish their efficacy, as the current *in vivo* evidence for OA remains limited (Zhang *et al*., 2016; Sharun *et al*., 2021).

GFs are signalling molecules that act in the cellular environment to stimulate cell proliferation, growth, expansion, and even differentiation. These GFs contribute to a variety of cellular processes, such as enhancing bone formation, promoting angiogenesis, and stimulating tissue regeneration (Qian *et al*., 2017; Harrison, 2018; Sharun *et al*., 2021).

In addition to GFs, platelets contain significant amounts of chemokines, cytokines and proinflammatory factors, which regulate the inflammatory cascade. These factors are activated upon platelet degranulation, stimulating the recruitment, migration, proliferation and differentiation of MSCs to regenerate and repair tissues. Moreover, activated platelets secrete stromal cell-derived factor 1-alfa (SDF-1α), which enhances primary adhesion and migration of cells (Qian *et al*., 2017; Alderman & Alexander, 2011).

Since GFs are stored in the alpha granules of platelets, they can be released into the plasma compartment prior to use through a process called platelet activation

or platelet lysis, which occurs physiologically *in vivo*. Platelet activation can be induced by physical or chemical methods. Physical methods include extreme freeze-thaw cycles, which have the advantage of avoiding the addition of external products to the PC. Chemical activation, on the other hand, involves adding substances like calcium chloride ($ClCa_2$) and thrombin as platelet-disrupting agents (Astori *et al*., 2016; Qian *et al*., 2017; Shih and Burnouf, 2015; Franklin and Birdwhistell, 2018; Perinelli *et al*., 2020).

Among the various PC products studied, differences exist in their composition and preparation methods. PRP is particularly rich in platelets and, consequently, rich in GFs when platelets are activated. However, due to the lack of standardised protocols and the availability of numerous commercial PRP kits, PRP products exhibit significant intrinsic variability. Both PRP and PL are derived from whole blood and are characterised by platelet counts above the baseline levels (Franklin *et al*., 2017; Wang *et al*., 2019).

PRP is obtained through processes of centrifugation and phase separation of whole blood. When PRP is prepared to exclude leukocytes, it is referred to as leuko-reduced PRP. Leuko-reduced PRP is particularly beneficial in applications where avoiding external inflammation is critical, such as in cell culture systems. When PRP is activated and platelets are lysed, the resulting product is PL, which is characterised by the release of GFs from disrupted platelets (Franklin *et al*., 2017; DeMello *et al*., 2022).

In human medicine, it has been suggested that PRP should ideally contain a fivefold concentration of platelets compared to their physiological levels in blood (Alsousou *et al*., 2009). However, this standard has not yet been established or considered for canine PRP, leading to increasing variability among PC preparations.

Canine platelets can be cryopreserved using 6% dimethyl sulfoxide (DMSO), which offers a significant advantage for long-term storage. Furthermore, studies have demonstrated that PC can be preserved without any cryoprotectant while maintaining stable GF concentrations conserved for minimum of six months (Appleman *et al.*, 2009; DeMello *et al*., 2022).

A 2017 study by Franklin demonstrated a correlation between platelet concentration and Transforming growth factor beta 1 (TGF-β1) levels in canine PRP prepared using commercial kits. This finding suggest that platelet concentration could potentially serve as a feasible proxy for estimating GFs levels in PCs preparations (Franklin *et al*., 2017).

1.3.3. Xeno-free and allogeneic cMSCs ready-to-use product for canine OA

Residual bovine components in FBS pose significant risks when MSC preparations are administered therapeutically. These risks include immune reactions triggered by the production of immunoglobulin G (IgG) antibodies against bovine proteins, which can potentially lead to anaphylactic reactions or reduced therapeutic efficacy. Additionally, the use of FBS introduces the potential for zoonotic infections, including bovine viral diarrhoea, bovine parvovirus, and prions responsible for Bovine Spongiform Encephalopathy (BSE) (EMA, 2013; EMA, 2011).

Despite these concerns, FBS and other allogeneic materials remain widely used due to their advantages as standardised products. However, some studies have highlighted variability in these products, which can influence cell culture experiments and potentially reduce their reproducibility and standardisation to levels below expectations (Liu *et al.*, 2023; Rowland *et al.*, 2021; Astori *et al.*, 2016; EMA, 2011).

The risks associated with xenogeneic components in cell-based therapies remain incompletely characterised. Nevertheless, regulatory bodies have taken a precautionary stance. In 2008 and 2011, the European Medicines Agency (EMA) Committee for Medicinal Products for Human Use discouraged the use of animal-derived products due to their potential to harbour infectious agents and induce undesirable immunological responses in recipients. Similarly, the World Health Organization (WHO) and other regulatory authorities have recommended restrictions or outright bans on materials of bovine origin, particularly for therapeutic applications such as cell therapy protocols (Cowper *et al.*, 2019; WHO, 2006; Bernardi *et al.*, 2017; EMA, 2011; EMA, 2008; Shih and Burnouf, 2015).

As a result, PL has gained popularity for *ex vivo* MSC expansion. PCs, including PL and PRP, are valid substitutes for FBS due to their high content of GFs, which can be efficiently released. Several studies have demonstrated that PCs have potential comparable to FBS as growth culture supplements (Bernardi *et al.*, 2017; Shih and Burnouf, 2015; Cowper *et al.*, 2019; Montolío, 2020).

Another significant challenge in cell-based therapies is the high morbidity and cost associated with procuring autologous MSCs, which requires an initial surgical intervention to extract primary tissue. While these cells can be cryopreserved for future use, the process of freezing and thawing presents a risk of cell damage. Freezing remains the gold standard for cell and tissue conservation, requiring protocols that typically include cryoprotective agents like Dimethyl sulphoxide (DMSO). Research has shown that, with accurate freeze-thaw protocols, MSCs can retain their biological properties and be ready for use within 4-7 days (Uhrig *et al.*, 2022; Jaiswal and Vagga, 2022; Pavón *et al.*, 2017).

Transportation time is also critical. Cell viability may decrease significantly during transit from the laboratory to the patient due to suboptimal carrier media

and temperature conditions. Developing a safe and effective injection-ready-to-use medium is essential to ensure that cells maintain their viability and therapeutic potential (Ngo *et al.,* 2021; Garvican *et al.*, 2014; Sultana *et al.*, 2022).

To address these challenges, the use of allogeneic MSCs cultured with serum-free media and stored in ready-to-use carrier media has emerged as a promising solution for OA treatment. These cells can be sourced from donor individuals undergoing elective procedures, cryopreserved and maintained until required for use (Li *et al.*, 2021; Ivanovska *et al.*, 2022; Devireddy *et al.*, 2019; Herrera, 2022; Douce *et al.,* 2017).

Although cMSCs and PCs have emerged as promising xeno-free therapeutic options for treating canine OA, their widespread use is hindered by limitations in standardisation, ready-to-use formulations, and product availability. Developing a suitable carrier medium for allogeneic, ready-to-use intra-articular cMSC-based product, is essential to enhance cell viability, therapeutic efficacy, and practicality. Standardizing such a medium would not only optimise the benefits of cMSC therapy but also facilitate broader clinical application, making regenerative treatments more accessible for canine OA management (Ivanovska *et al.,* 2022; Alsousou *et al.,* 2009; Devireddy *et al.*, 2017; Garvican *et al.*, 2014; Guercio *et al*., 2012; Choudhary and Choudhary, 2021).

1.4. Regulatory frameworks for cell-based products in Europe

The regulatory framework governing the application of allogeneic cell-based therapies in veterinary medicine, particularly for canine patients, exhibits notable variability across European countries. This variability stems from differing

national legislations and the evolving nature of European Medicines Agency (EMA) guidelines.

1.4.1. European Union Overview

At the European Union (EU) level, there is no dedicated regulatory framework exclusively for cell-based medicinal products intended for veterinary use. Existing regulations primarily focus on human applications, leading to a reliance on case-by-case assessments for veterinary products. This approach underscores the need for specific legislation to address the unique challenges posed by veterinary cell-based therapies (Kuhlmann-Gottke and Duchow, 2015; EMA, 2009; EMA, 2019).

In response to this gap, the EMA's Committee for Medicinal Products for Veterinary Use (CVMP) has initiated efforts to provide guidance. Notably, the Ad Hoc Expert Group on Veterinary Novel Therapies (ADVENT) released a question-and-answer document focusing on the sterility of allogeneic stem cell-based products for veterinary use. This guidance emphasises the importance of sterility in products administered via injection and outlines considerations for manufacturing processes to ensure safety and efficacy (EMA, 2019).

1.4.2. National Regulations: The Case of Italy

Italy adopts a strict regulatory framework concerning the use of cell-based therapies in veterinary medicine, particularly for allogeneic applications. According to the guidelines established by the Ministry of Health and the State-Regions Conference (Gazzetta Ufficiale, No. 277, 26 November 2013), only autologous, minimally manipulated mesenchymal stem cell therapies are permitted in animals. These procedures must comply with defined sanitary,

traceability, and quality control requirements. The use of allogeneic or substantially manipulated cell-based products in veterinary contexts is not explicitly authorised and is instead subject to the provisions of Legislative Decree No. 193/2006, which regulates veterinary medicinal products. As a result, the absence of specific provisions for allogeneic therapies often leads to a restrictive interpretation and limited practical application in the veterinary field (Ministero della Salute, 2013; Sipp and Robey, 2015).

1.4.3. Variability Across Other European Countries

In contrast, other European nations exhibit more flexible approaches, primarily due to the absence of specific national legislation governing veterinary cell-based therapies. This regulatory ambiguity allows for a more permissive environment regarding the application of such therapies in animals. The EMA has recognised the need for overarching guidelines and has approved documents addressing concerns related to sterility and manufacturing processes for veterinary stem cell medicines, aiming to harmonise practices across member states (EMA, 2009; EMA, 2019; Kuhlman-Gotte and Duchow, 2015).

1.4.4. Xenogeneic Products and EMA Approvals

The EMA has also provided guidance on xenogeneic cell-based medicinal products, which involve cells derived from a different species than the recipient. While this guidance primarily pertains to human medicine, it sets a precedent for the evaluation and potential approval of such products, indicating a pathway that could be considered for veterinary applications in the future (EMA, 2009).

In the context of xenogeneic products and EMA approvals, a notable example is DogStem, a veterinary medicinal product. This product is an injectable

suspension containing 7.5 million equine umbilical cord-derived mesenchymal stem cells, specifically indicated for the reduction of pain and lameness associated with canine OA (European Commission, 2022).

The European Medicines Agency (EMA) granted a centralised marketing authorisation for DogStem on June 15, 2022, making it the first licensed stem cell treatment for dogs with OA in the European Union.

Regarding its availability across European countries, DogStem's centralised authorisation allows it to be marketed in all EU member states. However, the actual availability may vary depending on national implementation and distribution agreements. For instance, in the United Kingdom, DogStem has been authorised for use in Northern Ireland, as indicated by the Veterinary Medicines Directorate (VMD, 2022a; VMD, 2022b).

It is important to note that while the EMA provides centralised approvals, individual countries may have specific regulations or restrictions concerning the use of xenogeneic cell-based therapies in veterinary medicine. Therefore, veterinary professionals should consult local regulatory authorities to ensure compliance with national laws and guidelines before administering such treatments.

The regulatory landscape for allogeneic cell-based therapies in canine veterinary medicine within Europe is complex and heterogeneous. While the EMA has initiated steps to provide guidance, significant disparities remain at the national level, exemplified by Italy's stringent regulations compared to more lenient approaches elsewhere. This underscores the pressing need for harmonised, species-specific regulations to ensure the safe and effective application of advanced cell-based therapies in veterinary practice.

Hypothesis and objectives

This study hypothesises that allogeneic cMSCs, when expanded under good manufacturing practices (GMP), will be a clinically safe and effective allogeneic intra-articular treatment for canine OA. Additionally, incorporating joint-beneficial substances into a ready-to-use medium will enhance cell viability and therapeutic efficacy.

The objectives of this research are to:

- Validate in-house PL and PRP as xeno-free, GF-rich supplements for xeno-free cell culture supplement and carrier media supplement in cMSCs-based product.
- Determine the non-immunogenic potential of cMSCs through cell surface marker analysis to confirm their safety for allogeneic use.
- Identify a ready-to-use carrier medium that supports joint health and maintains cMSCs viability for a mid-term storage period.
- Evaluate the effects of storage conditions (media, duration and temperature) on cMSCs viability and functionality.
- Confirm that xeno-free, ready-to-use cMSCs product is a clinically safe treatment for canine OA patients.

The principal aim of this research is to develop a standardised, xeno-free, ready-to-use carrier medium for intra-articular injection of canine allogeneic mesenchymal stem cells, optimizing their therapeutic potential for canine OA treatment.

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Chapter 2. Comparative Analysis of Growth Factor Concentrations in Canine Platelet-Rich Plasma and Platelet Lysate made by an in-house method for its use in xeno-free mesenchymal stem cell cultures and in stem cell-based product.

In vitro study. The role of platelet count as a predictor of growth factor concentration.

Part of this work has been published as an opened article in the Clinical Comparative Pathology Journal as "Growth factor concentration in canine platelet-rich plasma and platelet lysate is correlated with platelet number" (DOI: 10.1007/s00580-025-03674-x). Full text can be download at: https://link.springer.com/article/10.1007/s00580-025-03674-x).

Abstract

Growth factors-concentrated blood derivatives such as Platelet Lysate (PL) and Platelet-rich Plasma (PRP) have gained increasing clinical relevance and usage in both human and veterinary medicine in recent years. These therapies are indicated based on anti-inflammatory and regenerative potential due to the presence of multiple growth factors (GFs) and cytokines stored in platelet cytoplasmic granules, which can be physically disrupted and harvested. Furthermore, this growing interest is also due to their potential to maintain xeno-free cell cultures. Currently, there is a lack of standardisation, and no established protocols or specific consensus exist regarding the preparation, production, or manufacturing of PRP and PL in canines. This absence contributes to significant variability across studies and outcomes.

The aim of this study is to confirm the GFs content of the product to support its intended use. In addition, the study seeks to correlate the expression levels of

three key growth factors—Vascular endothelial growth factor A (VEGF-A), Transforming growth factor beta-1 (TGF-β1), and Platelet-derived growth factor BB (PDGF-BB) —which are involved in cell proliferation and bone regeneration, with platelet concentration in PCs obtained using an in-house method.

In accordance with experimental ethics protocols established by the Autonomous University of Barcelona (UAB) (procedure #5075), eight healthy adult canines were selected as blood collection donors, with assessments conducted at two time points separated by a 4-month interval. Blood samples were collected into EDTA and sodium citrate tubes in order to obtain whole blood samples and plasma derivatives: plasma, leukocyte-reduced PRP, platelet poor plasma, and PL. After two centrifugation cycles with an open sterile technique, leukocyte-reduced PRP was obtained. Furthermore, PL was obtained by applying multiple freeze-thaw cycles to PRP. The presence and concentration of three GFs were analysed using canine-specific Enzyme-linked Immunosorbent Assay (ELISA) kits.

Results obtained in this study suggest that the in-house method employed is effective in concentrating platelets and growth factors—specifically TFG-β1 and PDGF-BB—in blood-derived products such as PRP and PL. A statistically significant difference in TGF-β1 levels between PRP and PL was observed, indicating that platelet lysis can enhance the release of GFs. Additionally, a strong positive correlation (r^2=0.7195) was found between TGF-β1 concentration and platelet count in this set of samples, suggesting that platelet concentrations may serve as a reliable predictive indicator of specific growth factor levels. Given that platelet counts are simple, fast and widely accessible, they could serve as a practical tool to guide the selection of GF-rich plasma preparations. This could benefit applications such as in xeno-free stem cell cultures, cell-based therapeutic products, or musculoskeletal treatments, including bone regeneration, joint

therapies, and tendon repair procedures, where estimating the GFs content is critical to optimising therapeutic outcomes.

Introduction

Allogeneic PCs are used in both human and veterinary medicine to treat various haematological disorders and imbalances (Burnouf *et al*., 2023; Callan *et al*., 2009). PL, along with other PCs such as PRP, are biological products enriched in platelets and/or in growth factors (GFs) at concentrations significantly higher than physiological levels (Pavlovic *et al.,* 2016). This enrichment underlies their therapeutic potential in certain diseases and pathological conditions, as well as their use as substitutes for xenogenic components in xeno-free cell cultures (Sharun *et al.*, 2021; Burnouf *et al.*, 2023).

Over the past few decades, PCs such as PRP have gained increasing relevance prevalent in veterinary medicine, particularly for the treatment of orthopaedic conditions. These applications include enhancing bone regeneration in complex cases of non-union fractures or fractures involving substantial bone loss, as well as alleviating symptoms associated with tendinopathies and OA. Moreover, their use may help prevent the need for radical procedures, such as amputations or invasive surgical procedures (Sharun *et al.,* 2021; Arnhold and Wenisch, 2015). One of the most relevant applications of PCs lies in their demonstrated capacity to serve as a functional source of nutrients for xeno-free cell cultures. Due to their high concentration of GFs, PCs have emerged as a viable alternative to FBS in the culture of cMSCs intended for clinical use, thereby reducing the risk of immune cross-reactivity associated with residual xenogeneic components (Bernardi *et al.*, 2017; Wang K *et al*., 2019). Additionally, it has been demonstrated that PRP-

derived GFs enhance stem cell differentiation in musculoskeletal regeneration (Qian *et al*., 2017).

Most GFs can be obtained from platelets through various PCs (Wang X *et al*., 2019; Franklin and Birdwhistell, 2018). These biological products are either rich in intact platelets or in lysed platelets, with concentrations exceeding physiological levels. As a result, they contain elevated levels of GFs, enhancing their clinical relevance as therapeutic agents, supplements in the management of diverse health conditions or supplements for cell cultures and cell-based products (Harrison, 2018; Wang K *et al*., 2019).

In vitro degranulation of platelets, commonly referred to as PRP activation, leads to the release of GFs from platelet granules, thereby increasing their concentration (*Figure 1*). PRP activation can be induced through various chemical and physical processes that replicate the natural *in vivo* activation process (Franklin *et al*., 2017). Chemical agents such as Calcium chloride ($CaCl_2$) and thrombin (Franklin and Birdwhistell, 2018; Perinelli *et al*., 2020) are often employed; however, their use may result in coagulation and rapid fibrin clot formation (Toyoda *et al*., 2018). Alternatively, freeze-thaw cycles serve as a physical method to activate platelets without the addition of exogenous substances (Franklin *et al*., 2017). Upon complete platelet lysis, the product is referred to as PL.

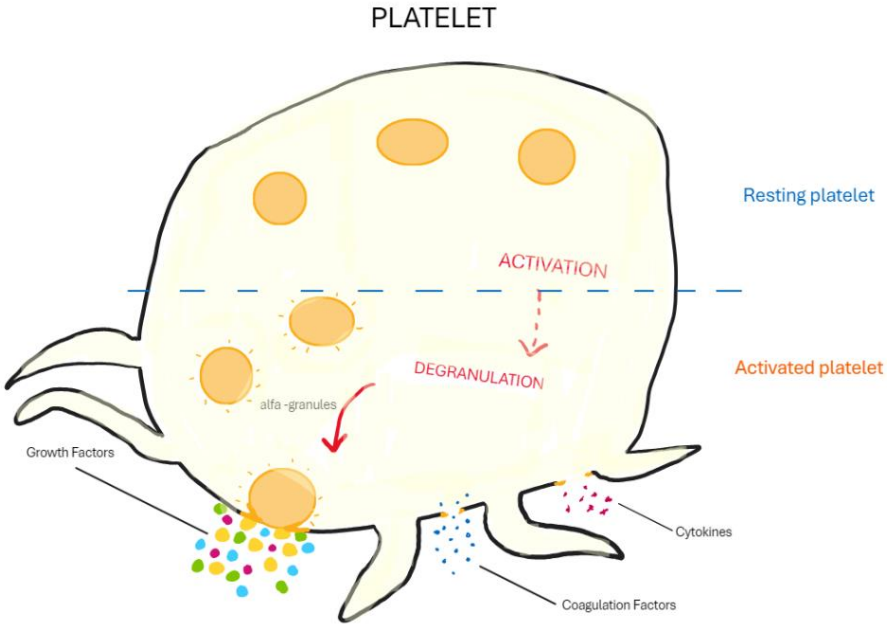

Figure 1. *Schematic representation of* ***platelet activation and degranulation*** *leading to the release of growth factors (GFs).*

Although TGF-β1 and PDGF-BB are found in higher concentrations than VEGF in canine PRP (Franklin *et al.*, 2017), VEGF plays a critical role throughout the bone repair process. It promotes macrophage recruitment during the initial inflammatory phase of bone healing and stimulates endothelial cells to induce angiogenesis. VEGF also enhances osteoblasts proliferation and differentiation, contributing to intramembranous ossification (Hu and Olsen, 2016). Importantly, VEGF is essential for the neovascularisation, supporting tissue repair through the proliferation, migration and organisation of endothelial cells into new blood vessels. This vascular network is vital for delivering oxygen, nutrients, GFs and MSCs to the injury site (Beheshtizadeh *et al.*, 2023).

TFG-β1 plays a central role in bone maintenance and repair by upregulating regenerative pathways. Bone Morphogenic Proteins (BMPs), which are key regulators of bone formation and osteoblast differentiation, belong to the TFG-β

superfamily (Rahman *et al*., 2015). PDGF-BB, secreted by preosteoclasts during bone formation, enhances osteogenesis by stimulating angiogenesis—particularly in CD31 Emen vessels—thereby facilitating vascular development and subsequent bone regeneration (Xie *et al*., 2014). Collectively, TGF-β1 and PDGF-BB are essential components in tissue regeneration and bone healing (Barrientos *et al*., 2008).

PCs remain widely used as regenerative therapies or co-therapies in both human and veterinary medicine, largely due to their low incidence of adverse effects and the ease of preparation and application at the point of care. However, several controversies persist regarding their efficacy. While some studies report limited effectiveness of PCs in managing canine cranial cruciate ligament rupture (Sample *et al.,* 2018), others suggest beneficial outcomes in the treatment of canine OA and in enhancing fracture healing, particularly when combined with mesenchymal stem cells (Lee *et al*., 2019; Sharun *et al.,* 2021). Additionally, certain investigations have demonstrated improved bone regeneration with PC application alone (Dehghan *et al.* 2015; López *et al.,* 2019; Zhang *et al.,* 2021). Despite their widespread use, standardised characterisation and dosing guidelines for PCs therapies and in cell cultures and cell-based products remain lacking (Wang K *et al*., 2019). These inconsistences may stem from the substantial variability in PC preparation protocols and the absence of consistent qualitative or quantitative assessments. Furthermore, interindividual differences in donor blood characteristics contribute to heterogeneity in final PC compositions. The use of pooled samples in heterologous PC applications may help mitigate this variability (Gilbertie *et al.,* 2018; Franklin and Birdwhistell, 2018; Sharun *et al.,* 2021; Liebig *et al.,* 2020). Overall, the concentration of GFs in PC preparations can vary widely, potentially influencing their therapeutic efficacy (Devireddy *et al*., 2019).

Objectives

The primary aim of this study is to compare the presence and concentration of three key GFs (TGF-β1, VEGF-A, and PDGF-BB) which are critically involved in musculoskeletal regeneration and cell proliferation, in two types of PCs: PRP and in PL. Both products were produced using an in-house protocol developed by the *Bone Regeneration Research Group* (UAB). An additional objective is to assess the potential correlation between platelet concentration and GF levels, to determine whether platelet count could serve as a predictive indicator for estimating GF content. The relevance of this research lies in validating the in-house protocol as a safe, reliable and reproducible method for generating a GF-rich platelet concentrate, suitable for use as a clinical therapeutic product or as a xeno-free supplement in cell culture systems and cell-based products.

Our hypothesis is that canine PL, activated and processed using this in-house method, will contain significantly higher concentrations of GFs than PRP, making PL a more efficient alternative. Furthermore, we expect that platelet counts in PRP may serve as a useful predictor of GFs concentration.

Materials and Methods

Donors

This study was conducted between July and November 2023 at the animal and experimental facilities of the Autonomous University of Barcelona (UAB). Eight adult Beagle dogs of both sexes were approved as blood donors in accordance

with the guidelines of the Animal Research Ethics committee of the UAB (Authorisation procedure number #5075). The age of the donor animals ranged from 1 to 9 years.

To meet the study's inclusion criteria, all donors were required to have a complete health and vaccination record, a normal physical examination, up-to-date deworming status, absence of systemic diseases, negative serology for Leishmania and Rickettsia, and no haematological abnormalities (Wardrop, 2016). Additionally, a baseline platelet concentration within the established reference range for canine whole blood was required for inclusion in platelet level analysis.

Blood extraction

To obtain plasma (P), blood was collected from eight canine donors at two separate time points, spaced four months apart. Aseptic blood collection was performed by a veterinarian using jugular venipuncture technique with a butterfly catheter system and vacuum extraction (Blood collection set safety lock 21G – 18cm.; BD Vacutainer. Franklin Lakes, USA). For each collection, seven 4.5mL sodium citrate tubes (Buffered sodium citrate blood collection tubes 4.5mL 1.29M; BD Vacutainer. Franklin Lakes, USA) were used per dog, yielding a total of 31.5 mL of blood per donor (*Figure 2a*). Additionally, 1mL of blood was collected from each donor into an EDTA tube (EDTA K3 1mL tube Aquisel, Everest Tecnovet; Barcelona, Spain), which was gently rotated for 2 minutes to facilitate complete blood count (CBC) analysis and establish baseline haematological parameters.

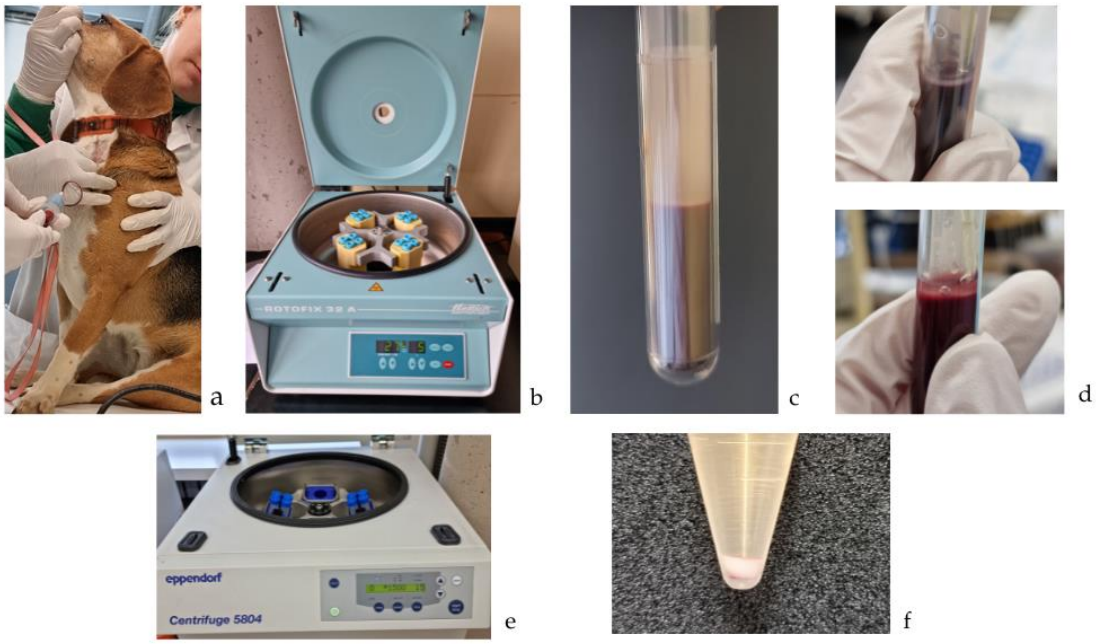

Figure 2. PRP preparation process*. (a) Blood collection via jugular venipuncture using a vacuum tube system; (b) Initial centrifugation (soft spin); (c) Separation of blood components following the first spin, showing plasma, buffy coat, and red blood cell layers; (d) Manual aspiration of plasma using pipettes while carefully preserving the buffy coat; (e) Second centrifugation (hard spin); (f) Formation of the platelet pellet at the bottom of the tube after the second spin.*

PRP obtention

Blood samples stored in citrate tubes were processed between 40 and 50 minutes after extraction to allow adequate interaction between platelets and citrate, thereby preventing aggregation. Samples should be processed between 20 and 60 minutes post-extraction at room temperature, or stored at 4-7°C for up to 24 hours if delayed. PRPs were obtained using a modified double-centrifugation protocol based on Cowper *et al*. (2019), and further adapted by Montolío *et al.*

(2025). All procedures were performed under sterile conditions within a laminar flow hood, using sterile equipment and cleanroom garment protection.

The first centrifugation (soft spin) was carried out at 800G for 5 minutes (Rotofix 32A centrifuge; Hettich Tuttlingen, Germany). After that, P was carefully aspirated using a 200µl and 1000µl manual pipettes, avoiding disruption of the buffy coat to minimise white blood cell contamination. The collected P from each donor was transferred into a 14 ml Falcon tube. A second centrifugation (hard spin) was then performed at 1500G for 15 minutes (5804 centrifuge; Eppendorf. Hamburg, Germany), yielding a platelet pellet and platelet-poor plasma (PPP) (*Figure 2*). Platelet pellets were resuspended in a calculated volume of autologous PPP to achieve a theoretically target concentration of 1 x 10^9 plt/µl, thereby producing PRP (*Figure 3*). Additionally, a pooled PRP sample was prepared by mixing equal volumes from all individual PRP samples.

$$\frac{C_1 \cdot V_1}{C_2} = V_2$$

Figure 3. Formula used to calculate the final plasma volume *required to obtain desired theoretical platelet concentration (C_1 = initial platelet concentration in Falcon tube after soft spin; V_1= Initial plasma volume after soft spin; C_2 = target theoretical platelet concentration ($1x10^6 pl/\mu L$); V_2 = Final volume (X)). V_1-V_2 = volume of PPP to be discarded.*

PL obtention

Activation of PRP was carried out through a series of freeze-thaw cycles to induce platelet lysis and generate PL. The first cycle involved freezing the PRP at -80°C for 10 minutes, followed by thawing in a 37°C water bath for 7 minutes. A second cycle was performed by freezing at -80°C for 15 minutes and thawing again at

37°C for 7 minutes. The final cycle consisted of freezing at -80°C for 10 minutes and a final thaw at 37°C for 7 minutes *(Figure 4)*. Upon completion of the lysis process, individual aliquots of PL were stored in sterile Eppendorf tubes for subsequent GF analysis.

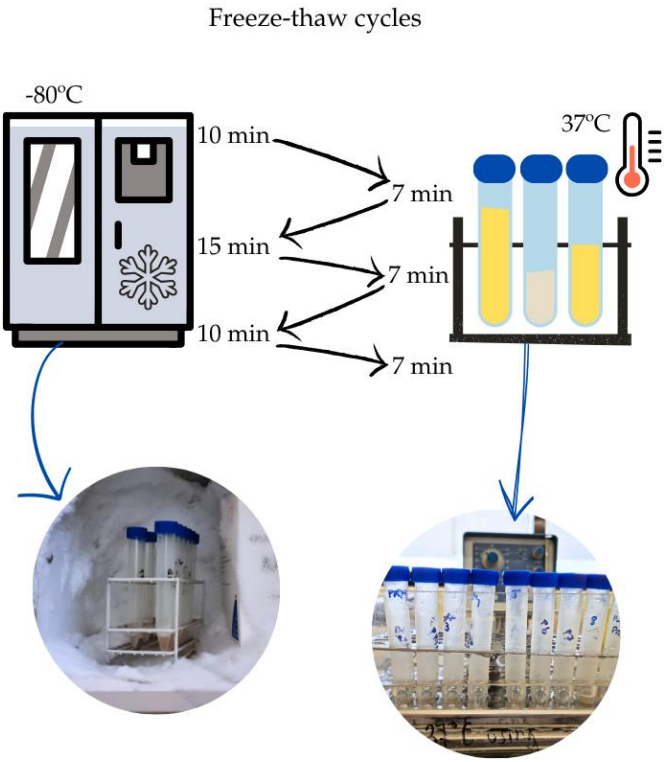

Figure 4. Protocol for platelet lysate (PL) preparation *using three freeze–thaw cycles at -80°C and 37°C temperatures.*

Blood, PRP and PL hemogram analysis

Basal blood counts—including platelets, red blood cells, and white blood cells concentrations—were performed for each canine donor using samples stored in EDTA tubes and analysed with an automated haematology analyser (XN-1000, Sysmex. Kobe, Japan). During the PRP preparation process, basal blood counts were also assessed for P (P was obtained from whole blood samples through

centrifugation in order to perform basal GFs analysis), PPP, and PRP. For this purpose, 300µL aliquots of each sample were collected and stored in Eppendorf tubes for analysis. Additionally, six PL samples were evaluated using the same automated analyser to determine actual platelet concentrations, as these may differ from the previously calculated theoretical values. However, due to limited sample volumes, not all PL samples could be assessed for platelet content.

Growth Factors quantification via ELISA

Duplicate samples of PRP, PL, PPP and P were processed for the quantification of three canine-specific growth factors using commercial ELISA kits, according to the manufacturer's protocols. Optical density was measured using a multiplate reader (VICTOR3, PerkinElmerWaltham, USA).

For PDGF-BB, the Canine PDGF-BB sandwich ELISA kit test (#ECPDGFB PDGF-BB Canine ELISA Kit; Invitrogen, ThermoFisher® Waltham, USA), was used. Samples were diluted 1:20. The diluted samples, along with standards and controls, were incubated for 2.5 hours on plates pre-coated with capture antibodies. After washing unbound protein complexes, a biotin conjugated detection antibody was added and incubated for 1 hour, followed by Streptavidin-HRP for 45 minutes. A TMB substrate solution was then added for 30 minutes to develop a colourimetric signal. Finally, a stop solution was added, and absorbance was measured at 450 nm within 30 minutes.

VEGF-A concentrations were determined using the Canine VEGF-A ELISA kit (# EC18RB Canine VEGF-A ELISA Kit; Invitrogen, ThermoFisher® Waltham, USA). The procedure followed the same protocol as for PDGF-BB, with a sample dilution of 1:10.

TGF-β levels were assessed using an ELISA kit (#DB100C Human/Mouse/Rat/Porcine/Canine TGF-beta 1 Quantikine ELISA; Bio-Techne R&D Systems®). Samples were diluted 1:2 and incubated for 2 hours on pre-coated plates with TFG-β1 antibodies. After washing away unbound substances, an enzyme-linked polyclonal antibody was added for an additional 2 hours, followed by a substrate incubation for 30 minutes. The reaction was terminated with stop solution, and absorbance was read at 450nm within 30 minutes.

Statistics and data analysis

Tables and statistical analyses were performed using GraphPad Prism software (*version 10.1.2, GraphPad Software, Boston, USA; RRID:SCR_002798*) and R (*version 4.3.3, R Project for Statistical Computing, Vienna, Austria; RRID:SCR_001905*).

Pearson correlation tests were used to evaluate the relationship between platelet concentration and GF levels. Differences in GF concentration among groups were assessed using the Kruskal-Wallis test. A *p-value* of less than 0.05 ($\alpha = 0.05$) was considered statistically significant.

Results

All canine donors were of the Beagle breed, with ages ranging from 3 to 8 years (mean ± SD: 6.13±2.10 years). Their body weights ranged from 11.4 to 18.34 kg, with a mean of 14,25±2.65 kg (*Table 1*).

Donor ID	Age (years old)	Weight (kg)	Sex
1	8	12.30	Male
2	8	12.56	Male
3	6	17.70	Male
4	7	12.66	Female
5	3	18.34	Male
6	8	11.40	Female
7	6	15.75	Female
8	3	13.30	Female

Table 1. *Table displaying the* ***information—age (years), weight (kg), and sex—of the canine donors*** *at the second sampling time point.*

From the initial eight canine donors sampled at two time points, two first-time-point samples were excluded from the platelet statistical analysis due to not meeting the inclusion criteria for minimum platelet levels in the baseline hemogram. A pooled PRP sample was included, resulting in a total of 15 samples for the PRP platelet analysis (P n=14; PPP n=14; PRP n=15; PL n=8). The target platelet concentration was achieved in all PRP samples. All 16 individual samples, along with the pooled sample, were included in the GFs analysis. The three analysed GFs were detected in all samples, with notably higher concentrations in PL and PRP samples compared to P and PPP samples *(Figure 5).*

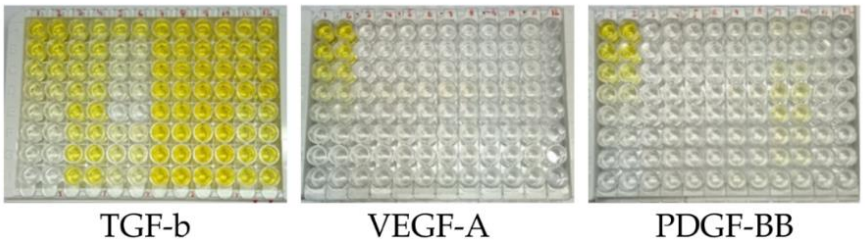

Figure 5. Representative 96-well ELISA plates *used for colourimetric quantification of* ***TGF-β1, VEGF-A, and PFDG-BB****. The layout includes standards, controls, and duplicate test samples for each GF assay.*

The mean platelet concentration in PRP was 3.87 ± 1.13 times higher than that of whole blood (P), with observed increases ranging from a minimum of 1.96-fold to a maximum of 5.36-fold (*Figure 6*).

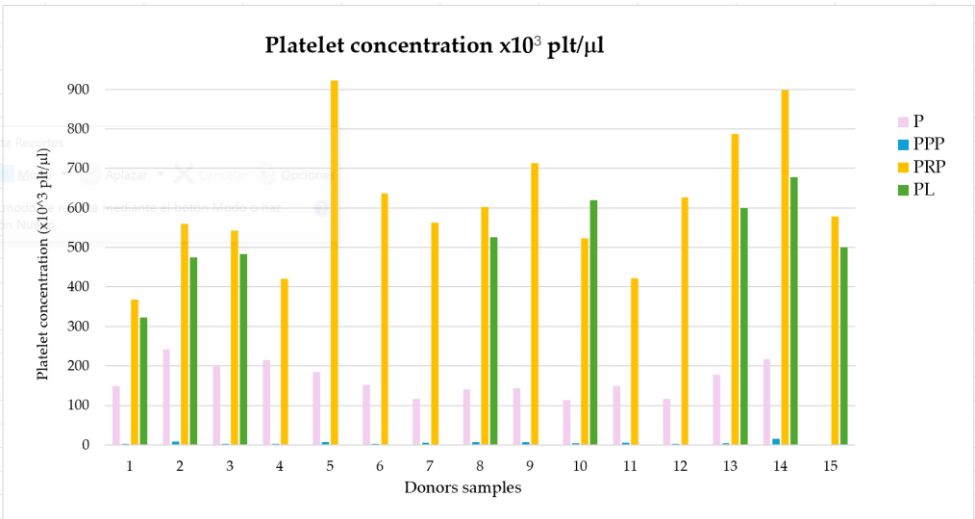

Figure 6. Platelet concentration (x10³ Plt/µl) *across different sample types -Plasma, PPP, PRP and PL-. The X-axis represents individual samples from 14 canine donors, with 15 samples corresponding to the pooled PRP sample. The Y-axis displays platelet concentrations as measured by an automated haematology analyser. Different colours indicate the respective sample groups. Sample sizes were as follows: P (n=14); PPP (n=14); PRP (n= 15); PL (n=8).*

The mean platelet concentrations were as follows: 165.5 ± 41.12 x10^3 plt/µl in P samples, 5.43 ± 3.34 x10^3 plt/µl in PPP, 613.43± 168.78 x10^3 plt/µl in PRP, and 528.71 ± 117.76 x10^3 plt/µl in PL. Comparative statistical analysis revealed that PRP differed significantly from both P and PPP groups. However, no statistical difference was found between PRP and PL. Additionally, P showed statistically significant differences when compared to both PRP and PL. The PPP group was significantly different from all other groups, except when compared to P (*Figure 7*).

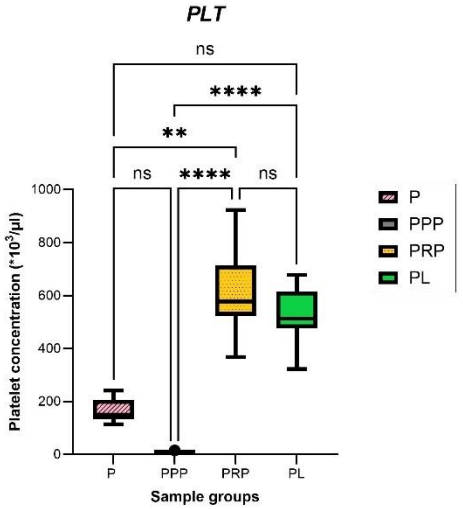

Fig. 7 Platelet concentration and intergroup comparisons among P, PPP, PRP and PL samples. *X-axis represents platelet concentration (10^3 plt/µl) as measured by automated haematology analyser. The Y-axis displays the different sample groups (P n=14; PPP n=14; PRP n= 15; PL n=8). Distinct colours are used to differentiate the sample groups. Asterisks indicate levels of statistical significance: "**" denotes p < 0.01, "***" denotes p < 0.001 and "****" denotes p < 0.0001. Data are presented as mean ± standard deviation. Adapted from Montolío et al., 2025.*

The three GFs analysed in this study—TGF-β1, PDGF-BB, and VEGF-A—were detected in varying concentrations across PL and PRP samples (*Figure 8*). TGF-β1 was consistently detected in PL (n=17), PRP (n=17), PPP (n=16) and P (n=16)

samples. Its mean concentration followed a progressive increase across groups: PPP (7676 ± 8834.62 pg/µL), P (21332.50 ± 14106.07 pg/µL), PRP (100690.17 ± 55344.71 pg/µL), and finally, PL (124869.23 ± 59135.17 pg/µL) *(Figure 8a)*. In pooled samples, TGF-β1 concentration was 144120 pg/µL for PRP and 168240 pg/µL for PL.

PDGF-BB was detected in the majority of PL (n=13) and PRP (n=16) samples. The mean concentration was 169.09 ± 76.77 pg/µL in PRP and 607.00 ± 566.42 pg/µL in PL (*Figure 8b)*. PDGF levels in the pooled samples were 120 pg/µL for PRP and 250 pg/µL for PL.

VEGF-A was detectable in only five donor samples; concentrations in the remaining samples were below the assay's detection threshold (6 pg/mL). Among detectable samples, mean VEGF-A concentrations were 36.30 ± 64.83 pg/µL in PPP, 39.15 ± 64.21 pg/µL in P, 46.20 ± 70.38 pg/µL in PRP, and 39.55 ± 75.11 in PL (*Figure 8c).*

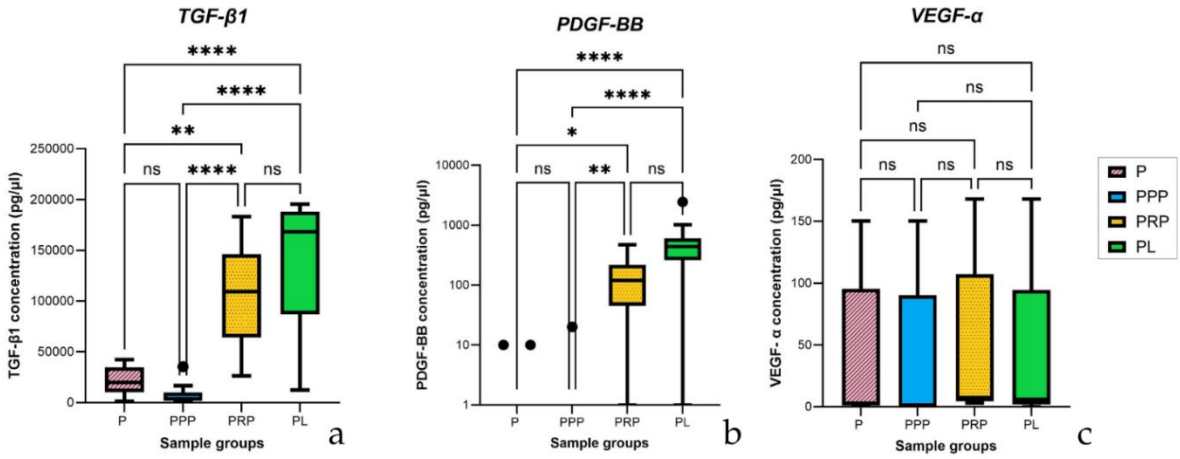

Figure 8. Concentration of Growth Factors and statistical comparison between sample groups *(**a**) TGFβ-1 concentration (pg/µL) found in P, PPP, PRP and PL groups along with the significance of differences between them. (**b**) PDGF-BB concentration in the same groups and corresponding statistical analysis. (**c**) VEGF-A concentration across P, PPP, PRP and PL groups, with comparative significance.*

Different colours represent the respective sample groups. Statistical significance is indicated as follows: "" denotes p < 0.05, "**" denotes p < 0.01, "***" denotes p < 0.001 and "****" denotes p < 0.0001. Data are presented as mean ± standard deviation. Pearson correlation tests were performed. Adapted from Montolío et al., 2025.*

The correlation between platelet concentration and GF levels was calculated for all samples included in the study, excluding the PL group, as platelets in these samples had been previously lysed and were not reliably quantifiable. A strong positive correlation was observed between platelet count and TGF-β1 concentration (r = 0.7195; p = 7.111 x 10^{-10}) (*Figure 9*). In contrast, a weak but statistically significant correlation was found between platelet concentration and PDGF-BB levels (R = 0.204; p = 0.0016) (*Figure 9*). No significant correlation was detected between platelet count and VEGF concentration (R = -0.019142; p = 0.9853).

The correlation between different GF concentrations was calculated separately for PRP and PL samples. In PRP samples, the correlation coefficient was 0.204 (p = 0.2272) for TGF-β1 and PDGF-BB, while in PL samples, it was 0.09561 (p = 0.2175).

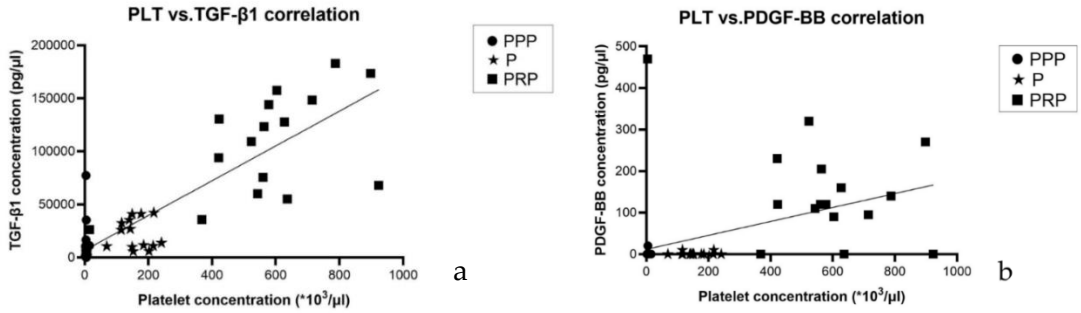

Figure 9. Positive correlation between platelet count and GF concentration *(a) Scatter plot showing the correlation between platelet count (x10³ plt/µl) and TGF-β1 concentration (pg/µl), with a regression line. Circles represent PPP samples, stars represent P, and squares represent PRP samples. (b) Scatter plot showing the correlation between platelet count (x10³ plt/µl) and PDGF-BB concentration (pg/µl) with a*

regression line. Symbols represent the same sample groups as in panel (a). Adapted from Montolío et al., 2025.

Discussion

Although GFs were not detected in all samples, analysis of PCs confirmed the presence of all three target GFs in both PL and PRP samples. PDGF-BB was not detected in PPP and was only identified in one plasma sample. In contrast, it was detected in the majority of PL (n=16) and PRP (n=13) samples, which corresponded to samples with higher platelet concentrations. Notably, PDGF-BB levels increased following platelet lysis, suggesting that freeze-thaw activation enhances the release of GFs in these preparations. This aligns with findings from a previous study by *Franklin et al*., where PDGF-BB was only detected in PRP samples activated by $CaCl_2$, a chemical activation method, which is comparable to our freeze-thaw degranulation system.

TGF-β1 levels also appeared elevated in PL compared to PPR, consistent with the expected release of GFs upon platelet lysis. However, this difference was not statistically significant, likely due to the limited sample size. Compared with the previous study mentioned using the same ELISA kit (2017), TGF-β1 concentrations obtained by our in-house method were higher than those produced using commercial kits. This is clinically relevant, given the demonstrated bone-regenerative potential of TGF-β1 in both animal models (Ehrhart *et al*., 2005) and *in vitro* studies (Yamamoto *et al*., 2000).

VEGF-A was detected in only five donor samples and did not show a consistent increase in PL relative to PRP. No statistically significant differences in VEGF-A concentration were observed between groups. This may reflect an insufficient

sample size, as VEGF-A levels were below the detection threshold in most samples.

Platelet concentrations increased throughout the concentration process, reaching their highest levels in PRP samples. A subsequent decrease was observed in PL compared to PRP, as expected, due to platelet lysis induced by the freeze-thaw cycles.

PL samples were excluded from the platelet correlation analysis, as most platelets in these samples were likely lysed and therefore not accurately quantifiable. A strong positive correlation was observed between platelet count and TGF-β1 concentration (R^2= 0.7195, $p < 0.05$), indicating that TGF-β1 levels may be reliably predicted based on platelet concentration. The predominance of TGF-β1 over PDGF-BB and VEGF-A in the samples likely contributed to the strength of this correlation.

In contrast, the correlation between platelet count and PDGF-BB concentration was low, although statistically significant, suggesting a weaker predictive relationship. No meaningful correlation was found between platelet count and VEGF-A, as indicated by a non-significant *p-value*. Additionally, VEGF-A was detectable above the assay's sensitivity threshold (6 pg/µl) in only a few samples, suggesting it is present in low concentrations in both PRP and PL. This finding is consistent with previous studies, such as *Franklin et al. (2017)*, which reported undetectable VEGF-A in PRP produced by various commercial systems.

The relationship between GFs was also assessed. A weak positive correlation between TGF-β1 and PDGF-BB was observed in both PRP and PL samples, but it did not reach statistical significance. These results suggest that the GFs may act independently in these preparations.

The principal limitation of this study was the limited volume of PL obtained per donor and the relatively small number of donors. Due to ethical constraints, blood volumes collected from university-owned dogs were restricted, limiting the total PL yield. In clinical scenarios involving healthy patients, larger blood volumes can be collected, potentially increasing PL availability. Additional donors are needed to draw more robust conclusions regarding VEGF-A, which appears to be present in quantities near the detection threshold.

This study demonstrated that a GF-rich PC can be generated without the use of commercial kits. Moreover, platelet quantification, being straightforward, cost-effective and widely accessible, can serve as a reliable surrogate for estimating GFs concentrations, particularly TGF-β1. This approach could reduce the reliance on time-consuming and costly ELISA assays in clinical settings. Nonetheless, strict adherence to GMP is essential to ensure product sterility and safety during preparation for therapeutic use (Brecher and Hay, 2005).

Conclusions

While previous studies have compared GF levels across various commercial preparation systems, to our knowledge, no other research has specifically analysed and compared canine GFs in PRP and PL obtained using a non-commercial, in-house protocol. The importance of this study lies in demonstrating that veterinary professionals with access to standard laboratory equipment and working under GMP can reliably produce GF-rich plasma concentrate without relying on proprietary kits.

The observed correlation between TGF-β1 levels and platelet count suggests that platelet quantification may serve as a useful tool for predicting the GF content in PC products.

Given the well-established role of TGF-β in promoting bone regeneration, and enhancing stem cell differentiation in musculoskeletal regeneration, the elevated levels identified in the PL produced through our in-house method indicate its potential utility in clinical musculoskeletal pathologies and in stem cell-based products for canine patients. These findings support the feasibility of producing high-quality, GF-enriched PCs using simplified, cost-effective methodologies suitable for both orthopaedic clinical applications and as supplements for cell culture systems. Nevertheless, strict adherence to GMP is essential to ensure product safety and sterility for clinical use.

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Chapter 3. Verification of cMSCS characteristics and safety following cryopreservation for allogeneic use

Including Assessment of Plastic Adherence, Morphology, Surface marker Expression, MHC Class II Absence, and Microbiological Sterility

Introduction

Canine Mesenchymal Stem/Stromal Cells (cMSCs) have been employed in both experimental and clinical applications over the past two decades. Nevertheless, their validation and standardisation remain ongoing challenges in veterinary regenerative medicine (Baouche *et al.*, 2023).

The most recent guidelines for cMSCs validation, published by Guest *et al.* in 2022, propose a minimum set of criteria to ensure the identity and functionality of cMSC lines. These include: (1) specific surface antigen expression, (2) plastic adherence, and (3) capacity of tri-lineage differentiation. Additionally, a fourth criterion—the absence of MHCII expression—is considered essential when the cells are intended for allogeneic use (Ivanovska *et al.*, 2017; Guest *et al.*, 2022; Rivera Orsini *et al.*, 2024).

Cells used in this study were sourced from the *Bone regeneration research group cell bank* (UAB). Prior to cryopreservation, these cells underwent comprehensive characterisation to confirm their mesenchymal identity. They exhibited plastic adherence, fibroblast-like morphology, proliferative capacity, and a defined immunophenotype, with positive expression of CD90 and CD44, and absence of CD34 and CD45. In addition, thorough microbiological testing confirmed the absence of mycoplasma and other potential pathogens. Genetic profiling further validated the stemness of the cell line, demonstrating mRNA expression of key

pluripotency markers OCT4 and NANOG, in alignment with the most recent recommendations for cMSC validation (Herrera, 2022; Guest *et al*., 2022).

In this study, cMSCs are intended for allogeneic application. Therefore, an additional validation criterion is required to ensure the absence of immune-mediated responses after cryopreservation. Specifically, expression of MHCII molecules must be evaluated. Stem cells should not express MHCII or express minimal MHCII positivity, which will prove their capacity to lack immunogenicity when injected into a different individual (patient). When cMSCs are used allogeneically, MHCII must be analysed beforehand to ensure they are negative for this surface marker and cannot produce immunogenicity when injected (Rivera Orsini *et al*., 2024; Guest *et al*., 2022; Ivanovska *et al*., 2022).

MHCII molecules play a fundamental role in the adaptive immune response by binding extracellular antigenic peptides and presenting them on the cell surface for recognition by T-cell lymphocytes. This process is critical for identifying and activating the immune responses against pathogens and non-self cells. MHCII expression is typically restricted to professional antigen-presenting cells, including dendritic cells, macrophages, and B lymphocytes (Janeway *et al*., 2001; Janeway and Travers, 1997).

As donor-derived cells undergo laboratory processing and manipulation prior to clinical application, there is an inherent risk of contamination or the potential for these cells to act as vectors for pathogenic transmission. To mitigate such risks, it is imperative to adhere to GMP, as recommended by the European Medicines Agency (EMA) and the International Society for Stem Cell Research (ISSRC). Additionally, comprehensive microbiological testing must be performed to ensure the sterility and safety of the final product. These measures are critical not only to prevent infection in recipients but also to minimise the risk of transmitting zoonotic pathogens, thereby safeguarding both animal and public health (Lovell-Badge *et al*., 2021; Pekker *et al*., 2023; EMA, 2019).

Objectives

The primary objective of this study is to validate the defining characteristics and biosafety of cryopreserved, banked cMSCs, ensuring their suitability for allogeneic clinical use. This includes confirming the retention of MSC properties post-thaw, with particular emphasis on their non-immunogenic profile and absence of MHCII expression, which is critical for safe intra-articular administration in allogeneic applications. Additionally, the study aims to verify the microbiological sterility of the cells and product following processing and culturing under GMP conditions, thereby ensuring their safety for therapeutic applications. Furthermore, a streamlined post-thaw characterisation protocol is proposed to re-validate cell identity and confirm their fitness for clinical application.

Cryopreserved cMSCs obtained from Cell Bank—which were characterised prior to freezing—are expected to retain their identity and defining properties upon thawing, including non-immunogenicity. Additionally, given their processing under GMP and sterile handling in a laminar flow hood, cells are anticipated to remain free of microbial contamination, ensuring their biosafety and clinical suitability without risk of pathogen transmission to patients.

Materials and Methods

Adipose-derived cMSCs used in this study were obtained from the *Bone Regeneration Group Cell Bank* and stored at -80°C in the Antibodies and Cell

Culture Service (SCAC) facilities at the Autonomous University of Barcelona (UAB).

Two cryovials labelled "G4" (passages 1 and 2), each containing $1x10^6$ cells, were thawed in a 37°C water bath for 2 minutes. Following thawing, 9mL of culture medium (Low Glucose DMEM + 2nM L-glutamine + 10% v/v FBS + 1% anti-anti (*antibiotic-antimycotic 100x; Gibco. Thermo-Fisher. Carlsbad, US*)) was added. The suspension was then centrifuged at 300G for 5 minutes (Eppendorf centrifuge 5702. Hamburg, Germany) to remove the cryoprotectant. The resulting cell pellet was resuspended in 14mL of fresh culture medium and cultured in a 75T flask for incubation at 37°C in a humidified atmosphere under 5% CO_2.

Cell morphology, adherence, and confluency were assessed every 24 hours, with culture medium refreshed daily. Once confluency was reached at 48 hours, cells were subcultured at a 1:2 ratio. Trypsinisation was carried out using 2.5 mL of trypsin solution, followed by a 2-minute incubation period. Enzymatic activity was neutralised with 2.5 mL of PBS. Cell suspension was then centrifuged at 300G for 5 minutes, the pellet resuspended in 4 mL of fresh culture medium, and evenly distributed into two new culture flasks. After one additional week of culture and an additional passage, cells were harvested and prepared for flow cytometry analysis.

For flow cytometry, cells were detached and manually counted using 4',6-diamidino-2-phenylindole (DAPI) blue staining. Cells were then resuspended at a concentration of 1×10^6 cells/mL in either PBS or Ringer Lactate (RL), with 0.5 mL aliquots transferred into 1 mL Eppendorf tubes. Samples were incubated for 60 minutes with canine-specific antibodies: anti-dog MHC Class II (MCA1044F, Bio-rad, California, US), Anti-dog CD45 (MCA1042PE, *Bio-rad, California, US*), and CD90 (CyFlow CD90 PE; Clone 5E10, *Sysmex Europe, Norderstedt, Germany*), following the manufacturers' instructions. After incubation, samples were analysed using an automated flow cytometer (BD FACSCanto Clinical Flow

Cytometry System; New Jersey, USA). Additionally, two samples were reserved for microbiological testing to verify sterility and confirm the absence of infectious agents.

Results

Cells demonstrated plastic adherence from the initial day of microscopic observation and maintained a spindle-shaped, fibroblast-like morphology throughout the entire culture period (*Figure 1 and Table 1*).

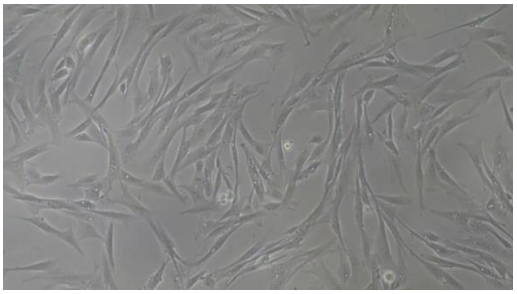
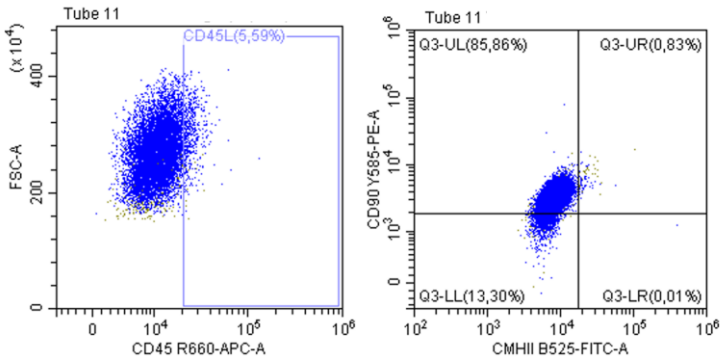


Figure 1. Microscopic observation *of cMSCs culture showing plastic adherence, proliferation, and a fibroblast-like morphology. Scale bar: 50µm.*

Flow cytometry analysis confirmed the absence of MHCII expression (0,01%). Conversely, the CD90 marker showed positive expression (85,86%) (*Figure 2b*), while CD45 was minimally expressed (5,59%) (*Figure 2a*; *Table 1*).



a b

Figure 2. *(a)* ***Graphical representation of the flow cytometry analysis*** *showing negative expression of CD45 in cMSCs. (b) Graphical representation of flow cytometry*

analysis showing positive expression of CD90 and negative expression of MHCII in cMSCs.

Additionally, microbiological analysis, performed using standard conventional microbiological techniques, confirmed that the samples were sterile and free from *Enterobacteriaceae, Staphylococcus aureus*, anaerobic bacteria, mesophilic aerobic bacteria, mycelial fungi, and yeasts, thereby ensuring the safety and innocuity of cMSCs *(Table 1).*

Analyses	Result	Before cryopreservation	After cryopreservation
Plastic adherence	+	√	√
Proliferation capacity	+	√	√
Spindle-shape morphology	+	√	√
Mycoplasma analysis	-	√	x
Microbiological analysis	-	√	√
MHCII	-	x	√
CD90	+	√	√
CD45	-	√	√
CD44	+	√	x
CD34	-	√	x

Table 1. Profiling analyses of cMSCs *to ensure their characteristics and safety for allogeneic application.*

Discussion

Microscopical observation of cell cultures confirmed that cMSCs maintained their plastic adherence capacity and fibroblast-like spindle morphology after cryopreservation. Additionally, phenotypical analysis using cell surface markers demonstrated that cells retained their immunophenotype, with CD90 expression and CD45 absence, consistent with MSC characteristics, confirming

mesenchymal stem/stromal identity of cells and further validating the non-hematopoietic nature of cMSCs.

Flow cytometry analysis of MHCII expression provided crucial insights into the immunogenicity of these cells. Lack of MHCII expression suggests that this cMSC population does not elicit immune cross-reactivity, supporting their suitability for allogeneic therapeutic applications.

Although this profiling could coincide with fibroblast cell lines—and some authors (Guest *et al*.) suggest that a minimum of two positive and two negative cell markers, along with trilinear differentiation, and mycoplasma screening, are necessary to fully characterise cMSCs—these tests had already been performed prior to cryopreservation of this specific cell lineage. Cryopreservation does not alter cell lineage. Characterisation prior to clinical use and before cryobanking has been recommended as the preferred method to ensure a ready-to-use product available (Rivera Orsini *et al*., 2024). Since the same methodology and GMP were applied, it can be assumed that cell characteristics remain unchanged. This assumption is further supported by a shortened cell marker profiling (CD90+, CD45-, MHCII-), the observation of plastic adherence and characteristic spindle-shaped morphology, and by the trilinear differentiation potential confirmed before cryopreservation. Microbiological analyses further ensured cell safety. Moreover, this limited post-thaw characterisation is proposed as a double-check making the clinical application of cMSCs both safer and more cost-effective.

Conclusions

Given that this cMSC cell line has been fully characterised, including confirmation of the absence of MHCII expression, it meets the essential criteria

for allogeneic clinical use, provided that GMP are consistently maintained during cell handling and preparation.

Additionally, a streamlined marker profiling approach—along with assessments of cell morphology, microbiologic analyses, and MHCII expression—can be employed to revalidate the identity of cMSC after thawing for clinical allogeneic application, provided that the cell line has undergone full characterisation prior to cryopreservation in a cell bank.

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Chapter 4. Factors Affecting the Viability and Proliferation of Canine Mesenchymal Stem/Stromal cells in Ready-to-Use Media for Intra-Articular Osteoarthritis Therapy

Abstract

Canine Mesenchymal Stem/Stromal Cells have shown potential for treating canine OA. However, the choice of storage conditions significantly impacts their viability and, therefore, their therapeutic efficacy. Currently, no standardised storage protocols are available. This study evaluates four ready-to-use delivery media: (i) Ringer's Lactate, (ii) Ringer's Lactate supplemented with Hyaluronic Acid, (iii) Ringer's Lactate supplemented with Platelet Lysate, and (iv) Ringer's Lactate supplemented with both Hyaluronic Acid and Platelet Lysate, stored for up to 72 hours at 4°C and 25°C temperatures. Cell viability, colony-forming unit assay, and post-storage proliferation were assessed to determine the most suitable conditions for maintaining cells. Results suggest all media maintained acceptable viability at 4°C for 24 hours. Ringer's Lactate-based media enriched with Hyaluronic Acid and/or Platelet Lysate also preserved viability at 25°C. Furthermore, all media retained more than 80% viability up to 48 hours at 4°C. These findings suggest that RL-based media enriched with HA and/or PL could provide a biocompatible and joint-safe solution for maintaining cMSCs viability and functionality for up to 48 hours, with storage time and temperature affecting cell viability and potential.

Introduction

Mesenchymal Stem Cells (MSCs) have represented a significant advance and improvement in therapeutic approaches in recent years. Canine MSCs have been shown to exert anti-inflammatory effects due to their immunomodulatory properties, particularly when administered intra-articularly in osteoarthritic joints (Weiss and Dahlke, 2019; Zhang *et al*., 2016; Carrade and Borjesson, 2013; Russell *et al*., 2016). However, the lack of standardisation protocols for MSC processing and the existence of multiple product-related variables, still render this treatment unreliable (Brondeel *et al*., 2021).

Although the process of freezing cells can lead to cell damage, it remains the gold standard for preserving cells and tissues. This process typically requires a well-established freezing protocol, often involving cryoprotective agents such as Dimethyl sulphoxide (DMSO) (Jaiswal and Vagga, 2022). Nonetheless, evidence suggests that by following an appropriate freezing and thawing protocol, cells can be prepared for clinical use within 4 to 7 days while retaining their biological properties (Uhrig *et al*., 2022; Franch *et al*., 2023).

Transportation of cells from the lab to the clinical setting is a critical period during which improper handling can compromise cell viability. Therefore, validated ready-to-use media and standardised transport protocols are essential to ensure the delivery of the required cell dose while preserving cell functionality and therapeutic potential (Ngo *et al*., 2021; Brondeel *et al*., 2021).

The Role of Mesenchymal Stem/Stromal Cells in Osteoarthritis

MSCs applied in canines have demonstrated promising results in OA preclinical studies and veterinary applications, due to their ability to secrete bioactive molecules that reduce inflammation and stimulate tissue repair through differentiation into other cells (Beerts *et al.,* 2023; Wang *et al.,* 2023). The ability of MSCs to secrete bioactive molecules facilitates immunomodulation and reduces inflammation (Wang *et al*., 2023; Weiss and Dahlke, 2019; Carrade and Borjesson, 2013; Russell *et al.,* 2016; Brondeel *et al*., 2021), while the chondrogenic properties can contribute to cartilage repair (Brondeel *et al*., 2021; Li *et al*., 2018). When administered intra-articularly into osteoarthritic joints, cMSCs have been shown to alleviate pain and improve clinical outcomes (Wang *et al.,* 2023; Weiss and Dahlke, 2019; Carrade and Borjesson, 2013; Russell *et al*., 2016; Brondeel *et al*., 2021; Li *et al*., 2025; Nicpon *et al.,* 2014).

However, the mechanisms of action and clinical effects are still not fully described, as the clinical efficacy of these cells depends not only on their inherent therapeutic properties but also on the environment into which they are delivered (Przadka *et al*., 2021; Brondeel *et al*., 2021).

For MSCs to survive, function optimally, and exert their regenerative effects, they require a supportive medium or biomaterial scaffold that maintains cell viability and enhances their cellular therapeutic potential (Bharti *et al*., 2021). Additionally, the efficacy and viability of cMSCs depend not only on their intrinsic biological properties but also on the conditions in which they are cultured and administered (Rowland *et al*., 2021; Sultana *et al*., 2022; Andreoli *et al*., 2024; Fitzgerald *et al.,* 2023).

Necessity of a Suitable Medium for MSC Injection

One of the critical challenges in using MSCs for intra-articular injections in canine OA is finding a medium that is both biocompatible with the joint environment and capable of acting as a carrier to preserve cell viability and enhance their functions.

Osteoarthritic joint environment presents a significant challenge for cell survival due to chronic inflammation and the presence of various pro-inflammatory mediators. These include cytotoxic and chondrotoxic agents such as tumour necrosis factor-alpha (TNF-alpha), interleukin-1β (IL-1β), matrix metalloproteinase-2 (MMP-2), and C-reactive Protein, as well as infiltrating immune cells like T cells, macrophages, and natural killer cells (de Bakker *et al*., 2021; Li *et al*., 2017).

Moreover, allogeneic cMSCs may offer advantages over autologous sources, as MSCs derived from OA-affected individuals exhibit reduced proliferation rates and diminished chondrogenic capacity (Murphy *et al*., 2002). Adipose-derived MSCs, in particular, have shown several advantages over other tissue sources, including ease of harvest, lower donor site morbidity (Woods *et al*., 2020), higher proliferative rates, and superior chondrogenic potential (Humenik *et al*., 2022; Rashid *et al*., 2021).

It has been demonstrated that osteoarthritic synovial fluid can exert cytotoxic effects on MSCs (Kiefer *et al*., 2015). However, certain components, such as PRP, may offer a protective effect to synovial tissue when intra-articular administration of cytotoxic agents like lidocaine is necessary (Bianchini *et al*., 2018). Hyaluronic acid (HA), a key component of healthy synovial fluid, tends to decrease in concentration in inflamed or metabolically compromised joints such

as those affected by OA. Intra-articular administration of HA has been widely used to alleviate OA symptoms (Budsberg *et al.,* 2006; Lee *et al*., 2023).

Without a suitable delivery medium, MSCs may exhibit reduced viability, impaired functionality, or unintended differentiation—factors that can significantly compromise therapeutic efficacy (Sultana *et al.,* 2022). Therefore, the chosen carrier medium must not only maintain cell viability and phenotype but also counteract these detrimental factors in joint environment, fostering conditions favourable to anti-inflammatory and regenerative actions (Brondeel *et al*., 2021; Ivanovska *et al.*, 2022; Przadka *et al.,* 2021; Rowland *et al*., 2021; Sultana *et al*., 2022; Fujita *et al.*, 2020; Bharti *et al*, 2021; Ngo *et al*., 2021).

Optimised carrier media for canine osteoarthritis treatment

An optimised injection medium should meet several critical criteria to ensure both the safety of the host joint and the efficacy of the MSCs:

1. **Safety and Non-Toxicity:** The medium must be free of potentially cytotoxic elements and free from infectious agents, including bacteria (EMA, 2017; EMA, 2019). Moreover, it is recommended to avoid xenogeneic components in order to eliminate possible zoonotic pathogens and to prevent potential reduction in the therapeutic efficacy of MSCs (Fernández-Santos *et al*., 2022; Rowland *et al*., 2021; EMA, 2009; EMA, 2019). Xeno-free media formulations are being developed and are increasingly preferred in clinical settings to ensure that the treatment is both safe and compliant with regulatory standards (Shih and Burnouf, 2015; Astori *et al*., 2016; Ivanovska *et al*., 2022).

2. **Cell Viability and Potency:** The medium should maintain cell viability at an acceptable level. Fresh MSCs products typically exhibit viability between 85-90%; however, viability may decrease during storage and transport. In this study, a threshold of > 70% viability at the time of administration is established as acceptable. Research indicates that factors such as osmolarity, nutrient availability and the type of carrier solution play critical roles in maintaining stem cell viability, integrity and functional potency during preparation and storage (Lima *et al.*, 2019; Fujita *et al.,* 2020; Rowland *et al*., 2021; Sultana *et al.*, 2022; Andreoli *et al*., 2024). Furthermore, the inclusion of GFs in the medium has been shown to improve cell viability and enhance the regenerative chondrogenic potential of MSCs (Kazemi *et al*., 2017; Bianchini *et al*., 2018; Gilbertie *et al*., 2018).

3. **Anti-Inflammatory Properties:** Since inflammation is a major component found in OA joints, the medium should ideally have anti-inflammatory properties that support the cells modulating the joint environment, reducing pain and preventing further damage to the cartilage (Yunus *et al.,* 2020). Incorporating anti-inflammatory or protective agents into the medium can synergise with the MSCs' own immune-modulatory effects to achieve better clinical outcomes (Burdick and Prestwich, 2011).

4. **Accessibility and standardisation:** As one of the challenges of regenerative medicine therapies is standardisation, the culture and carrier medium should be readily available and accessible across different countries to ensure widespread applicability (Kang and Park, 2020; Ivanovska *et al*., 2022).

Ringer Lactate, hyaluronic acid and Platelet Lysate

Ringer's Lactate (RL) is a widely used intravenous solution that provides water and three cations (calcium, sodium and potassium). It is commonly administered intravenously for hydration and electrolyte replacement in clinical situations (AEMPS, n.d.). Additionally, RL is extensively employed for irrigation purposes, including surgical lavages, wound irrigation, joint lavage, and as an arthroscopic fluid, as it does not harm cells (Shinjo *et al.*, 2002; Buffa *et al.*, 1997). Moreover, when compared to saline solution in studies on human meniscus tissue, RL demonstrated superior preservation of meniscal integrity (Shinjo *et al*., 2002).

Hyaluronic acid (HA), or hyaluronan, is a biological polysaccharide naturally present in mammals and other animals. It plays an essential role in various cellular functions and tissue formation. Since its discovery and subsequent production, HA has been widely used in clinical applications, particularly in aesthetic medicine as a dermal filler, and in orthopaedics as a joint lubricant (Burdick and Prestwich, 2011; Gupta *et al*., 2019). However, its levels are often diminished in inflamed or osteoarthritic joints (Budsberg *et al.*, 2006; Lee *et al*., 2023).

In canines and other animals, HA has long been considered the gold standard for intra-articular treatment for OA symptoms due to its chondroprotective properties and lubrication (Gupta *et al*., 2019; Lee *et al*., 2023; Marshall *et al*., 2000; Neuenschwander *et al*., 2019). When combined with cMSCs, HA may further enhance therapeutic outcomes, as it has been shown to possess regenerative effects, making it a valuable additive to support a reparative intra-articular environment (Li *et al*., 2018).

PCs such as PRP and PL are already used products in intra-articular therapies (Cuervo *et al*., 2014; Catarino *et al*., 2020; Lee *et al*., 2019). These components, rich in GFs, support cell proliferation and promote chondrogenesis (Lima *et al*., 2019; Kazemi *et al*., 2017), while also stimulating synoviocyte activity and HA production (Gilbertie *et al*., 2018), which may enhance cell viability and alleviate OA symptoms.

All three components—RL, PCs, and HA—could potentially be used in cell cultures and as carrier media, as they are non-cytotoxic and are presumed to support cell growth, promote cartilage regeneration, and reduce OA-related inflammation and symptomatology.

Objective

The principal aim of this research is to identify the variables that affect proliferation rates and viability of cMSCs when stored in ready-to-use media. Specifically, the study seeks to determine the optimal medium for use as a transportation medium prior to clinical application by comparing various ready-to-use formulations: (i) Ringer Lactate (RL), (ii) RL supplemented with Hyaluronic Acid (HA), (iii) Platelet Lysate (PL) at 6% in RL, and (iv) PL at 6% in RL supplemented with HA, intended for intra-articular injection of cMSCs as a treatment for canine OA.

Furthermore, this study aims to demonstrate that cMSCs can maintain their proliferative capacity, exhibit high viability rates, and preserve their identity as cMSCs after being stored in different ready-to-use suspension media for more than 24 hours, enabling direct application to the canine patient. The focus is on determining the duration for which cells can be stored in each medium and temperature while retaining their viability and key characteristics.

The hypothesis of this study is that cMSCs stored in an optimised carrier medium, such as RL supplemented with PL and/or HA, will maintain a viability rate exceeding 80% within the first 48 hours under cold storage conditions. If confirmed, this approach could provide a cost-effective and practical solution for cMSCs storage, transport, and intra-articular administration, ultimately enhancing the therapeutic potential of stem cell-based treatments for OA.

Materials and Methods

Study approval, cMSCs culture and characterisation

Canine Mesenchymal stem cells (cMSCs) used in this study were thawed from *Bone Regeneration Research Group's Animal Stem Cell Bank UAB* (Universitat Autònoma de Barcelona; Bellaterra, Spain) where they had been previously cryopreserved.

The origin of canine adipose-derived mesenchymal stem/stromal cells (cAD-MSCs) was guaranteed from a healthy canine donor. The cells were obtained laparoscopically from falciform ligament fat during an elective ovariectomy. Prior to their use in this study, cMSCs were characterised according to the minimal criteria established for canine MSCs (Guest *et al*., 2022), which include tri-linear differentiation potential, plastic adherence, lack of Major MHCII expression, two positive surface antigen expression (CD90, CD44) and two negative surface antigen expression (CD34, CD45). Additionally, cells were assessed for morphology, genetic profile (NANOG and OCT4), and viability, as previously described by *Herrera et al*. (Herrera *et al*., 2023). After resuspension in the carrier medium, cells were characterised at 24 and 72 hours by flow cytometry to evaluate cell surface markers (CD90, CD45 and MHCII), in order to confirm

the maintenance of their stem cell identity. Finally, following recovery from cell culture, cells were re-characterised for positive CD90, CD44, and negative CD34, CD45, and MCHII expression.

Culture seeding and expansion of cMSCs

Cells were thawed out from sixteen cryovials of canine adipose-derived mesenchymal stem cells from the same donor at a concentration of 1 million of cells per vial at second passaging. Media culture was 500 ml low-glucose DMEM (*Dubelcco Modified Eagle Media; Gibco. Thermo-Fisher. Carlsbad, US*) with 10% FBS (Foetal Bovine Serum; *Gibco. Thermo-Fisher. Carlsbad, US)*, 100ml of anti-anti (*antibiotic-antimycotic 100x; Gibco. Thermo-Fisher. Carlsbad, US*), and 5ml of L-glutamine (L-glutamine 200mm; *Gibco. Thermo-Fisher. Carlsbad, US*). Cells were cultured in T75cm flasks. At 24h, media was changed and the 70 - 80% of confluency was reached at 72h *(Figure 1)* when cells were reseeded at 1:3 ratio. On the 7th day of expansion, cells were ready to be used on the experimental study with different ready-to-use carrier media.

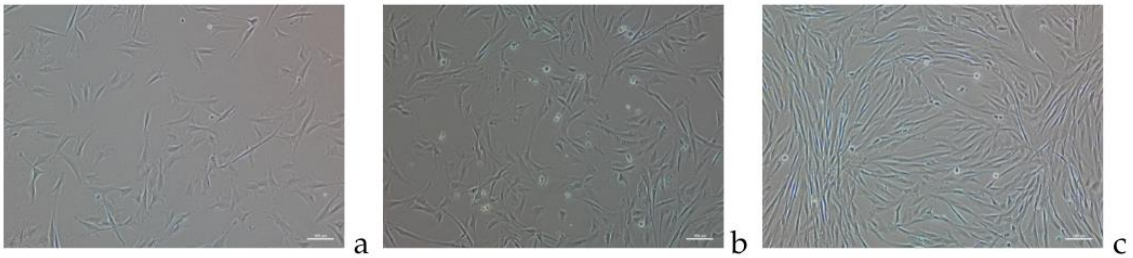

***Figure 1. Microscopical images** of **canine mesenchymal stem cell culture** expansion showing its spindle-shape morphology, plastic adherence, proliferation and confluence (scale bar: 100mm) (a) 24 hours after thawing (b) 48 hours after thawing (c) 72 hours after thawing.*

Ready-to-use and carrier medium

Four different carrier ready-to-use media were prepared aseptically in a laminar flow cabinet with sterile micropipettes and stored into sterile Falcon tubes. All components used in media preparations were either inherently sterile or underwent sterilisation processes to ensure their safety. Moreover, selected media were all designed to be biocompatible and beneficial for the canine joint environment.

Ringer's Lactate (RL)

First carrier media used was Ringer's Lactate (Lactate-RingerVet, Braun; *Melsungen, Germany*), an injectable solution containing lactate and bicarbonate ion with an osmolarity similar to plasma.

Ringer's Lactate + 0.04% HA (HA)

This medium was prepared by adding 1.18mL of HA preparation which contains 17mg HA/ml (H*Y-50-VET; Dechra. Northwich, UK*) into 50ml of Ringer Lactate. Thus, obtaining a Ringer Lactate supplemented with HA at 0.04% media.

Platelet Lysate 6% Ringer's Lactate (PL)

Platelet Lysate (PL) used for this treatment was a pooled canine PL obtained by a standardised protocol (Montolío *et al*., 2025). All canine donors were approved as blood donors (Authorisation procedure number #5075). This PL was obtained from a leukoreduced-PRP via two-step centrifugation process to avoid potential inflammation, and platelet lysis was performed by freeze-thaw cycles. Their growth factors (PDGF-BB, VEGF-A and TGF-β) were measured to ensure this

was a rich-GF product (Montolío *et al*., 2025 and Chapter 2 thesis). Additionally, PL was filtered through a 0.22-µm microporous syringe filter (Whatman UNIFLO syringe filters; Buckinghamshire, UK).

For this medium preparation, 0,9 ml of PL were solved into 15ml of RL. Thus, obtaining a RL at 6% PL.

Platelet Lysate 6% + HA 0.04% Ringer's Lactate (PL+HA)

For this carrier medium, 0.9 ml of PL and 0,35 mL of HA preparation were diluted into 15ml of RL.

Product storage and carrier media assay

Cultured and expanded cMSCs were prepared by centrifugating and resuspending them at the desired concentration of 2 x 10^6 cells/ml into Eppendorf tubes for the different media, temperature condition and times. Each condition was prepared for two temperatures—at room temperature (21.5°C ± 1.5) and at cold temperature (5°C ± 1) —for four time points (0, 24, 48, and 72 hours), and in triplicate *(Figure 2)*. Each tube consisted on 0,5ml of media at a concentration of 2x 10^6 cells/ml.

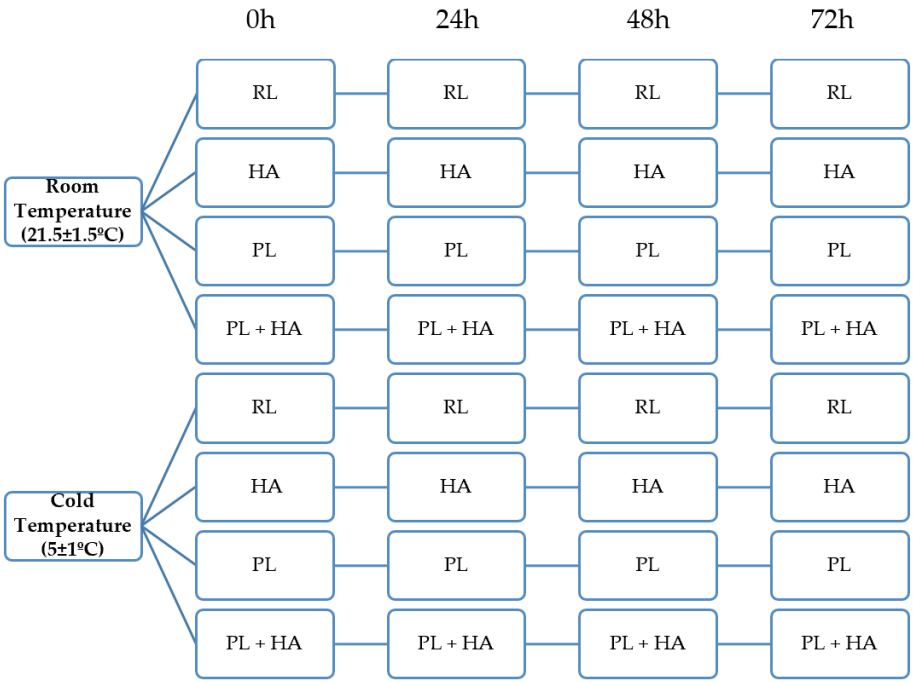

Figure 2. Carrier ready-to-use media groups *(RL; RL+HA; RL+PL; RL+PL+HA) at four times (0 to 72 hours) and two temperature (cold and room temperature) conditions. Each condition consisted of 3 triplicate samples.*

Viability (Trypan blue + 7-aad)

During the experimental study, cell viability was measured every 24 hours up to 72 hours (each time point) for each temperature and medium condition. It was measured in triplicate by two distinct parallel methods: Trypan Blue exclusion staining with manual counting in a Neubauer chamber under microscopy (*Eclipse TS100; Nikon, Japan*) made in duplicate and, 7-aminoactinomycine-D (7-AAD) staining, detected by flow cytometry (*Cytoflex LX; Beckman Coulter. California, USA*), which marked and detected dead or damaged cells (non-viable). Viability was calculated using this formula:

Cell viability (%) = (total cell count – non-viable cell count) / (total cell count) x 100

Cell recovery assay (Confluence capacity)

Following storage under each experimental condition (time, temperature and medium), cell recovery was assessed by confluence. cMSCs were collected and seeded into 24-well plate at a 20.000 cells/well density, and incubated at 37°C with 5% CO_2. Confluency was evaluated every 24 hours over a 120-hour (5-day) period by analysing randomly selected images obtained manually by microscopy (*Eclipse TS100; Nikon, Japan*). Percentage of confluency was quantified automatically after applying a preset for thresholding the images using ImageJ software (1.x., NIH), with a mean of 3 different randomised images confluency of each sample condition (*Figure 3*).

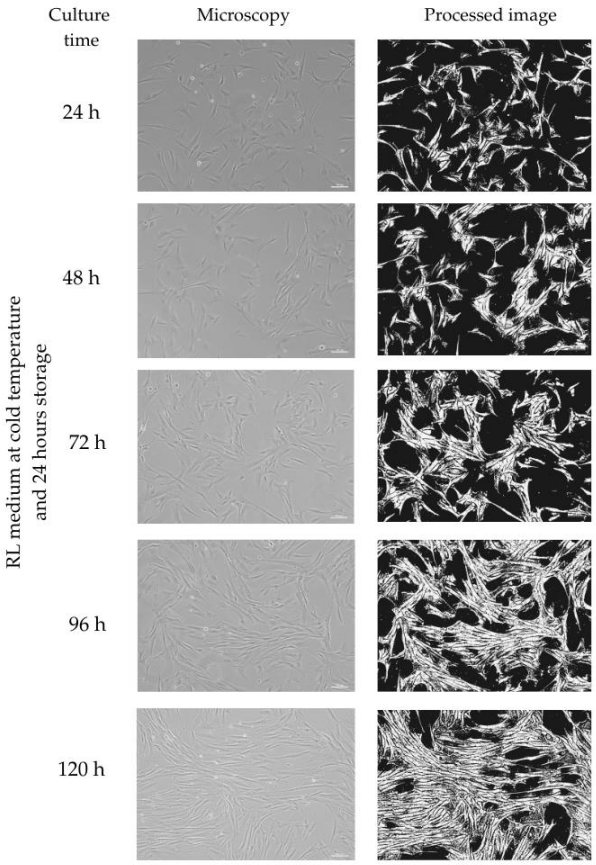

Figure 3. Microscopic images of cell recovery culture *after 24-hours storage in RL medium at cold temperature (4°C), along with* ***processed images*** *using ImageJ software for automatic calculation of cell confluency percentage. Scale bar: 100µm.*

Colony-Forming Unit (CFU) assay

Colony-Forming Units capacity was evaluated by seeding cells from each condition at a density of 500 cells/well in 6-well plates (9.4 cm^2 per well = 2127.6596 cells/cm^2), which were cultured for 3 weeks at 37°C in 5% CO_2. On the 10^{th} day, the medium was changed and CFUs were evaluated the 21^{th} day. Culture was stained with 0.3% Crystal Violet, which was prepared from 0.15 g of Methylrosanilinium chloride (*Genciana Violet – Metilrosanilina Clorur 97.5% purity; Roig Farma SA. Terrassa, Barcelona)* solved in 40ml of distilled water and 10ml of Methanol (MeOH). One millilitre of Crystal Violet solution was applied per well and incubated for 30 minutes at room temperature. After that, each well was washed with 2ml of PBS and colonies were photographed by a macro telephoto lens and counted by ImageJ Software (1.x., NIH). Colonies were counted manually including these round-shaped clusters which were formed by 50 cells or more (*Figure 4*).

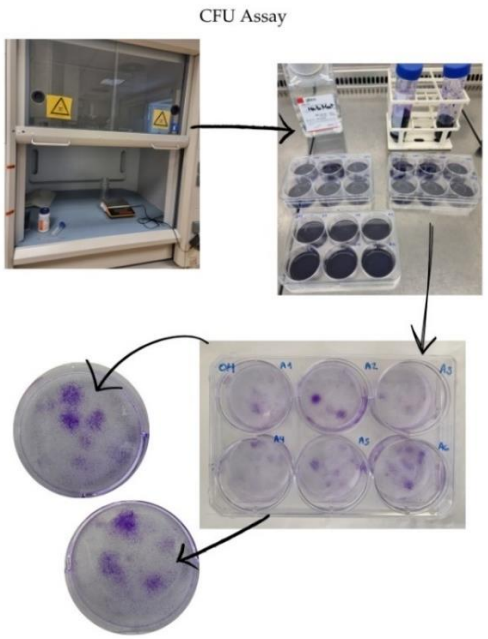

***Figure 4. CFU assay procedure**, including Gencian Violet staining, culture fixation, and macroscopic imaging using a macro lens. All steps were performed under a laminar flow cabinet following GMP.*

Statistical analysis

All data collected were processed and analysed using IBM SPSS statistics (29.0 version. IBM, New York, USA). Normality of data was evaluated using the Shapiro-Wilk test for sample sizes below 50 and the Kolmogorov-Smirnov test for larger datasets. As the data did not follow a normal distribution, group comparisons were conducted using the Kruskal-Wallis test, a non-parametric test, and pairwise comparisons were conducted using the Mann Whitney U test as a post-hoc analysis. Statistical significance was defined as $p\text{-}value < 0.05$.

Results

Cell characterisation

Cells exhibited plastic adherence and a characteristic spindle-shaped morphology in cell culture (*Figure 5*). Flow cytometry analysis was performed at 24 and 72 hours after resuspension in carrier media to evaluate the expression of CD90, CD45 and MHCII surface markers. Cells were positive for CD90 at both 24 hours (76.29% mean expression in cell population) and 72 hours (74.73% mean expression), while they remained negative for CD45 (0.3%) and MHCII (1.72%) at 72 hours.

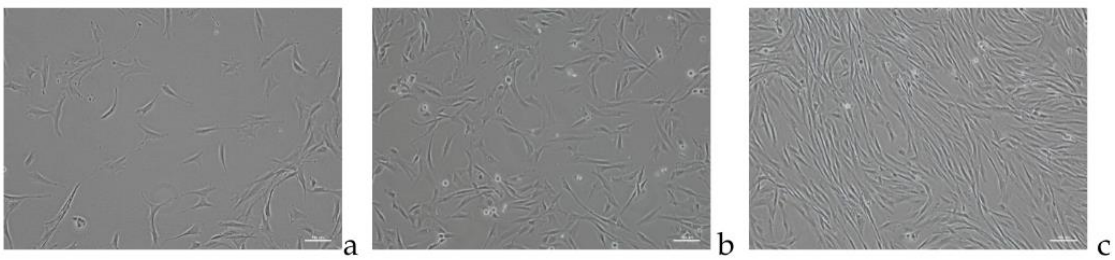

Figure 5. ***Microscopic images of cMSC culture expansion*** *demonstrating spindle-shaped morphology, plastic adherence, proliferation, and confluence at different time*

points post-thawing: (a) 24 hours, (b) 48 hours, and (c) 72 hours after thawing (scale bar: 100µm).

Additionally, flow cytometry analysis was performed to assess the expression of CD45, CD44, CD90 and CD34 in cells after reaching confluency, eight days post-seeding. The results showed positive expression for CD44 (99%) and CD90 (73%), and negative expression for CD34 (0%) and CD45 (0%) (*Figure 6*).

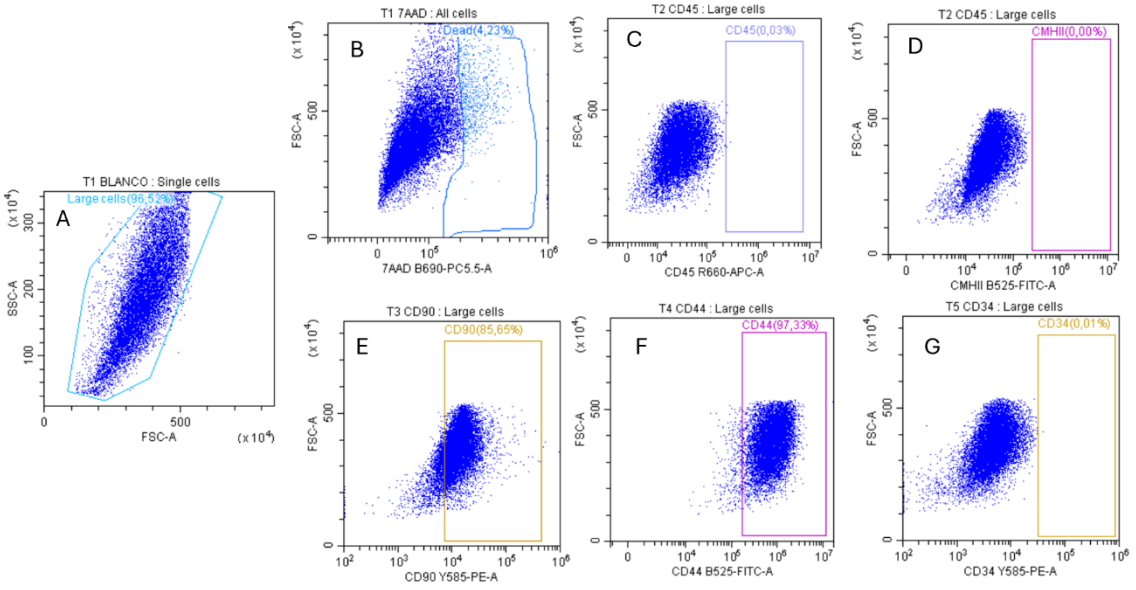

Figure 6. Representative flow cytometry plots *showing the expression of specific surface markers and viability staining in canine mesenchymal stem/stromal cells (cMSCs): (a) Unstained control cells, (b) 7AAD, (c) CD45, (d) MHCII, (e) CD90, (f) CD44, and (g) CD34.*

Viability

Viability of cells was measured by two different methods and percentage of viability was obtained for each condition and time point. Initial time point (0h) was used as control between two methods resulting in a mean of 96.17% via manual counting and 93.12% via 7-AAD staining. Although the results presented

a minor difference, showing a slightly lower viability with the 7-AAD method, both methods presented a correlation (r = 0.847; p = 0.000) and produced comparable results (*Figure 7*). Overall mean viability of cells determined by manual counting was 87.72±13.08%, while mean viability by 7-AAD staining was 85.40±10.44%.

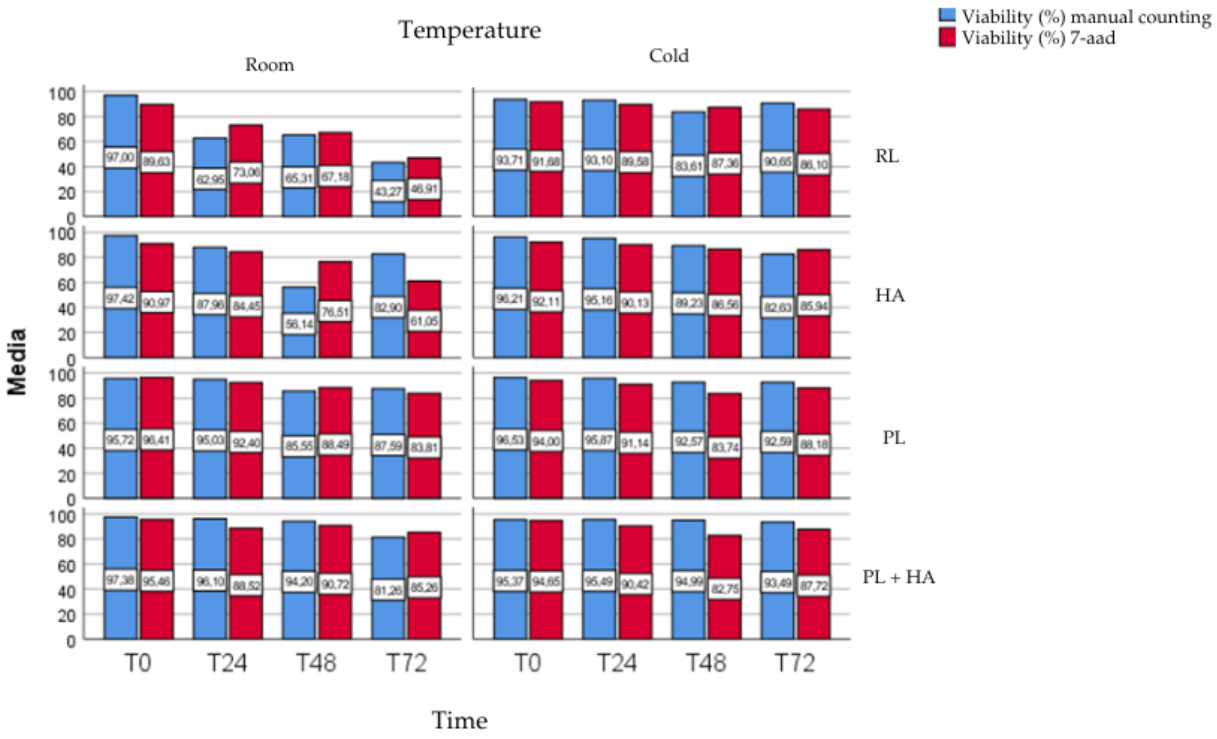

Figure 7. Cell viability *assessed by manual counting and 7-AAD staining across all experimental groups (ready-to-use carrier media), time points, and temperature conditions.*

Cell viability, measured by both manual counting and 7-AAD staining, was higher in groups stored at low temperatures than in those supplemented with 6% PL. Conversely, viability progressively decreased with increased storage duration (*Figures 7, 8 and 9*).

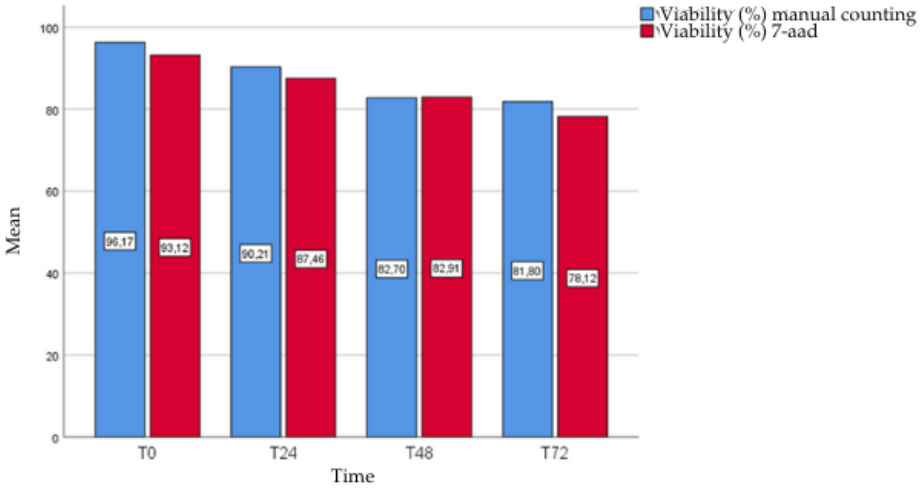

Figure 8. Viability media *of each time, comparing both viability-counting methods: 7-AAD flow cytometry, and manual counting by trypan blue staining. Viability obtained by both methods decreased over time.*

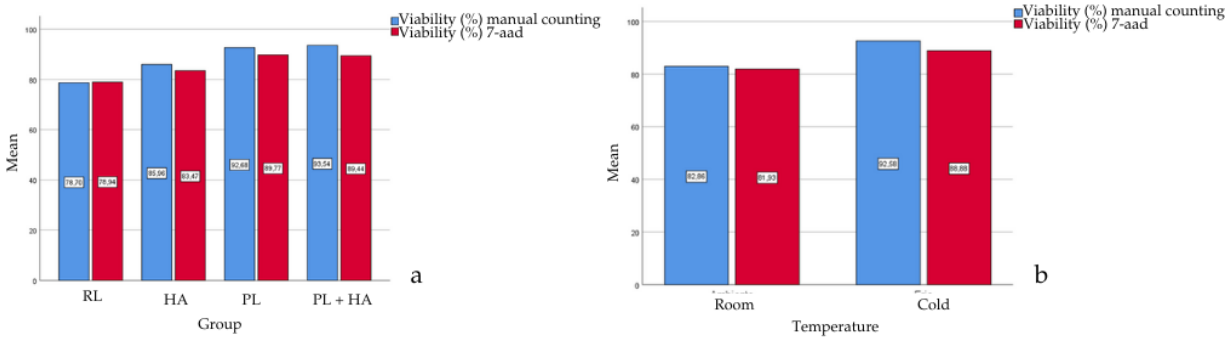

Figure 9. Percentage of cell viability*. (a) Mean viability over different groups (carrier ready-to-use media) obtained by manual counting and 7-AAD. (b) Mean viability of samples preserved at room temperature and at cold temperature, obtained by manual counting and 7-AAD staining.*

Trypan blue manual counting

Mean cell viability calculated by trypan blue manual counting was similar between four groups at both temperatures at time 0h, ranging from 93.71% to 97.42% (*Figure 7*). Manual counting viability decreased over the time period,

independent of the group or temperature condition. Time is a significant variable in manual cell counting viability ($p = 0.000$) (*Figure 7*).

Samples maintained at cold temperature showed a higher viability in most of cases, independent of the group and time, except at 0h *(Figures 7 and 9b)*. Although this difference was not statistically significant ($p = 0.309$).

Viability assessed by trypan blue manual counting was compared among the different delivery media groups. The lowest viability was observed in the RL group (T0h: 97.00 ± 2.74%; T24h: 62.95 ± 2.27%; T48h: 65.31 ± 27.12%; T72h: 43.27 ± 39.59%), followed by the HA group (T0h: 97.42 ± 2.93%; T24h: 87.96 ± 6.19%; T48h: 56.14 ± 9.51%; T72h: 82.90 ± 12.50%), particularly when stored at room temperature. The highest viability was maintained in the Platelet Lysate (PL) group under cold temperature conditions (T0h: 96.53 ± 1.47%; T24h: 95.87 ± 1.98%; T48h: 92.57 ± 1.61%; T72h: 92.59 ± 5.43%). However, these differences between groups did not reach statistical significance ($p = 0.246$) (*Figures 7 and 9a*).

7-aad cytometry

Cell viability assessed by 7-AAD flow cytometry was comparable to that obtained through manual counting. At time 0h, all groups—whether stored at cold or room temperature—exhibited similar viability percentages measured by 7-AAD, ranging from 89.63% to 96.41% (*Figure 7*).

The viability decreased over the time, regardless of the carrier media group or temperature conditions. Time was identified as a significant variable influencing cell viability, as measured by 7-AAD ($p = 0.000$) (*Figures 7 and 8*). Although groups stored at low temperatures consistently showed higher viability than those kept at room temperatures—particularly in RL and RL+HA groups—temperature did

not have a statistically significant effect on cell viability assessed by 7-AAD manual counting ($p = 0.274$) *(Figures 7 and 9b).*

No significant differences in cell viability were detected between the various carrier media groups ($p = 0.246$). Although RL and HA tended to exhibit lower viability—particularly under room temperature conditions, where a progressive decline was noted over time—these variations did not reach statistical significance (*Figures 7 and 9a*).

At 24 hours, the highest viability assessed by 7-AAD cytometry at room temperature was observed in the PL group (92.40±2.25%). At cold storage conditions, PL (91.14±1.95%), HA (90.13±2.55%) and PL+HA (90.42±4.12%) groups exhibited comparable values. By 48 hours, the highest viability at room temperature was maintained in the PL (88.49±2.32%) and PL+HA (90.72±1.90%) groups, while viability in RL (67.18±28.88%) and HA (76.51±2.88%) groups dropped below 80%. Under cold storage, RL (87.36±3.23%) and HA (86.56±1.21%) maintained acceptable viability rates, similar to PL (83.74±3.11%) and PL+HA (82.75±2.22%). At the 72-hour time point, PL (83.81±1.24%) and PL+HA (85.26±1.25%) were the only groups at room temperature maintaining acceptable viability, as RL (46.91±36.21%) and HA (61.05±25.88%) exhibited a significant decline. In contrast, under cold conditions, all groups demonstrated similar viability at 72 hours: RL 86.10±1.00%, HA 85.94±5.46%, PL 88.18±3.25%, PL+HA 87.72±1.95% (*Figure 7*).

Colony Forming Unit (CFU) Assay

CFU capacity data was collected and statistically analysed (*Annex 1*). CFU data obtained showed that it was not influenced by time ($p = 0.906$). Over time, CFU

capacity decreased in the RL and HA groups at room temperature, and CFU mean also decreased (*Figure 10).*

Different media influenced CFU capacity. The highest CFU mean was observed in PL+HA group (4.63±0.73), while RL group exhibited the lowest CFU formation (2.4±1.49). HA (3.46±1.57) and PL (3.94±0.76) groups showed similar CFU mean. This difference was statistically significant (p = 0.009). RL group showed significant lower CFU formation than PL+HA group under both room temperature (p = 0.017) and cold storage conditions (p = 0.02). Additionally, under cold storage, HA group also exhibited a significant lower CFU count compared to PL+HA group (p = 0.039) (*Figures 10 and 11*).

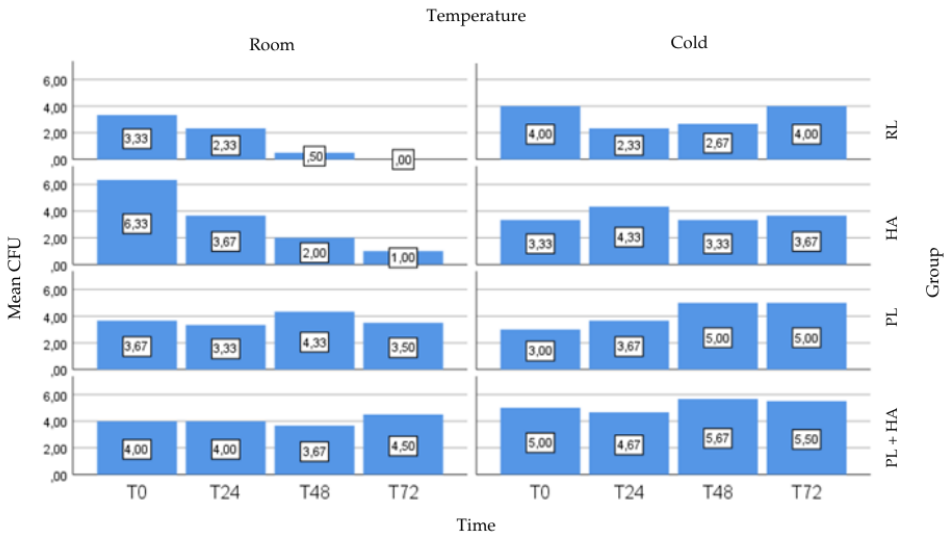

Figure 10. Mean CFU formation *at different storage time points and temperature conditions across media groups (ready-to-use media).*

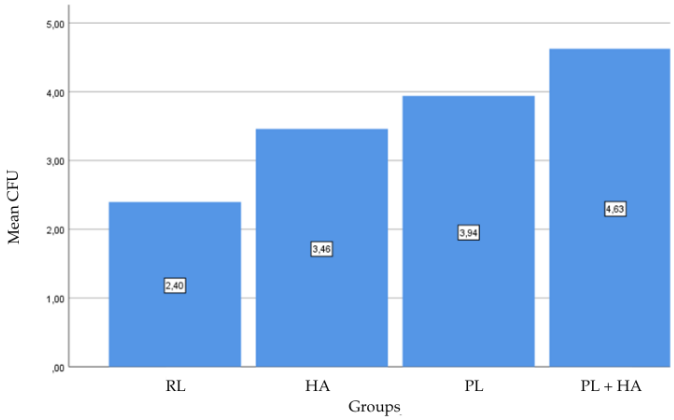

***Figure 11**. **Mean CFU** among groups with different ready-to-use carrier media.*

Storage temperature was another factor affecting the CFU formation. Cold temperature groups exhibited a higher mean CFU count (mean: 4.07±1.01) compared to room temperature groups (mean: 3.14±1.62) (*Figures 10 and 12)*. Temperature had a significant effect within specific media groups, namely PL (p = 0.043) and PL+HA (p = 0.02), showing higher CFU. Additionally, the RL group at room temperature exhibited significantly lower CFU counts than both PL (p = 0.021) and PL+HA groups (p = 0,000) stored under cold conditions. Similarly, HA and PL groups at room temperature showed significantly lower CFU formation compared to PL+HA group stored at cold temperature (p = 0.023 and p = 0.049, respectively) *(Figure 10)*.

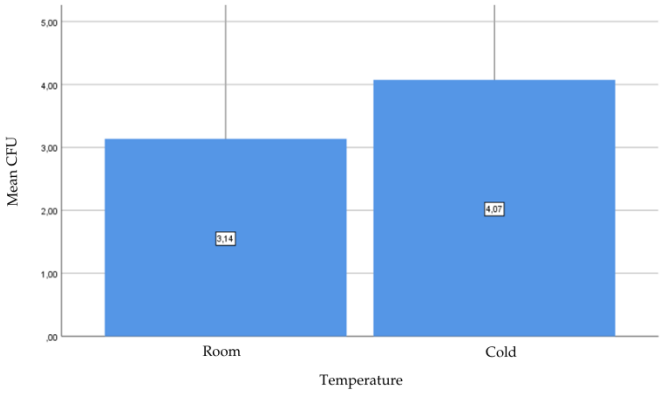

***Figure 12**. **Mean CFU** at room temperature and cold temperature conditions.*

HA+PL at cold storage (T0: 5±2; T24h: 4.67±1.53; T48h: 5.67±1.15; T72: 5.5±0.71) showed the highest CFU capacity, followed by HA+PL at room temperature (T0: 4±3; T24h: 4±0; T48h: 3.67±0.58; T72: 4.5±0.71) and PL group at cold storage (T0: 3±1; T24h: 3.67±1.53; T48h: 5±1; T72: 5±0). RL stored at room temperature (T0: 3.33±1.53; T24h: 2.33±1.53; T48h: 0.5±0.71; T72: 0±0) exhibited the lowest CFU capacity. Furthermore, RL and HA groups at room temperature showed similar CFU capacity to others at 0 and 24 hours, but it decreased at 48 and 72 hours *(Figure 10).*

Confluence and proliferation capacity assay

Proliferation assays to assess recovery capacity after storage showed a progressive increase over the culture period, regardless of the ready-to-use media, storage duration, or temperature conditions (*Figure 13, Table 1 and Annex 1*).

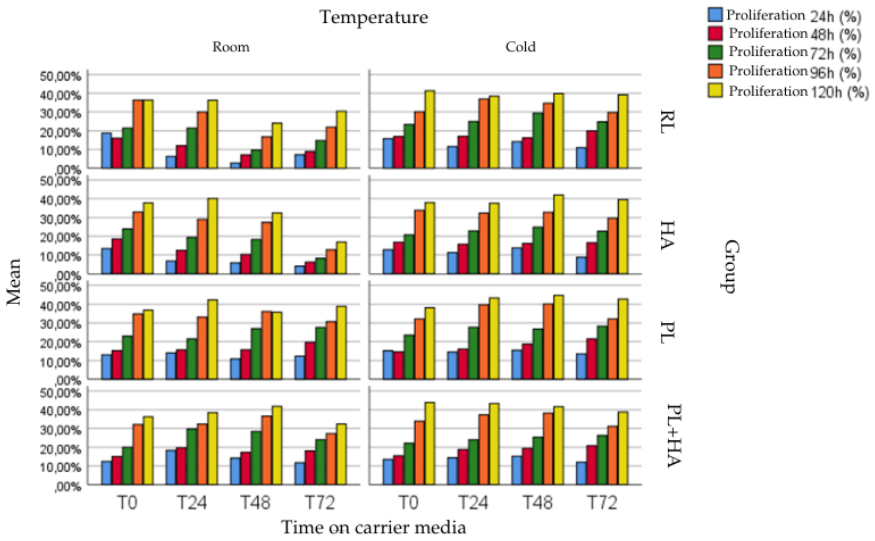

Figure 13. Graphical representation of mean proliferation rate (%) *at each time point, categorised by carrier ready-to-use media group, temperature conditions and storage duration.*

Storage time period (hours)	Carrier media	RL	RL	HA	HA	PL	PL	PL+HA	PL+HA
	Storage temperature	Room	Cold	Room	Cold	Room	Cold	Room	Cold
	Proliferation time (hours)								
0	24	18.75 ±4.3	15.84 ±3.76	13.44 ±3.48	12.83 ±1.34	13.01 ±2.2	15.22 ±4.39	12.40 ±2.98	13.52 ±2.45
	48	16.03 ±2.98	16.90 ±3.12	18.61 ±3.35	16.78 ±4.68	15.16 ±1.98	14.60 ±2.17	14.99 ±1.17	15.42 ±1.73
	72	21.35 ±3.86	23.31 ±2.29	23.86 ±3.54	20.68 ±3.14	22.96 ±3.91	23.55 ±5.65	20.01 ±1.72	22.07 ±2.74
	96	36.37 ±6.22	30.00 ±5.71	32.87 ±5.69	33.80 ±4.38	34.80 ±5.37	32.14 ±2.44	32.18 ±7,58	33.90 ±2.21
	120	36.36 ±5.87	41.31 ±5.93	37.81 ±3.78	37.94 ±6.37	36.89 ±4.08	38.10 ±4.85	36.24 ±4.00	43.83 ±9.38
24	24	6.30 ±1.27	11.60 ±1.47	6.79 ±1.67	11.35 ±1.68	14.05 ±4.83	15.53 ±2.68	18.30 ±4.61	14.36 ±2.90
	48	12.05 ±2.72	17.05 ±2.63	12.43 ±3.00	15.76 ±3.29	15.64 ±2.00	16.13 ±1.92	19.71 ±6.96	18.75 ±3.60
	72	21.41 ±4.47	24.93 ±2.31	19.42 ±3.39	22.90 ±4.99	21.56 ±5.94	27.67 ±5.14	29.75 ±8.50	23.94 ±3.23
	96	29.95 ±5.40	36.91 ±6.33	28.92 ±2.42	32.31 ±2.77	33.11 ±6.72	39.67 ±4.95	32.40 ±4.66	37.30 ±4.00
	120	36.29 ±4.66	38.43 ±3.46	40.09 ±5.48	37.58 ±3.67	42.26 ±4.26	43.30 ±2.14	38.39 ±5.04	43.36 ±6.11
48	24	2.78 ±0.47	14.20 ±3.41	5.72 ±2.40	13.77 ±2.10	10.84 ±2.26	15.45 ±1.68	14.22 ±4.64	15.16 ±3.38
	48	7.09 ±1.23	16.18 ±2.95	10.29 ±3.78	16.17 ±2.46	15.65 ±2.77	18.70 ±2.57	17.25 ±4.91	19.39 ±3.17
	72	9.64 ±4.90	29.32 ±5.90	18.26 ±4.52	24.82 ±4.37	27.02 ±5.29	26.73 2.58	28.44 ±3.55	25.38 ±2.89
	96	16.71 ±9.05	34.70 ±3.64	27.35 ±7.02	32.71 ±2.63	36.09 ±2.68	40.11 ±3.89	36.58 ±5.54	38.18 ±4.54
	120	24 ±8.52	39.81 ±3.32	32.42 ±6.68	42.02 ±4.12	35.66 ±6.57	44.76 ±5.25	41.76 ±4.28	41.60 ±3.98
72	24	7.20 ±2.00	10.96 ±1.16	4.06 ±0.64	8.78 ±1.34	12.25 ±6.39	13.52 ±1.25	11.69 ±3.96	11.95 ±3.01
	48	8.95 ±2.53	19.94 ±2.85	6.18 ±1.43	16.52 ±3.56	19.72 ±8.03	21.56 ±3.59	17.99 ±4.91	20.79 ±1.57
	72	14.76 ±4.64	24.73 ±4.91	8.19 ±3.12	22.74 ±3.72	27.55 ±9.58	28.28 ±2.77	24.07 ±5.25	26.20 ±5.92
	96	21.94 ±5.60	29.74 ±3.97	12.83 ±7.07	29.62 ±2.19	30.64 ±4.70	32.16 ±2.83	27.24 ±6.84	31.13 ±2.47
	120	30.36 ±3.23	39.15 ±6.16	16.86 ±7.55	39.50 ±3.44	38.82 ±4.53	42.71 ±6.06	32.42 ±4.86	38.77 ±3.88

Table 1. *Mean confluence rate (%) categorised at each time point, categorised by carrier ready-to-use media group, temperature conditions and storage duration (see Annex 1 for individual sample and repetition data).*

Time of preservation in carrier media was an independent factor affecting cell proliferation. Proliferation rates decreased when cells were stored in carrier media for extended periods. However, statistical significance for this effect was only observed at the 96-hour time point ($p = 0.019$). Cells stored for 72h showed a significant decrease in proliferation compared to those stored for 0h ($p = 0.016$), 24h ($p = 0.011$), and 48h ($p = 0.007$) (*Figure 13, Table 1*).

The type of carrier media also influenced cells' confluence capacity. Higher confluence capacity was observed in PL and PL+HA groups compared to RL and HA, particularly under room temperature. This difference was statistically significant at 72 hours ($p = 0.036$) (*Figure 13, Table 1*). At 72 hours, HA group stored at room temperature exhibited significantly lower confluence than PL+HA group ($p = 0.024$). Similarly, RL group at room temperature showed lower confluence rates compared to both PL ($p = 0.038$) and HA+PL group ($p = 0.014$).

Temperature during storage in carrier media was another factor influencing cell confluency. Cells preserved at cold temperatures showed higher confluence rates (*Figure 13, Table 1*). This effect was statistically significant at 48h ($p = 0.18$) and 120h ($p = 0.01$) groups. Specifically, in the RL group, temperature was a significant independent factor at 48h ($p = 0.021$), 72h ($p = 0.021$) and 120h ($p = 0.021$) (*Figure 13, Table 1*). In the PL group, statistical significance was observed at 24 hours ($p = 0.043$). Additionally, proliferation at both 72h and 120h in the RL group was significantly higher under cold storage compared to room temperature ($p = 0.013$ and $p = 0.032$).

Proliferation rates increased progressively over culture period, independent of the carrier media group, storage duration in carrier media, or storage temperature (*Figures 13 and 14, Table 1*).

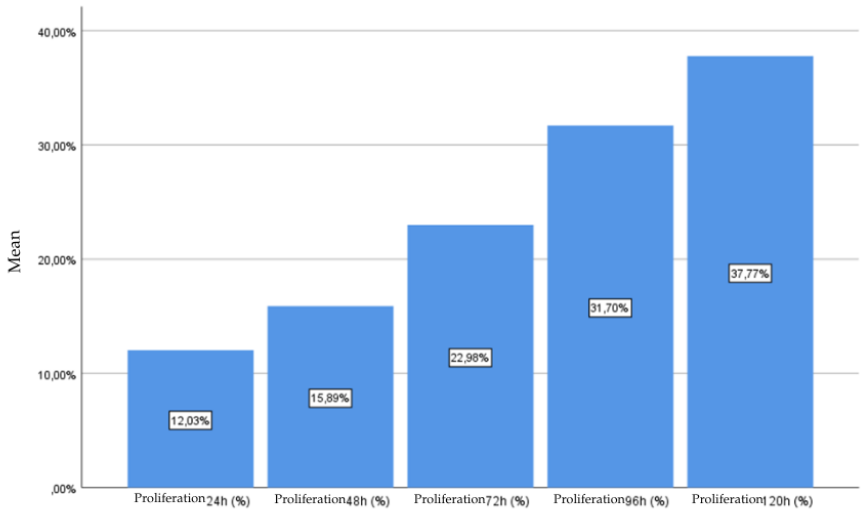

Figure 14. Mean proliferation *media of each proliferation time during recovery capacity culture (24 – 120 hours).*

The highest confluency rates were observed in PL and PL+HA groups stored at cold temperatures. However, up to 72 hours, proliferation rates in these groups were similar between room and cold storage conditions. Lowest confluency rates were found in RL and HA groups at room temperature, whereas RL and HA groups stored at cold temperatures exhibited confluency levels similar to those of PL and PL+HA groups stored at room temperature. Additionally, at the same time point, the RL group at room temperature showed significantly lower confluency compared to the PL (p = 0.016) and PL+HA (p = 0.018) groups, both stored at cold temperature (*Table 1 and Figure 13*).

Cultured cells retained their characteristic spindle-shape morphology during post-storage proliferation (*Figure 15*).

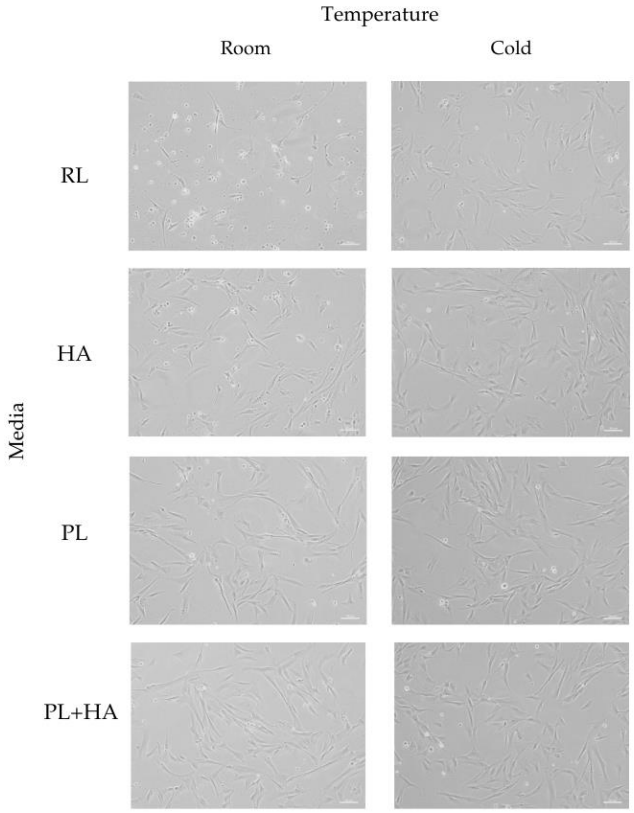

***Figure 15**. **Morphology and confluency of cMSCs** after 48 hours of culture, following 48 hours of storage in different ready-to-use delivery media (scale bar: 100 μm).*

Discussion

Developing a specialised ready-to-use medium and determining time and temperature conditions protocol for intra-articular injection of canine MSCs are essential for optimising the therapeutic effects of stem cell therapy in treating canine osteoarthritis as well as other pathologies. Despite the various preservation media proposed for cMSCs (Brondeel *et al*., 2021; Jaiswal and Vagga,

2022; Uhrig *et al*., 2022), xeno-free media especially designed for intra-articular applications have not been thoroughly evaluated in terms of their ability to preserve cell viability. In this study, RL was selected as the base delivery medium due to its established use in previous research, its accessibility, and safety profile (Andreoli *et al*., 2024; Ngo *et al*., 2021).

Such a medium should not only ensure the viability and functionality of the cells but also contribute positively to the joint environment by reducing inflammation and promoting cartilage repair as PL and HA could potentially help. By focusing on a safe, biocompatible, and bioactive medium, we can enhance the clinical efficacy of MSC therapies, ultimately leading to better management of osteoarthritis in canine patients. Further research into the composition and properties of these media is crucial for advancing regenerative medicine in veterinary applications.

The study demonstrated that temperature is a factor that influences both proliferation and CFU capacity. Cold storage conditions resulted in increased viability compared to room temperature, particularly from 48 hours onward. Although temperature appeared to affect confluency rates, statistical significance was not achieved due to limited sample size and *p-values* close to the significance threshold ($p = 0.05$). Specifically, borderline *p-values* were observed at 24h ($p = 0.06$), 72h ($p = 0.055$) and 96h ($p = 0.06$). Proliferation rate, measured as confluency, was affected by temperature in specific groups and time points, suggesting a potential benefit of cold storage in maintaining or enhancing proliferative capacity upon cell.

Cold temperature also increased CFU compared to room temperature in all groups, although this was statistically significant in only one group, which is a factor to consider. However, the inconsistency of this effect across groups may

be attributed to insufficient statistical power and the differential impact of carrier media. Despite these limitations, the observed trend suggests that cold storage should be prioritised to conserve cell potential as it has been seen that cold temperature promotes CFU, especially for prolonged cMSC preservation, in line with previous studies (Robinson *et al*., 2014; Pal *et al*., 2008).

Storage duration and time are factors that affect viability and proliferation. As storage time increased, cell viability decreased, likely due to nutrient depletion and absence of structural scaffold (Andreoli *et al.,* 2024). Furthermore, cell confluency seems to be affected, reaching statistical significance when cells were seeded for prolonged durations (96 hours), probably because of a lack of nutrients (Jung *et al.,* 2010), suggesting that storage time should be restricted to the first 48 hours in order not to affect cell potential. As time passed, the viability of cells decreased (statistically significant for 7-aad viability).

Carrier media is another factor influencing cMSCs behaviour. Confluence capacity varied depending on the type of medium, and CFU formation differed significantly across media types, with PL+HA yielding the highest number of colonies and RL the lowest, a difference that reached statistical significance at the 72-hour time point. Supplementation with PL and HA also promoted higher confluence rates compared to RL alone. These differences may be directly related to cell viability, although no statistically significant effect of the media on viability was observed, as well as to each medium's capacity to support cell adhesion and maintain functional potential under conditions of oxidative stress and/or nutrient deprivation. Previous studies have shown that MSCs exposed to nutrient-poor environments may retain viability but exhibit reduced colony-forming efficiency, likely due to diminished proliferative capacity or the onset cellular senescence (Zhang *et al*., 2017; Jung *et al*., 2010).

To achieve optimal viability and proliferation rates, maintaining cells at low temperatures (4°C) is recommended. In cases where storage at room temperature is necessary, the duration in carrier media should be kept to a minimum—ideally under 24 hours—to minimise viability loss and the associated decline in proliferative and CFU potential. The choice of delivery medium is also critical. Media supplemented with HA and/or PL demonstrated superior performance in CFU formation compared to RL alone.

Ideally, FBS-based culture systems should be avoided in favour of xeno-free cell culture media. However, when FBS is used, pre-injection lavage and replacement with a xeno-free medium is essential to improve biocompatibility and maintain cell viability (Ivanovska *et al*., 2022; Rowland *et al*., 2021).

Furthermore, selecting a carrier medium that not only preserves cMSCs but also provides joint-protective properties—such as anti-inflammatory or cartilage-regenerative effects—could substantially enhance therapeutic efficacy of intra-articular MSCs injections for canine OA.

A study conducted by Fujita *et al*. demonstrated that human MSCs stored at low temperature in RL supplemented with dextran and trehalose exhibited high viability rates -94.9% and 97.6% at 24 hours- (Fujita *et al*., 2020). These values are comparable to those observed in our study after 24 hours for RL alone (93.10%), RL supplemented with HA (95.16%), and PL (95.87%), and are consistent with findings reported by Kiet *et al*. (Kiet *et al*., 2024). Another study on cMSCs storage (Sultana *et al*., 2022) employed Hartmann's solution and saline solution, both of which share compositional similarities with RL. Additionally, dextrose solution and heparinised saline have also been employed as transport media. While these media achieved high viability—saline maintaining over 85% viability at 12 hours, a level comparable to RL at 24 hours in our study—our findings indicate that RL

supplemented with HA or PL may further enhance cell viability. Conversely, heparin in saline has been reported to enhance chondrogenic potential after 12 hours of storage (Sultana *et al.,* 2022), highlighting the need for further investigation into chondrogenic differentiation capacity of cMSCs stored in RL supplemented with HA and/or PL. Andreoli *et al*. reported that storing cMSCs in RL, saline, Platelet-rich plasma (PRP), or Platelet-poor plasma (PPP) at room temperature for 24 hours may influence their therapeutic potential (Andreoli *et al*., 2024). Therefore, further research is warranted to explore the impact of various storage conditions on gene expression profiles and functional characteristics of cMSCs.

Our findings are consistent with a previous study made by Rivera Orsini *et al*., which showed that cMSCs could be maintained in saline for up to 48 hours before clinical application (Rivera Orsini *et al*., 2024). Our results suggest that RL could serve as an accessible and standardised transport and delivery medium for short-term cold storage of cMSCs. Additionally, RL supplemented with PL may be suitable for prolonged cold storage beyond 48 hours or for up to 48 hours at room temperature, although its broader use may be limited by availability and standardisation of PL. As an alternative, RL supplemented with HA could be considered for shorter storage durations. For this reason, HA, PL and PL+HA media tested in this study could be included in future experiments as feasible delivery media for the first 48 hours, with HA being the most feasible for standardisation.

These findings suggest that a bioactive, ready-to-use medium such as RL and its supplemented variants may not only preserve cell viability and support proliferation, but also enhance the therapeutic potential of intra-articular cMSC injections by improving the joint microenvironment. However, to confirm this clinical relevance, further *in vivo* studies are required. Such studies should assess

not only clinical outcomes, but also additional functional parameters beyond viability, including the immunomodulatory capacity of MSCs, even in apoptotic states (Weiss and Dahlke, 2019). Additionally, the mechanisms of action of cMSC secretome—which may be critical for both optimising storage strategies and enhancing OA treatment—are still not fully understood and warrant further investigation. Notably, even the composition and function of exosomes may be influenced by the choice of transport medium, emphasising the importance of medium selection in both research and clinical applications (Merlo *et al.*, 2023; Saba *et al*., 2024).

The main limitation of this study was the sample size, particularly given the inclusion of three independent variables: storage time, carrier medium, and temperature. Although each experimental condition was tested in technical triplicates, achieving statistical significance in some analyses remained challenging due to the complexity introduced by these variables. Sample availability was constrained by the need to obtain cells from the same donor and passage, in order to maintain homogenous experimental conditions. Another limitation was the use of a single Ad-cMSCs lineage. While this cell source offers advantages such as ease of collection and reduced donor-site morbidity, other tissue sources may provide higher therapeutic and chondrogenic potential (Reich *et al.*, 2012; Sasaki *et al*., 2019; Yaneselli *et al.,* 2018). Additionally, future studies should include multiple lineages from diverse donors and tissue origins to minimise donor-related variability.

Cell proliferation was assessed indirectly through confluency measurements derived from automated image analysis using ImageJ software. Although this method provides an objective estimate of surface coverage and morphological integrity, it does not capture more nuanced aspects of cellular viability or functional status. Therefore, future studies should incorporate complimentary

proliferation and viability assays to better evaluate cellular responses under different storage conditions.

Conclusions

This study addresses key challenges in the short-term preservation and handling of canine MSCs following thawing under non-cryogenic conditions. By evaluating the effects of different storage media, temperatures, and timeframes, we identified practical conditions that can help preserve cell viability and phenotypic integrity. The significance of these findings lies in its translational potential: the proposed conditions are simple, cost-effective, and compatible with routine clinical workflows. As such, this work contributes to the optimisation and standardisation of MSC preparation and transport protocols, supporting more reliable and reproducible applications in veterinary regenerative medicine and cell therapy.

Our results suggest that RL-based media enriched with HA and/or PL provide a biocompatible and joint-safe solution for maintaining cMSC viability and functional potential for up to 48 hours, prior to intra-articular injection. While PL appears to be a promising delivery medium, its clinical implementation is challenged by issues related to availability and standardisation for its widespread clinical use. Among the tested formulations, HA-enriched media may represent a particularly promising alternative for developing standardised, xeno-free, ready-to-use solution for stem cell-based canine OA therapy, ensuring the preservation of cell quality during short-term storage.

Despite these advancements, further research is needed to refine the composition of carrier media—exploring varying concentrations of HA and PL—and to evaluate cells from diverse donors. Future studies should also assess the post-

storage bioactivity, chondrogenic potential of cMSCs, in order to support the development of effective regenerative therapies in veterinary medicine. Finally, establishing a standardised biobanking system for cMSCs intended for OA treatment will be essential to enhance the clinical applicability and long-term therapeutic outcomes.

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Chapter 5. Clinical safety of intraarticular therapy with an allogeneic canine adipose-derived mesenchymal stem/stromal cell-based product in dogs with elbow osteoarthritis

Introduction

Advances in veterinary medicine have underscored the need for improved strategies to manage pain and slow the progression of OA, a prevalent and irreversible DJD in dogs (Anderson *et al*., 2018*)*. OA affects an estimated 20% of dogs over one year of age in the United States, with prevalence rising to 80% in dogs over eight years of age (Johnston, 1997). More than half of diagnosed cases occur in dogs aged 8 to 13 (Esteban, 2007). Reported annual prevalence ranges from 2.5–6.6% in the United Kingdom and 6.1% in the U.S. among dogs seen in primary care settings (Anderson *et al.,* 2018; O'Neill *et al.,* 2014). More recently, a study using a clinical screening checklist estimated OA prevalence at approximately 38% in dogs visiting first opinion practices (Wright *et al*., 2022). However, the true prevalence is likely underestimated due to underdiagnosis and inconsistencies in veterinary medical record and report systems (O'Neill *et al.,* 2014).

In this context, MSC therapy has emerged as a promising alternative for managing DJD in dogs, with evidence of both its efficacy and safety (Kriston-pal, 2020; Kang *et al*., 2012; Punzon *et al*., 2022; Cuervo *et al*., 2014; Arnhold & Wenisch, 2015; Kiefer *et al*., 2016; Guercio *et al*., 2012; Li *et al*., 2025), including in systemic administration (Cho *et al*., 2024; Brondeel, 2023; Beerts, 2023). Most MSC-based treatments rely on autologous or xenogeneic cells (Punzón, 2022). However, autologous MSCs present challenges such as increased morbidity, cost and logistical complexity; while xenogeneic MSCs may pose zoonotic risks or induce

post-injection inflammation, potentially reducing their therapeutic benefit (Rowland *et al.,* 2021; Bieback *et al.*, 2010; EMA, 2008; EMA, 2011).

Cell viability and therapeutic efficacy are also influenced by storage conditions and the composition of the carrier medium used during delivery. Since intra-articular environment plays a central role in OA pathophysiology, selecting a biocompatible and joint-beneficial carrier is critical. Ringer's Lactate (RL) has shown promise in maintaining MSC viability and enhancing therapeutic outcomes (Andreoli *et al*., 2024; Fujita *et al*., 2020), particularly when supplemented with hyaluronic acid (HA), which can maintain cell viability and characteristics, as demonstrated in some studies (Ngo *et al.,* 2021) and in chapter 4 of this thesis.

Despite this, there is currently a lack of commercially available, standardised, pre-formulated carrier media specifically designed to support both MSC viability and joint health. Most clinical protocols rely on media designed for cell culture or general transport purposes, which may not be optimised for intra-articular application. HA-enriched media offer not only a supportive matrix for MSC survival but also exert anti-inflammatory and chondroprotective effects within the joint (Moreno *et al.*, 2015). Studies have demonstrated that HA can enhance MSC adhesion, proliferation, and chondrogenic differentiation, suggesting a synergistic potential when used as a carrier (Li *et al*., 2018; Chiang *et al*., 2016; Meriç *et al.,* 2025). Additionally, our study described in chapter 4 suggested that this medium maintained cell viability and function when conserved at cold temperature for short periods. The absence of such purpose-built media limits standardisation and may reduce the therapeutic consistency of MSC-based treatments. Therefore, the development of xeno-free, joint-compatible, ready-to-use formulations represents an important step toward improving clinical

outcomes and ensuring translational viability in veterinary regenerative medicine.

For these reasons, allogeneic adipose-derived cMSCs—when well-characterised and stored in a cell bank—offer a feasible and standardised therapeutic option for canine OA treatment. Delivering these cells in a xeno-free medium enriched with joint-beneficial supplements, such as HA, may enhance their therapeutic effects while ensuring safety and convenience in clinical applications.

Objectives

The main objective of this pilot *in vivo* protocol is to assess the clinical safety of an allogeneic cMSCs product formulated in a ready-to-use carrier medium supplemented by HA. In addition, the study aims to evaluate therapeutic potential of the cMSC product on clinical symptomatology in dogs diagnosed with OA following intra-articular administration.

Clinical safety will be assessed through a combination of owner-reported outcomes via standardised questionnaires, clinical evaluation by a veterinarian, measurements of joint range of motion (ROM) and radiographic analysis.

The hypothesis of this study is that cMSCs product will not result in a higher incidence of adverse effects compared to intraarticular HA injection alone. The MSC-based product is expected to reduce joint pain and improve symptoms in dogs affected by OA.

Material and methods

cMSCs ready-to-use product

To support the *in vivo* experimental study and, verify the characteristics and non-immunogenicity of the cMSCs, cells were thawed and cultured under aseptic laboratory conditions, as described in Chapter 2, 3, and 4. All procedures were conducted in a laminar flow cabinet using sterile gloves, materials and techniques.

Three cryovials containing passage 2 adipose-derived cMSCs (1 x 10^6 cells/ml) were thawed in a 37°C water bath. To eliminate residual cryopreservation medium, 9 ml of complete culture media (Low Glucose DMEM (*Dubelcco Modified Eagle Media; Gibco. Thermo-Fisher. Carlsbad, US*)) supplemented with 5ml of L-glutamine (L-glutamine 200mm; *Gibco. Thermo-Fisher. Carlsbad, US*), 10% FBS (Foetal Bovine Serum; *Gibco. Thermo-Fisher. Carlsbad, US)*, and 100ml of anti-anti (*antibiotic-antimycotic 100x; Gibco. Thermo-Fisher. Carlsbad, US*)) were added. The cell suspension was centrifuged at 300G (*Eppendorf centrifuge 5702. Hamburg, Germany*) for 5 minutes. After discarding the supernatant, 14ml of fresh culture medium were added, and cells were seeded into T75 flasks and incubated at 37°C with 5% CO_2.

Cell cultures were monitored daily under microscopy. Two days post-seeding, once cells reached optimal 80% confluency, they were subcultured at 1:2 ratio. After aspirating the medium using a sterile Pasteur pipette, 5ml of PBS were added and subsequently removed. Then, 2.5ml of trypsin solution were added for cell detaching, and cells were incubated for 2 minutes at 37°C with 5% CO_2. Upon detachment, 2.5ml complete culture medium were added to neutralise trypsin. The suspension was centrifugated into a 15ml Falcon tube at 300G for 5 minutes, the supernatant was discarded, and the cell pellet was resuspended in

4ml of culture medium. The suspension was equally divided into two flaks (2ml per flask), which were incubated for an additional 4 days. The medium was changed once during this period.

After the final medium change, cell viability and concentration were assessed using manual counting with a Neubauer chamber and DAPI staining.

The ready-to-use carrier medium consisted of Ringer's Lactate (RL) supplemented with 0.04% HA. This solution was prepared aseptically by adding 1.18ml of a veterinary HA preparation containing 17mg HA/ml (H*Y-50-VET; Dechra. Northwich, UK*) into 50ml of RL (Lactate-RingerVet, Braun; *Melsungen, Germany*), as described previously in chapter 4. A final cell concentration of 1 x 10^6 cells/ml was prepared in 2ml sterile Eppendorf tubes containing the HA-enriched RL solution. The formulated product was stored at cold temperature (4-7°C) for up to 48 hours. The control group was injected with the veterinary HA preparation (H*Y-50-VET; Dechra. Northwich, UK*).

To confirm the phenotypic stability post-expansion, flow cytometry was performed on supplicate samples for MHCII, CD90, and CD45 surface markers. Plastic adherence and spindle-shaped morphology were also visually confirmed under microscopy. In addition, samples of both the culture supernatant and the final cMSC product were submitted for microbiologic testing to ensure culture sterility and confirm the absence of contamination.

Patients and inclusion criteria / Study population

Animals from three veterinary clinics and hospitals were enrolled to this study (*Table 1*). Patients had to meet the inclusion criteria: elbow osteoarthritis with failure in other previous pharmacological treatments and absence of active infectious disease in the affected joint.

Patient	Sex	Treatment	Breed	Weight (kg)	Years
1	Male	MSC-based product	American Staffordshire Terrier	26.5	6
2	Male	MSC-based product	Mix-breed	10	10
3	Male	MSC-based product	Golden Retriever	43.2	11
4	Male	HA	Boxer	28	10
5	Female	HA	Chow Chow	22	7

Table 1. *Demographic and clinical characteristics of canine patients, including sex, type of intra-articular therapy, breed, body weight, and age.*

Five dogs were enrolled in the study and all of them were treated for both OA elbows.

Anti-inflammatory treatment by NSAIDs or corticosteroids was limited from one month before the treatment and to the duration of the study. Pain relief after procedure was limited to opiate drugs such as butorphanol in order not to interfere in cMSCs immune-modulatory effect.

Clinical Procedure

The treatment was carried out in a surgical suite under aseptic conditions. All canine patients were sedated following a standardised protocol consisting of buprenorphine and medetomidine. Anaesthesia was induced with propofol, and intravenous fluid therapy was maintained throughout the procedure.

The affected limb was aseptically prepared shaving the area and using chlorhexidine and ethanol washes. Sterile surgery drapes were applied to ensure sterile field. The intra-articular injection was performed by an experienced orthopaedic veterinarian via a lateral arthrocentesis approach to the elbow, with the needle inserted between the olecranon and lateral epicondyle.

An 18G x 50mm needle was used to access the joint cavity. First, synovial fluid was aspirated and conserved for subsequent analysis. Without removing the needle, the syringe was exchanged, and the therapeutic agent—either the MSC-based product or HA—was administered. A minimum of 2ml was injected per elbow, adjusted according to the individual dog's weight (Figure 1). This procedure was replicated in all affected elbow joints per animal, ensuring uniformity and consistency across treatments.

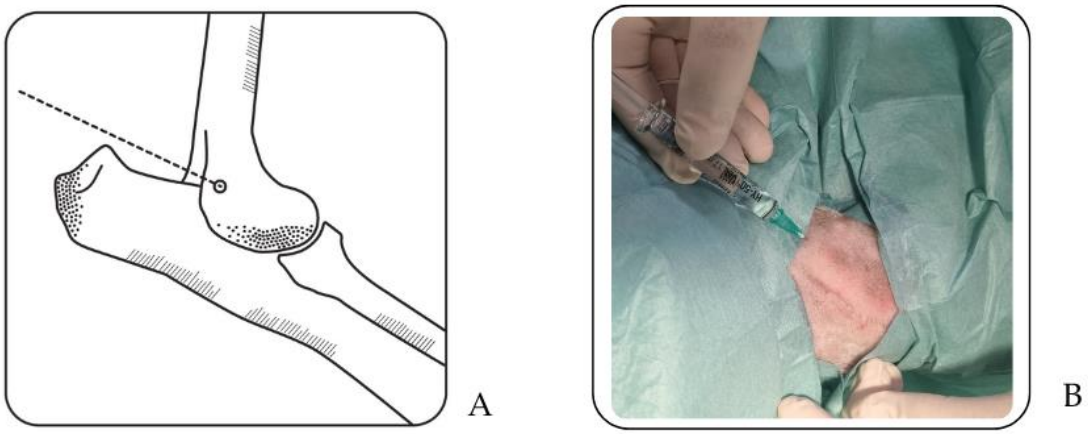

Figure 1. *Elbow arthrocentesis procedure for intra-articular treatment injection. The injection was performed using a lateral arthrocentesis approach, with needle insertion between the olecranon and the lateral epicondylar crest.*

Evaluation and follow-up assessments

All patients included in the study were evaluated using both a subjective and objective assessment method. The subjective evaluation consisted of the Liverpool Osteoarthritis in Dogs (LOAD) questionnaire (MB. Walton *et al*, 2013; M. Olcoz *et al*., 2017), completed by the owners (*Supplementary material, S1*). The objective assessment involved measuring the Range of Motion (ROM) of the affected joint. In addition, each patient underwent a physical examination by an orthopaedic veterinarian, and reports of side effects as well as pre-existing elbow radiographs were reviewed. All evaluations were repeated at the end of the study, five months post-treatment.

Although a third objective assessment method—synovial fluid analysis using cytology and flow cytometry for inflammatory markers—was initially planned, it could not be performed due to insufficient synovial fluid volume in the joints of dogs affected by OA.

Results

cMSC-based product validation

All analyses confirmed that the cMSCs maintained their phenotypic and functional characterisation prior to cryopreservation, and met the established standards for clinical use, as detailed in chapter 3 and 4 of this thesis. Moreover, samples of the cMSCs seeded for the cMSCs-based product were tested for some surface markers resulting negative for MHCII and CD45, and negative for CD90. Additionally, microbiological analyses of product samples confirmed sterility,

with no microbial growth detected, thereby validating the aseptic conditions and microbiological safety of the final product.

Side effects and exclusions

In this study, two canine patients received traditional intra-articular therapy consisting of high-molecular-weight HA, and three canine patients were treated with the allogeneic cMSCs-based product. One month after treatment application, both dogs in the HA group were withdrawn from the study due to persistent pain and lameness that required analgesic therapy, which was incompatible with the study protocol.

Following the arthrocentesis procedure, one dog of each group (Patient #2 and #5) required additional analgesia for three days. This consisted of subcutaneous butorphanol, selected to avoid interference with the potential immunomodulatory and anti-inflammatory effects of MSCs.

No local adverse events such as swelling, heat, or redness were observed at the injection site in any patient. No systemic or unexpected side effects were reported throughout the five-months follow-up period.

During the study, ROM, physical examination, LOAD questionnaire and radiographies were performed for the product safety assessment (*Figure 2*).

Figure 2. Safety assessment protocol *for the cMSC-based product, incorporating the LOAD (Liverpool Osteoarthritis in Dogs) questionnaire, range of motion (ROM) measurements, radiographic evaluations, and clinical physical examinations.*

Range of Motion (ROM) results

Patient #1 could not be evaluated for ROM due to significant behavioural and handling difficulties.

The two patients (#2 and #3) treated with cMSC-based product showed an increase in joint ROM at four months post-treatment. Patient #2 demonstrated a 22° improvement in the right forelimb and a 24° in the left forelimb. Patient #3 showed a 46° increase in left forelimb and a 28° in the right forelimb (*Table 2*). Overall, all evaluated joints showed improvement in ROM.

Canine Patient	ROM time 1 – Right elbow	ROM time 1 – Left elbow	ROM time 2 – Right elbow	ROM time 2 – Left elbow
#2	87°-102°	96°-117°	76°-113°	98°-143°
#3	84°-126°C	96°-115°	76° - 146°C	80°-145°

Table 2. ROM of canine patients *completing the study, including measurements prior to therapy (time 1) and four months post-treatment (time 2), for both treated elbows.*

Radiography results

Radiographic evaluation did not reveal any evident progression of OA in the treated joints. Likewise, no signs of septic arthritis or soft tissue inflammation were observed. No additional radiological findings of clinical relevance were detected.

LOAD questionnaire results

Updated LOAD questionnaires were obtained for the dogs that continued the study. The responses perceived improvements in pain control and a reduction in lameness, although the overall scores remained within the "extreme" category. Specifically, patient #2 improved from an initial score of 39 to 32, and patient #3 from of 35 to 33. Additionally, owners reported a positive clinical perception following treatment.

After five months of the administration of the cMSCs-based therapy, all dogs treated with the cMSC-based product remained free of observable signs of pain or lameness and exhibited improved joint ROM compared to baseline values.

Discussion

Although only two dogs completed the clinical trial with all assessments performed, it is noteworthy that none of the dogs in the cMSCs-based product group exhibited lameness or adverse effects throughout the study period. Mild discomfort observed in one dog treated with MSC-based product and one dog treated with HA following the intra-articular procedure was most likely associated with the arthrocentesis itself. Intra-articular needle insertion can cause transient pain due to penetration of the joint capsule, as has been described in veterinary medicine (Clements, 2006).

The withdrawal of two patients from the HA group due to pain requiring rescue analgesia prevented direct comparative analysis between treatment groups. However, the results obtained from the cMSC-treated dogs support the safety and potential therapeutic benefit of this approach. Notably, no local or systemic side effects were reported during the five-month follow-up, reinforcing the clinical tolerability of the allogeneic cMSC-based product.

Although, synovial fluid analysis was initially considered as an additional objective tool to evaluate joint inflammation, it could not be performed due to insufficient synovial volume (<0,5 ml). This limitation is well-documented in small dogs and cats and in osteoarthritic joints, where chronic inflammation often results in reduced synovial production and joint space narrowing (Martinez and

Santangelo, 2017). The absence of this parameter limited the ability to directly quantify intra-articular immunological changes. Nevertheless, the significant improvements in ROM observed in the cMSCs-treated dogs suggest not only a mechanical benefit from the injection volume—as may occur with HA—but also a likely contribution from the anti-inflammatory and immunomodulatory effects of MSCs. These findings are consistent with those reported by Harman *et al*., where intra-articular administration of allogeneic adipose-derived MSCs in dogs with OA demonstrated greater efficacy than placebo in improving lameness and reducing pain (Harman *et al*., 2016). Other parameters, such as peak vertical force measured by force platform, could be evaluated, as it has been done in other studies (Vilar *et al*., 2014), combined with cytological analysis, which provides the most valuable information when synovial fluid volume is limited (Martinez and Santangelo, 2017).

These effects have been consistently reported in the literature, with MSCs having the ability to exert paracrine immunomodulatory activity via secretion of bioactive molecules, such as prostaglandin E2 (PGE2), interleukin-10 (IL—10), and TGF-β, and influence immune cell populations including macrophages and T cells (Carrade, 2013). These mechanisms may explain the reduction in pain and functional improvement observed, even in the absence of structural joint changes on radiographs.

Although this was a small study with a limited number of canine patients, the results suggest that the allogeneic cMSC product is safe for intra-articular use in dogs. No clinically relevant adverse effects were observed, supporting the product's tolerability in the treatment of elbow OA. The principal limitation of this study lies in the small sample size, particularly dogs with confirmed elbow OA. Additionally, the inability to perform synovial fluid analysis limited insight

into molecular mechanisms of action. Future trials should aim to include larger cohorts.

Furthermore, a broader challenge relates to the regulatory landscape. The framework for allogeneic cell-based veterinary therapies in the European Union remains fragmented and insufficient. Although the European Medicines Agency (EMA), through the CVMP and ADVENT initiative, has published general guidelines on sterility, quality, and safety (EMA, 2019; EMA, 2009; Kuhlmann-Gottke and Duchow, 2015), there is no harmonised regulation specific to veterinary regenerative medicine. Member states interpret and enforce these guidelines differently; for instance, Italy applies human-centred legislation to veterinary stem cell products (Ministero della Salute, 2013), while other nations take more permissive stances due to the lack of specific local policies.

An important precedent is the approval of DogStem®, an injectable formulation of equine umbilical cord-derived MSCs for canine OA, authorised via EMA centralised procedure in multiple EU countries, including Spain (European Commission, 2022). Although DogStem® is xenogeneic, its authorisation highlights the feasibility of stem cell-based veterinary treatments within the current legal framework. These developments underscore the pressing need for a harmonised, species-specific regulatory approach that supports innovation while ensuring patient safety.

Conclusions

This study provides preliminary evidence supporting the safety and clinical potential of an allogeneic cMSC-based product supplemented with HA for the treatment of canine osteoarthritis. The therapy was well tolerated, free of

significant side effects, and associated with improvements in joint mobility and functional outcomes in the treated animals.

As an allogeneic, xeno-free product, this formulation avoids the morbidity associated with autologous cell harvesting and offers a ready-to-use option that may be easily integrated into clinical practice. Despite the promising findings, the limited sample size restricts the generalisability of the results. Larger-scale clinical trials are needed to confirm efficacy, further characterise immunomodulatory mechanisms, and evaluate long-term outcomes.

Ultimately, the cMSC-based therapy may represent a valuable advancement in the management of canine OA and contribute to the broader development of cell-based therapies in veterinary medicine for diseases currently lacking effective treatments.

Supplementary material (S1) – LOAD Questionnaire

The Liverpool Osteoarthritis in Dogs (LOAD) questionnaire is a client-reported outcome measure developed and owned by the *University of Liverpool* and exclusively distributed by *Elanco Animal Health.* Due to copyright restrictions, the full questionnaire (including the validated Spanish version) is not reproduced in this thesis.

Researchers and clinicians interested in accessing the *LOAD* questionnaire may request it directly from *Elanco* at:
https://my.elanco.com/au/insights-centre/the-liverpool-osteoarthritis-in-dogs

Spanish LOAD questionnaire, completed by the owners of the canine patients that were enrolled in the study, both at the beginning and at the end, and named "Osteoartritis en perros de Liverpool: Cuestionario para tutores/propietarios de perros con problemas de movilidad" (Olcoz *et al.*, 2024), is published in *"Frontiers in Veterinary Science"* journal as *"Translation and linguistic validation into Spanish of the Owner-Reported Outcome Measure "Liverpool Osteoarthritis in Dogs""* (DOI: 10.3389/fvets.2024.1360926). Full questionnaire can be download: *https://www.frontiersin.org/journals/veterinary-science/articles/10.3389/fvets.2024.1360926/full*

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Chapter 6. General discussion and conclusions of the thesis

With our research, we have demonstrated that GF-rich PL can be produced using an in-house method. Additionally, we have introduced a practical tool to estimate GF content—particularly TGF-β—by correlating it with platelet concentration, thereby reducing the need for costly quantification methods. PL has proven to be a valuable xeno-free supplement for cell culture and, through this study, a feasible ready-to-use carrier media supplement for cMSC-based products, preserving cell viability and potential during mid-term storage. Together with supporting evidence, we confirm that PL offers a cost-effective and practical alternative to conventional supplements in cMSCs formulations, as well as for use in other orthopaedics regenerative strategies, given its demonstrated richness in GFs when prepared following validated and GMP-like methodologies.

Cryopreservation and cryobanking of cMSCs have been validated as a viable approach for the development of cost-efficient, standardised cMCS-based products. Having pre-characterised, frozen MSCs readily available allows for rapid and economical formulation of therapeutic doses, requiring only microbiological safety testing and minimal phenotypic reconfirmation—such as lack of surface MHCII expression—to reconfirm their immunogenicity prior to allogeneic use.

Allogeneic adipose-derived cMSCs, particularly when combined with joint-supportive agents such as HA, have proved to be a reasonable and clinically safe treatment for canine OA. This is primarily due to their low immunogenicity,

absence of adverse reactions, and their positive effects on clinical symptomatology in dogs. These therapeutic effects have been attributed—in previous literature—to the anti-inflammatory and immunomodulatory properties of MSCs.

A major challenge encountered in this study was the identification of a suitable ready-to-use carrier medium for intra-articular injection in canine OA patients. A medium that could preserve cell viability during product transport while simultaneously supporting the therapeutic activity of cMSCs and contributing positively to the joint microenvironment. Three xeno-free media were identified and demonstrated satisfactory viability for cMSCs in short to mid-term storage: RL supplemented with HA, RL supplemented with PL, and RL supplemented with both HA and PL. Among these, RL supplemented with HA emerged as the most practical and standardisable option.

For this reason, RL supplemented with HA was selected as the final carrier medium for intra-articular injection. Canine patients treated with this formulation showed no adverse effects over a 4-month follow-up period, aside from mild post-injection lameness likely due to the arthrocentesis procedure. The treatment outcomes were positive, with increased range of motion (ROM) and improved clinical signs of OA, including pain reduction.

This original research provides robust validation of a feasible, xeno-free, and accessible MSC-based treatment for canine OA, offering several advantages over current autologous MSC therapies: elimination of species-derived additives in the carrier medium, avoidance of surgical procedures for cell harvesting, and ease of clinical application. This approach could facilitate the treatment of OA in

dogs whose clinical settings or owners' economic limitations preclude access to autologous MSC therapies. Further studies are warranted to optimise product formulation, evaluate long-term outcomes, and define the ideal dosing regimen for large-scale clinical application, with the ultimate goal of translating this strategy into a standardised and reproducible therapeutic for canine OA.

Annex 1. CFU and Confluency data

CFU data description

Colony-forming unit (CFU) counts were obtained in triplicate for most samples, with the exception of 8 samples, which were analysed in duplicate. Samples 1–3 belong to the **RL** group, samples 4–6 to the **HA** group, samples 7–9 to the **PL** group, and samples 10–12 to the **HA+PL** group.

Sample labels indicate storage duration: **"A"** = 0 hours; **"B"** = 24 hours; **"C"** = 48 hours; **"D"** = 72 hours.

Samples prefixed with a **"C"** (e.g., "CA", "CB") were stored under **cold temperature conditions** for the respective time point, while those without prefix were stored at room temperature for the corresponding time point.

CFU data

Sample	CFU number			Group mean	SD
A1,A2,A3	2	3	5	3,33	1,53
CA1,CA2,CA3	4	3	5	4,00	1,00
A4,A5,A6	6	7	6	6,33	0,58
CA4,CA5,CA6	3	3	4	3,33	0,58
A7,A8,A9	3	4	4	3,67	0,58
CA7,CA8,CA9	3	4	2	3,00	1,00
A10,A11,A12	7	4	1	4,00	3,00
CA10,CA11,CA12	7	3	5	5,00	2,00
B1,B2,B3	2	1	4	2,33	1,53
CB1,CB2,CB3	3	0	4	2,33	2,08
B4,B5,B6	4	2	5	3,67	1,53
CB4,CB5,CB6	6	5	2	4,33	2,08
B7,B8,B9	3	2	5	3,33	1,53
CB7,CB8,CB9	5	3	3	3,67	1,15
B10,B11,B12	4	4	4	4,00	0,00
CB10,CB11,CB12	5	6	3	4,67	1,53
C1,C2,C3	0	1	-	0,50	0,71
CC1,CC2,CC3	3	1	4	2,67	1,53
C4,C5,C6	4	0	2	2,00	2,00
CC4,CC5,CC6	4	3	3	3,33	0,58
C7,C8,C9	6	2	5	4,33	2,08
CC7,CC8,CC9	5	4	6	5,00	1,00
C10,C11,C12	4	3	4	3,67	0,58
CC10,CC11,CC12	5	7	5	5,67	1,15
D1,D2,D3	0	0	-	0,00	0,00
CD1,CD2,CD3	2	6	-	4,00	2,83
D4,D5,D6	0	2	-	1,00	1,41
CD4,CD5,CD6	3	3	5	3,67	1,15
D7,D8,D9	4	3	-	3,50	0,71
CD7,CD8,CD9	5	5	-	5,00	0,00
D10,D11,D12	5	4	-	4,50	0,71
CD10,CD11,CD12	5	6	-	5,50	0,71
A1,A2,A3	2	3	5	3,33	1,53
CA1,CA2,CA3	4	3	5	4,00	1,00

Proliferation assay data description

Cell confluency measurements used to assess cell proliferation after storage was obtained in triplicate for most samples, with the exception of some samples that were obtained in duplicate due to cell availability limitation.

Samples 1–3 belong to the **RL** group, samples 4–6 to the **HA** group, samples 7–9 to the **PL** group, and samples 10–12 to the **HA+PL** group.

Sample labels indicate storage duration: **"A"** = 0 hours; **"B"** = 24 hours; **"C"** = 48 hours; and **"D"** = 72 hours.

Samples prefixed with a **"C"** (e.g., "CA", "CB") were stored under **cold temperature conditions** for the respective time point, while those without prefix were stored at **room temperature** for the corresponding time point.

Proliferation assay (confluency) data

24 hours culture				
Sample	**% confluency**			
A1	22,49%	12,29%	15,27%	
A2	15,40%	24,10%		
A3	17,25%	22,88%	20,32%	
A4	11,89%	11,77%	8,46%	
A5	11,45%	17,54%	17,338	
A6	18,30%	9,71%	12,40%	15,59%
A7	8,57%	11,53%	12,96%	14,43%
A8	16,45%	14,22%	11,39%	
A9	13,29%	14,83%	12,48%	
A10	9,86%	9,96%	8,09%	
A11	16,15%	11,82%	13,86%	
A12	10,23%	13,14%	17,53%	13,40%

CA1	15,15%	19,87%	15,64%	13,92%
CA2	23,38%	18,57%	15,48%	
CA3	14,09%	16,44%	12,21%	9,54%
CA4	11,79%	15,24%	12,182	
CA5	10,91%	12,48%		
CA6	14,00%	13,06%	13,01%	
CA7	13,21%	13,40%	12,65%	
CA8	14,99%	12,82%	14,82%	
CA9	23,27%	21,78%	10,08%	
CA10	15,89%	12,27%	10,46%	
CA11	17,01%	16,44%	13,89%	
CA12	11,23%	13,30%	11,22%	
B1	5,99%	5,77%	9,14%	
B2	5,80%	4,31%	6,48%	5,29%
B3	6,83%	6,23%	7,13%	
B4	5,99%	6,74%	10,44%	
B5	4,58%	6,15%	7,499	
B6	5,28%	7,20%	7,27%	
B7	15,70%	9,97%	11,20%	
B8	10,42%	10,71%	10,22%	
B9	12,71%	24,77%	18,95%	15,83%
B10	8,96%	18,61%	16,90%	25,01%
B11	18,61%	16,90%	25,01%	
B12	16,47%	22,54%	15,58%	16,70%
CB1	13,65%	12,73%	11,03%	
CB2	10,02%	10,84%	8,97%	
CB3	11,24%	13,24%	12,48%	11,77%
CB4	11,42%	12,18%	15,06%	
CB5	12,27%	10,84%	11,89%	
CB6	9,05%	10,75%	10,47%	9,55%
CB7	10,52%	20,67%	15,30%	
CB8	14,10%	13,68%	14,51%	
CB9	13,30%	13,90%	14,82%	
CB10	9,18%	13,20%	13,89%	
CB11	14,49%	16,87%	19,94%	
CB12	14,24%	14,18%	13,26%	
C1	2,58%	2,98%	2,14%	
C2	3,57%	2,98%	2,80%	2,41%
C3				
C4	9,94%	7,20%	10,28%	7,49%
C5	3,55%	4,76%	3,78%	4,69%
C6	4,52%	4,05%	4,32%	4,07%
C7	14,27%	13,23%	14,22%	
C8	8,84%	11,35%	9,39%	
C9	9,96%	9,48%	8,77%	8,89%
C10	10,83%	12,09%	11,33%	
C11	14,29%	11,43%	14,71%	

C12	11,09%	19,98%	24,79%	11,63%
CC1	14,53%	10,93%	14,06%	
CC2	9,45%	12,20%	12,70%	12,34%
CC3	19,73%	19,00%	17,06%	
CC4	12,43%	14,04%	12,75%	
CC5	13,93%	11,10%	11,77%	
CC6	16,45%	13,97%	17,52%	
CC7	17,24%	15,04%	17,44%	14,31%
CC8	16,82%	13,67%	16,65%	
CC9	13,55%	13,18%	16,64%	
CC10	16,06%	13,28%	18,00%	
CC11	15,48%	19,07%	16,03%	18,41%
CC12				
D1	5,94%	5,90%		
D2	8,91%	5,45%	9,79%	
D3				
D4	4,82%	4,41%	3,41%	
D5	3,36%	3,74%	4,60%	
D6				
D7	10,64%	7,28%	10,32%	
D8	25,02%	11,00%	9,26%	
D9				
D10	8,28%	9,02%	10,00%	
D11	19,07%	12,99%	10,79%	
D12				
CD1	11,93%	10,00%	12,65%	
CD2	10,36%	9,68%	11,14%	
CD3				
CD4	10,12%	9,11%	10,46%	
CD5	8,21%	7,61%	7,19%	
CD6				
CD7	12,76%	12,58%	14,15%	
CD8	13,82%	15,57%	12,24%	
CD9				
CD10	10,80%	9,70%	11,45%	
CD11	11,42%	10,37%	17,95%	

48 hours culture				
Sample	**% confluency**			
A1	10,55%	14,73%	17,64%	
A2	17,90%	13,98%	12,72%	18,42%
A3	19,56%	15,82%	19,01%	
A4	12,90%	16,76%	14,62%	
A5	18,94%	21,58%	19,44%	
A6	21,16%	23,24%	18,83%	
A7	17,68%	12,92%	17,56%	
A8	13,11%	15,18%	14,27%	
A9	17,70%	14,19%	13,80%	
A10	14,17%	15,17%	14,08%	
A11	17,24%	15,27%	13,43%	
A12	15,00%	16,18%	14,40%	
CA1	16,04%	19,72%	16,17%	
CA2	18,87%	12,74%		
CA3	12,35%	18,77%	20,55%	
CA4	16,85%	13,23%	17,93%	
CA5	13,62%		26,32%	
CA6	19,53%	15,33%	11,45%	
CA7	15,94%	14,22%	10,23%	
CA8	16,65%	15,84%	16,87%	
CA9	12,28%	14,23%	15,18%	
CA10	15,52%	15,99%	19,01%	
CA11	14,10%	13,95%	15,42%	
CA12	14,74%	13,31%	16,77%	
B1	16,04%	13,26%	13,33%	
B2	8,54%	8,90%	10,11%	
B3	12,44%	10,40%	15,39%	
B4	14,54%	11,16%	16,97%	8,92%
B5	11,69%	14,98%	9,98%	
B6	9,88%	16,37%	9,77%	
B7	11,93%	13,96%	15,11%	
B8	16,78%	14,83%	16,69%	
B9	17,00%	18,83%	15,61%	
B10	14,42%	19,52%	16,05%	
B11	15,90%	18,79%	15,34%	
B12	15,41%	35,41%	26,57%	
CB1	16,01%	18,73%	19,35%	
CB2	17,24%	13,55%	17,20%	
CB3	13,74%	21,72%	15,95%	
CB4	21,33%	19,18%	14,50%	
CB5	16,99%	11,11%	11,80%	
CB6	15,92%	14,25%	16,76%	
CB7	15,55%	15,24%	15,17%	
CB8	18,18%	15,52%	15,27%	

CB9	19,66%	17,30%	13,24%	
CB10	14,77%	22,00%	22,53%	
CB11	17,58%	17,90%	13,60%	
CB12	15,75%	22,12%	22,52%	
C1	8,64%	6,70%	7,95%	
C2	5,27%	7,51%	5,75%	7,80%
C3				
C4	15,76%	13,27%	16,37%	
C5	7,50%	6,79%	7,37%	
C6	8,93%	8,48%	8,13%	
C7	13,13%	16,82%	17,43%	
C8	9,97%	15,41%	17,32%	
C9	15,19%	16,09%	19,52%	
C10	15,13%	10,78%	14,74%	
C11	16,91%	14,40%	18,80%	
C12	28,11%	15,97%	20,37%	
CC1	19,20%	15,37%	11,78%	
CC2	15,37%	14,26%	14,31%	
CC3	15,31%	18,80%	21,19%	
CC4	11,87%	17,28%		
CC5	16,65%	17,91%	14,07%	
CC6	18,07%	19,07%	14,44%	
CC7	18,63%	20,55%	17,52%	
CC8	20,17%	23,72%	17,06%	
CC9	14,97%	16,66%	19,01%	
CC10	16,47%	19,90%	17,33%	
CC11	22,86%	15,26%	15,80%	
CC12	22,41%	22,47%	22,00%	
D1	12,40%	6,00%	6,96%	
D2	12,13%	7,11%	8,52%	9,51%
D3				
D4	4,85%	7,04%	4,54%	
D5	8,35%	6,47%	5,80%	
D6				
D7	15,91%	19,32%	13,57%	
D8	35,36%	14,69%	19,45%	
D9				
D10	22,42%	12,59%	15,13%	11,47%
D11	20,22%	20,24%	23,89%	
D12				
CD1	23,34%	23,13%	20,01%	
CD2	18,76%	16,07%	18,33%	
CD3				
CD4	12,47%	16,66%	21,00%	
CD5	20,15%	16,01%	12,84%	
CD6				

CD7	24,65%	16,59%	18,73%	
CD8	21,19%	26,25%	21,97%	
CD9				
CD10	17,86%	21,46%	21,76%	
CD11	22,28%	20,44%	20,96%	
CD12	12,40%	6,00%	6,96%	

72 hours culture				
Sample	**% confluency**			
A1	29,57%	23,82%	20,46%	
A2	21,04%	19,60%	20,89%	
A3	17,75%	17,65%		
A4	20,17%	21,31%	22,02%	
A5	22,10%	21,10%		
A6	28,71%	27,94%	27,50%	
A7	26,21%	24,97%	21,67%	
A8	19,80%	23,58%	23,95%	
A9	18,19%	18,24%	30,00%	
A10	19,77%	19,75%	18,36%	
A11	19,15%	19,32%	18,88%	
A12	20,78%	19,85%	24,23%	
CA1	23,37%	25,91%	21,75%	
CA2	25,98%	23,52%	20,05%	
CA3	26,05%	22,44%	20,74%	
CA4	18,51%	19,39%	18,57%	
CA5	18,28%	27,29%	18,52%	
CA6	20,34%	24,39%	20,82%	
CA7	22,37%	17,69%	21,15%	
CA8	32,11%	21,12%	23,43%	
CA9	32,91%	23,56%	16,69%	
CA10	18,79%	21,62%	24,13%	
CA11	18,62%	21,31%	22,22%	
CA12	25,62%	26,11%	20,17%	
B1	25,76%	22,19%	24,81%	
B2	24,31%	16,67%	16,23%	
B3	19,22%	16,01%	27,52%	
B4	26,95%	21,30%	16,15%	
B5	20,67%	18,81%	17,10%	
B6	15,81%	19,10%	18,87%	
B7	15,07%	33,24%	18,51%	20,35%
B8	29,49%	18,93%	19,03%	
B9	21,83%	17,42%	20,49%	
B10	25,66%	22,82%	23,45%	
B11	44,01%	28,75%	24,86%	
B12	44,74%	25,51%	27,96%	

CB1	25,08%	26,29%	25,42%	
CB2	25,46%	26,07%	21,61%	
CB3	23,54%	21,89%	29,01%	
CB4	23,71%	22,33%	18,03%	
CB5	34,13%	23,12%	26,99%	24,30%
CB6	19,04%	17,32%	20,03%	
CB7	20,04%	29,63%	26,23%	
CB8	25,49%	22,69%	33,00%	
CB9	36,21%	24,97%	30,73%	
CB10	23,38%	18,21%		
CB11	24,54%	25,16%	26,99%	
CB12	21,21%	23,49%	28,55%	
C1	9,01%	13,58%	12,66%	
C2	16,94%	3,74%	6,57%	4,99%
C3				
C4	22,65%	22,15%	22,24%	
C5	23,52%	13,31%	11,25%	
C6	17,45%	15,65%	16,09%	
C7	20,72%	26,76%	30,39%	
C8	24,45%	38,72%	22,86%	
C9	25,29%	29,06%	24,90%	
C10	28,47%	33,72%	25,42%	
C11	29,62%	21,96%	26,45%	
C12	30,94%	27,76%	31,58%	
CC1	26,31%	32,49%	27,38%	
CC2	22,03%	20,81%	29,96%	
CC3	31,15%	34,37%	39,34%	
CC4	28,18%	30,64%	30,21%	
CC5	23,26%	25,56%	16,37%	
CC6	24,15%	23,38%	27,01%	
CC7	28,63%	27,28%	31,32%	
CC8	27,36%	27,67%	26,89%	
CC9	23,83%	24,70%	22,91%	
CC10	26,60%	20,26%	21,68%	
CC11	25,07%	26,55%	25,84%	
CC12	28,26%	29,25%	24,89%	
D1	22,06%	16,49%	17,13%	
D2	11,27%	11,91%	9,70%	
D3				
D4	7,13%	6,07%	3,98%	
D5	8,48%	12,15%	11,31%	
D6				
D7	19,12%	20,92%	22,44%	
D8	45,13%	30,53%	27,18%	
D9				
D10	20,57%	16,73%	28,17%	

D11	26,97%	30,41%	21,56%	
D12				
CD1	24,22%	33,96%	23,58%	
CD2	22,56%	24,72%	19,36%	
CD3				
CD4	24,22%	25,65%	17,53%	
CD5	26,35%	18,65%	24,05%	
CD6				
CD7	25,46%	24,24%	29,88%	
CD8	29,07%	29,81%	31,23%	
CD9				
CD10	25,52%	23,35%	22,30%	
CD11	22,69%	37,97%	25,36%	
CD12				

96 hours culture				
Sample	**% confluency**			
A1	34,94%	42,35%	34,63%	
A2	37,42%	37,25%	29,15%	
A3	45,12%	34,12%	32,33%	
A4	28,45%	24,39%	26,34%	
A5	30,88%	31,55%	39,97%	31,80%
A6	37,31%	37,55%	40,48%	
A7	30,25%	35,17%	39,23%	
A8	24,29%	40,71%	31,51%	
A9	39,49%	34,25%	38,26%	
A10	17,88%	31,39%	28,43%	
A11	33,78%	33,52%	43,96%	
A12	41,14%	31,31%	28,18%	
CA1	26,53%	27,02%	18,24%	
CA2	25,02%	31,72%	37,82%	
CA3	41,99%	31,64%	34.93%	
CA4	32,13%	28,43%	29,51%	
CA5	39,59%	36,30%	42,19%	
CA6	31,22%	32,85%	31,69%	34,12%
CA7	33,67%	30,58%	31,44%	
CA8	29,45%	28,60%	34,10%	
CA9	33,13%	31,92%	36,35%	
CA10	36,32%	35,58%		
CA11	32,15%	30,22%	35,75%	
CA12	32,10%	33,67%	35,42%	
B1	35,11%	41,82%	29,95%	
B2	23,75%	29,29%	27,82%	
B3	28,06%	26,55%	27,21%	
B4	30,47%	32,34%	24,57%	

B5	30,56%	29,58%	28,15%	
B6	30,58%	27,02%	27,02%	
B7	35,83%	30,24%	26,39%	
B8	47,76%	33,98%	37,61%	
B9	30,15%	26,68%	29,33%	
B10	33,62%	31,44%		
B11	36,33%	32,50%	32,56%	
B12	22,12%	32,95%	37,72%	
CB1	28,99%	35,10%	34,02%	
CB2	40,89%	48,06%	31,80%	
CB3	41,11%	41,61%	30,58%	
CB4	35,82%	33,15%	34,89%	
CB5	32,83%	29,49%	31,71%	
CB6	35,19%	29,37%	28,31%	
CB7	38,27%	50,56%	37,36%	
CB8	38,22%	42,60%		
CB9	38,53%	34,31%	37,50%	
CB10	30,49%	36,73%	35,78%	
CB11	44,61%	38,34%	40,82%	
CB12	37,12%	37,96%	33,87%	
C1	22,94%	25,63%	25,72%	
C2	11,77%	6,51%	7,70%	
C3				
C4	39,60%	33,96%	35,06%	
C5	22,83%	22,72%	26,39%	
C6	22,87%	23,16%	19,54%	
C7	34,02%	38,08%	35,76%	
C8	34,12%	38,73%	31,43%	
C9	36,33%	36,15%	40,16%	
C10	39,02%	46,60%	36,33%	
C11	33,60%	27,87%	32,30%	
C12	33,36%	40,65%	39,53%	
CC1	32,03%	37,20%	38,96%	
CC2	33,94%	29,25%	32,19%	
CC3	40,44%	32,67%	35,62%	
CC4	29,68%	35,20%	36,58%	
CC5	31,37%	32,74%	34,18%	
CC6	36,44%	29,80%	32,26%	
CC7	40,24%	37,76%	45,95%	
CC8	33,12%	36,78%	39,91%	
CC9	41,03%	44,30%	41,86%	
CC10	31,55%	33,11%	40,17%	
CC11	42,71%	44,73%	33,88%	
CC12	37,19%	39,50%	40,79%	
D1	23,14%	29,32%	25,58%	
D2	22,66%	16,89%	14,08%	

D3				
D4	6,62%	7,40%	6,85%	
D5	13,43%	20,44%	22,22%	
D6				
D7	27,45%	25,01%	32,57%	25,21%
D8	34,45%	33,10%	36,72%	
D9				
D10	36,90%	22,87%	18,98%	
D11	23,04%	28,62%	33,05%	
D12				
CD1	33,98%	22,34%	31,01%	
CD2	29,33%	31,79%	29,97%	
CD3				
CD4	29,55%	28,78%	30,93%	30,12%
CD5	30,11%	25,39%	32,45%	
CD6				
CD7	35,97%	31,33%	29,26%	
CD8	31,03%	29,99%	35,38%	
CD9				
CD10	34,74%	31,10%	27,47%	
CD11	32,31%	29,57%	31,56%	
CD12				

120 hours culture			
Sample	**% confluency**		
A1	35,77%	37,96%	30,35%
A2	40,41%	25,68%	45,54%
A3	34,94%	35,86%	40,74%
A4	35,64%	40,33%	35,99%
A5	35,80%	34,03%	33,49%
A6	43,88%	38,22%	42,88%
A7	40,84%	31,46%	44,39%
A8	36,31%	37,11%	33,68%
A9	32,50%	37,76%	37,95%
A10	39,98%	30,93%	39,02%
A11	40,21%	35,92%	33,34%
A12	32,87%	41,50%	32,41%
CA1	50,81%	34,80%	45,97%
CA2	40,02%	45,86%	45,21%
CA3	33,21%	38,78%	37,13%
CA4	42,10%	31,68%	42,45%
CA5	40,88%	27,61%	44,96%
CA6	31,25%	43,94%	36,55%
CA7	35,40%	43,49%	41,71%
CA8	43,75%	36,19%	43,02%

CA9	31,52%	33,60%	34,18%
CA10	37,93%	39,94%	35,80%
CA11	37,36%	43,71%	41,11%
CA12	61,69%	57,90%	39,09%
B1	45,71%	37,12%	31,47%
B2	38,12%	35,55%	32,39%
B3	40,47%	33,74%	32,02%
B4	39,45%	37,72%	39,09%
B5	43,20%	47,95%	43,26%
B6	45,60%	32,56%	32,03%
B7	40,39%	45,25%	40,87%
B8	47,90%	37,13%	37,59%
B9	43,59%	48,52%	39,11%
B10	44,20%	43,93%	36,97%
B11	43,04%	38,62%	37,80%
B12	28,30%	37,75%	34,86%
CB1	42,74%	37,40%	35,94%
CB2	34,94%	37,92%	33,54%
CB3	38,52%	41,68%	43,24%
CB4	38,62%	38,94%	35,15%
CB5	39,74%	28,51%	39,32%
CB6	39,35%	39,00%	39,59%
CB7	42,12%	44,77%	39,78%
CB8	43,14%	45,61%	46,12%
CB9	44,20%	43,16%	40,78%
CB10	45,96%	47,03%	41,34%
CB11	32,52%	50,83%	51,18%
CB12	42,32%	37,41%	41,62%
C1	28,52%	28,29%	27,97%
C2	11,22%		
C3			
C4	37,94%	37,97%	35,92%
C5	20,78%	24,51%	31,47%
C6	37,68%	26,66%	30,71%
C7	40,03%	27,48%	43,70%
C8	46,12%	28,90%	30,60%
C9	32,05%	36,72%	35,32%
C10	45,33%	40,20%	40,73%
C11	38,19%	37,41%	42,84%
C12	50,99%	38,27%	41,90%
CC1	45,01%	38,13%	40,33%
CC2	39,23%	34,78%	38,99%
CC3	43,60%	41,97%	36,26%
CC4	37,49%	45,47%	46,13%
CC5	43,36%	37,35%	48,81%
CC6	40,31%	38,92%	44,46%

CC7	42,72%	54,68%	47,99%
CC8	39,88%	49,59%	45,57%
CC9	43,61%	39,84%	38,93%
CC10	36,32%	41,24%	45,14%
CC11	36,93%	41,04%	44,99%
CC12	37,75%	43,52%	47,43%
D1	32,61%	34,40%	28,41%
D2	25,44%	28,59%	30,77%
D3			
D4	8,78%	10,40%	12,95%
D5	19,31%	21,09%	28,62%
D6			
D7	34,85%	38,17%	40,29%
D8	38,47%	42,32%	48,11%
D9			
D10	32,93%	38,61%	34,55%
D11	30,50%	25,52%	
D12			
CD1	45,18%	45,68%	
CD2	35,83%	31,43%	37,65%
CD3			
CD4	44,68%	39,37%	38,80%
CD5	39,96%	34,69%	36,28%
CD6			
CD7	44,23%	40,70%	37,67%
CD8	53,55%	37,40%	39,97%
CD9			
CD10	34,77%	34,08%	36,85%
CD11	39,70%	43,95%	41,33%
CD12			

Annex 1. CFU and Confluency data- Allogeneic mesenchymal stem cell-based ready-to-use product for canine osteoarthritis