UNIVERSIDADE ESTADUAL PAULISTA "JULIO DE MESQUITA FILHO" FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS CÂMPUS DE JABOTICABAL

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UNIVERSITÉ DE MONTRÉAL

FACULTÉ DE MÉDECINE VÉTÉRINAIRE

CAMPUS SAINT-HYACINTHE

DEVELOPMENT OF A TISSUE ENGINEERING PLATFORM USING BOVINE SPECIES AS A MODEL: PLACENTAL SCAFFOLDS SEEDED WITH BOVINE ADIPOSE-DERIVED CELLS

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AUTHOR'S CURRICULUM DATA

AMANDA BARACHO TRINDADE HILL – The author was born in the city of Campinas, on the twenty-ninth of April in the year one thousand nine hundred and ninety. She began her course of study in Veterinary Medicine in the year two thousand and eight at the State São Paulo State University, in the city of Araçatuba, São Paulo; she completed four scientific initiation programs, received a study grant from the Brazilian National Council for Scientific and Technological Development (CNPQ), during the years of two thousand and ten and two thousand and eleven, in order to support her scientific initiation in the field of Inspection of Products of Animal Origin, and received honourable mention for the results of that project when presented at conference. She graduated, in December of the year two thousand and twelve, with a degree in veterinary medicine, on which occasion she received the Dr. Wagner Lavezzo prize for the highest achieving student in area of study of Animal Production. In March of the year two thousand and thirteen, she began a master's degree program under the supervision of Professor Dr. Joaquim Mansano Garcia, in the Veterinary Medicine program, with a concentration in Animal Reproduction, at the São Paulo State University, College of Agricultural and Veterinary Sciences, with the São Paulo Research Foundation (FAPESP) study grant number 13/14293-0. She completed an internship six months in length at the University of Montreal. She began her doctoral studies in a joint degree program between the University of Montreal and the São Paulo State University in 2016. She was awarded travel support twice by the Quebecois Reproduction Network (RQR) as well as a merit scholarship during her studies at the University of Montreal. To date, she has published four scientific articles as the first author in international research journals.

"Change your thoughts and you can change the world"

Norman Vincent Peale

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C E R T I F I C A D O

Certificamos que o projeto de pesquisa intitulado **"Gamete-like differentation of bovine stem cells"**, protocolo nº 018806/17, sob a responsabilidade do Prof. Dr. Joaquim Mansano Garcia, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 08 de fevereiro de 2018.

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DEVELOPMENT OF A TISSUE ENGINEERING PLATFORM USING BOVINE SPECIES AS A MODEL: PLACENTAL SCAFFOLDS SEEDED WITH BOVINE ADIPOSE-DERIVED CELLS

ABSTRACT - Stem cell technologies and biomaterial sciences have advanced and grown more popular all over the world. The researchers aim to investigate and evaluate different sources of cells and biomaterials that, in combination, could provide a low cost, highly scalable tissue engineering platform that could be used in drug tests, cell therapies and cell transplantation. The three most important components of tissue engineering systems in general are cell source, biomaterial source (scaffolding system), and the creation and maintenance of an environment that is conducive to tissue formation. When these three components are successfully managed, the tissue engineering treatment achieves a faithful imitation of the in vivo environment, allowing for the differentiation of cells into the desirable cell types. Decellularized bovine placenta has been demonstrated to be rich in extracellular matrix (ECM) and to have well-developed vasculature, representing a highly available, low cost, practically scalable biomaterial. However, it is not known if placental scaffolds have the potential to support recellularization with adipose-derived cells and their subsequent differentiation into different lineages. Thus, in order to provide information on the ability of the mesenchymal stem cell (MSC) - placental scaffold complex to be used in tissue engineering approaches, the objectives of this thesis were: to study the potential of bovine placental scaffolds to support adipose-derived cell recellularization and their differentiation into osteogenic and chondrogenic lineages. The first article of this thesis is a literature review that discusses the nature of mesenchymal stem cells, their applications in regenerative medicine, the importance of stem cell technologies to the livestock industry and the use of bovine species for translational medicine. The second article consists of an evaluation of scaffold recellularization and the differentiation of cells on the scaffolds. The bovine placentae were decellularized by umbilical vessel sodium dodecyl sulfate (SDS) perfusion and cell lines were established after the enzymatic digestion of adipose tissue from six cows and cell selection by rapid adherence to the culture plate. Then, cells were seeded onto the scaffolds and cultured in a 2D rocker system for 21 days in either differentiation or maintenance medium. The isolated cells, when cultured in the plastic dish, exhibited fibroblast-like morphology, CD90, CD73 and CD105 expression, and lacked CD34 and CD45 expression. Moreover, the cells were able to undergo differentiation into chondrogenic and osteogenic lineages, providing evidence of their mesenchymal

nature. Subsequently, the cells adhered to the scaffolds by cell projections, established cell-scaffold communication, and proliferated while maintaining cell-cell communication, which was evidenced by histological and scanning electron microscopy (SEM) assays. Throughout a 21-day culture period in the osteogenic medium, the cells exhibited proliferation and differentiation in a time-dependent manner, which can be observed by the greater abundance of cells in later periods, evidenced by cell nuclei staining (4′,6-diamidino-2-phenylindole - DAPI) and increased intensity of staining for COLLAGEN 1 (COL1) in the immunohistochemical assay, and by its expression as measured by real time polymerase chain reaction (qRT-PCR). This same pattern was observed by histological analysis. Widespread calcium accumulations were also more abundant on the scaffolds as time progressed, as evidenced by Von Kossa staining. The SEM analysis revealed that cells secreted globular/round structures when seeded under osteogenic induction conditions, in accordance with histological findings. Regarding chondrogenic differentiation, Safranin O and Fast Green staining revealed successful differentiation through staining of proteoglycans, chondrocyte-like cells and type II collagen on the scaffold. The SEM analysis showed that the cells changed morphology from fibroblast-like to globular when cultured with chondrogenic induction medium for 21 days. Additionally, cell-scaffold complexes expressed a cartilage marker, COLLAGEN 2 (COL2), which is conducive to the histological and SEM observations. Considering the results as a whole, this study demonstrated that placental scaffolds seeded with adipose-derived cells have the potential to be used in bone and cartilage tissue-engineering applications.

DESENVOLVIMENTO DE UMA PLATAFORMA DE ENGENHARIA TECIDUAL UTILIZANDO A ESPÉCIE BOVINA COMO MODELO: SCAFFOLDS PLACENTÁRIOS CULTIVADOS COM CÉLULAS DERIVADAS DO TECIDO ADIPOSO

RESUMO - A tecnologia de células-tronco e as ciências de biomateriais obtiveram um grande avanço nas últimas décadas e se tornaram mais populares em todo o mundo. Pesquisadores buscam investigar e avaliar diferentes fontes de células e de biomateriais que, em combinação, possam fornecer uma plataforma de engenharia tecidual de baixo custo e produzida em larga escala, para serem utilizadas em testes de drogas, terapias celulares e transplantes, com objetivo de fornecer suporte terapêutico à lesões e regeneração de tecidos danificados. Em geral, os três componentes mais importantes da engenharia de tecidos são: a escolha do tipo de célula, a fonte do biomaterial (scaffold), criação e manutenção de um ambiente propício à formação tecidual. Quando esses três componentes são gerenciados com sucesso, o microambiente celular *in vitro* é mais semelhante ao que a célula está exposta *in vivo*, permitindo que o crescimento e diferenciação celular ocorra de maneira mais fidedigna e eficiente. A placenta bovina descelularizada demonstrou ter uma rica matriz extracelular, vasos bem desenvolvidos, sendo um biomaterial com alta disponibilidade e baixo custo. No entanto, não há informação sobre o potencial dos scaffolds placentários em serem repovoados com células-tronco mesenquimais (MSC) derivadas do tecido adiposo, processo chamado recelularização. Ainda, também não há informação sobre a capacidade dos scaffolds placentários, de após recelularização, oferecer um ambiente adequado para diferenciação dessas células em diferentes linhagens. Assim, a fim de fornecer informações sobre a capacidade do complexo MSC - scaffold placentário em ser usado com sucesso na engenharia tecidual, os objetivos desta tese foram: estudar o potencial dos scaffolds placentários bovinos em oferecer suporte para recelularização por células-tronco derivadas do tecido adiposo bovino, bem como avaliar a capacidade de diferenciação celular em linhagens osteogênica e condrogênica. O primeiro artigo desta tese trata-se de uma revisão de literatura, que discute a natureza das células-tronco mesenquimais, suas aplicações na medicina regenerativa, a importância da tecnologia com células- tronco na indústria pecuária e o uso da espécie bovina na medicina translacional. O segundo artigo consiste na avaliação da recelularização e da diferenciação celular. As placentas bovinas foram decelularizadas por perfusão de SDS do vaso umbilical e as linhas celulares estabelecidas após digestão enzimática do tecido adiposo de seis

vacas e seleção por adesão rápida à placa de cultivo. Em seguida, as células foram cultivadas com os scaffolds em um sistema de agitação 2D por 21 dias em meio de diferenciação ou manutenção. Quando cultivadas na placa de cultivo, as células isoladas exibiram morfologia semelhante ao fibroblasto, expressão de CD90, CD73 e CD105, enquanto não expressaram os marcadores CD34 e CD45. Além disso, as células foram capazes de se diferenciar em linhagens condrogênicas e osteogênicas, fornecendo evidências de sua natureza mesenquimal. Posteriormente, quando cultivadas com os scaffolds, as células aderiram-se aos mesmos por projeções celulares, estabeleceram comunicação célula-scaffold e se proliferaram, fato evidenciado por análise histológica e microscopia eletrônica de varredura (SEM). Em seguida, o potencial das células em se diferenciarem em linhagem osteogênica quando cultivadas com scaffold foi avaliado. Durante um período de cultivo de 21 dias no meio osteogênico, as células se proliferaram e diferenciaram de maneira dependente do tempo, ou seja, a cada semana pode ser observado maior abundância de células, evidenciada pela coloração dos núcleos celulares e aumento da intensidade da coloração para COLAGENO 1 (COL1), que também foi expresso por reação quantitativa em cadeia da polimerase em tempo real (qRT-PCR). O mesmo padrão foi observado pela análise histológica; acúmulos generalizados de cálcio também foram mais abundantes nos scaffolds na terceira semana de cultivo, evidenciado pela coloração de Von Kossa. A análise SEM revelou que as células secretaram estruturas globulares quando cultivadas sob condições de indução osteogênica, condizente com a secreção observada pela análise histológica. Em relação à diferenciação condrogênica, os corantes Safranina e *Fast Green* revelaram sucesso na diferenciação, através da coloração de proteoglicanos, células semelhantes aos condrócitos e colágeno tipo II. A análise SEM mostrou que as células mudaram sua morfologia de fibroblastos para globulares quando cultivadas com meio de indução condrogênica por 21 dias. Além disso, os complexos células-scaffold expressaram um marcador de linhagem cartilaginosa, COLAGENO 2 (COL2), que são condizentes com as observações histológicas e SEM. Considerando os resultados, este estudo demonstrou que os scaffolds placentários bovinos cultivados com células-tronco derivadas de tecido adiposo bovino possuem potencial para serem utilizados na engenharia de tecidos ósseos e cartilaginosos.

Palavras-chave: Engenharia tecidual de cartilagem; engenharia tecidual óssea; placenta; células tronco mesenquimais; descelularização; recelularização; vacas; medicina translacional

LIST OF ABBREVIATIONS

AT-MSC: Adipose tissue-derived mesenchymal stem cells

AT: Adipose tissue

ASCs: Adult (somatic) stem cells

bMSC: Bovine mesenchymal stem cells

BM-MSC: Bone marrow-derived mesenchymal stem cells

CFU-F: Fibroblastic colony-forming units

CO2: carbon dioxide

DAPI: 4′,6-diamidino-2-phenylindole

DMEM: Dulbecco's Modified Eagle Medium

ECM: extracellular matrix

ESCs: Embryonic stem cells

FBS: fetal bovine serum

HE: Hematoxylin-eosin

iPS: Induced pluripotent stem cells

lfcinb: Lactoferricin

MSC: mesenchymal stem cells

NSP-MSC: Nasal septum-derived MSC

OA: Osteoarthritis

PBS: phosphate buffer solution

qRT-PCR: quantitative real time polymerase chain reaction

SDS: sodium dodecyl sulfate

SEM: Scanning Electron Microscopy

TGF- β 1: Transforming growth factor β

UC-MSC: Umbilical cord-derived MSC

VK: Von Kossa

INTRODUCTION

Generally, stem cells have two main characteristics: the capabilities to self-renew and to differentiate into functional cells under the correct stimuli (GAZIT, 2011). Over the past decade, the number of studies exploring the potential of stem cells has significantly grown, which has led to rapid progress and important advances in a number of areas in the field. Personalized therapies, tissue engineering, and treatments that mitigate chronic and degenerative diseases have all benefited greatly from these recent advances (Hill et al., 2019). Naturally occurring in the organism, there are two major classes of stem cells: embryonic stem cells (ESCs) and adult (somatic) stem cells (ASC) (Gattegno-Ho et al., 2012), mostly of mesenchymal nature, known as mesenchymal stem cells (MSC). During early embryogenesis, the trophectoderm differentiates into extraembryonic tissues, whereas the inner cell mass of the embryo forms the embryo itself, and, by the differentiation process, originates all tissues that compose a body (Gattegno-Ho et al., 2012). MSC are known to have a more restricted differentiation potential than ESC. However, there is an ongoing disagreement among researchers as to whether these differences regarding the differentiation potential, previously thought to exist, are narrower than previously thought or if they even exist at all (Zipori, 2004), given that MSC derived from several different species have been shown to differentiate into mesodermal, endodermal and ectodermal lineages (Jiang et al., 2002; Long et al., 2005; Ualiyeva et al., 2014; Dueñas et al., 2014; Xiong et al., 2014; Hill et al., 2017; Hill et al., 2018).

New therapeutic techniques based on stem cells represent potential solutions for various chronic diseases for which current pharmacologic treatments have been shown to be ineffective (Mount et al., 2015), as well as for some surgical procedures. The biggest advantages of the use of stem cells derived from adult tissues in therapeutics are their plasticity, their low immunogenicity, and their high anti-inflammatory potential (Meirelles et al., 2009; Peroni & Borjesson, 2011). MSC can be found in many (Gazit, 2011), if not all (Meirelles et al., 2009), major body tissues due to their principal and widely required responsibility to replace dead cells during the cell renewal process and engage in regular tissue turnover (Gazit, 2011). The bioactive mediators and adhesion molecules characteristically produced by mesenchymal stem cells actively increase angiogenesis, stimulate recovery of intrinsic progenitor cell functionality, and assist in the inhibition of scar formation and apoptosis (Meirelles et al., 2009; Peroni & Borjesson, 2011), processes that are organized by the microenvironment of the cells, referred to as the niche (O'Brien & Bilder, 2013).

There are several methods for the delivery of cells into the body, with the appropriate choice depending on the condition that is being treated (Choi, et al, 2014). One of the possible delivery methods is intravenous injection, which derives its effectiveness from MSC's ability to migrate across the endothelium and home to injured tissues (Gazit, 2011). However, a portion of the injected cells can be trapped in organs other than the desired target, for example the lungs (Chamberlain et al., 2007). Nevertheless, the most widely used methodology is direct injection into the injured tissue (Choi et al., 2014), in order to generate a high concentration of MSC at the injury site. Moreover, there are some cases in which relevant structural defects are present, including articular cartilage, bone, and soft tissue defects, in which cells need to be delivered by a carrier, particularly because a substrate is required to control cell adhesion and, importantly, to form the template for new tissue formation (Choi et al., 2014).

The necessity to mimic the cellular microenvironment has fomented the rise of tissue engineering, in which the main goal is to repair the functionality of injured tissue (Evans et al., 2006; Arenas-Herrera et al., 2013; Frese et al., 2016). In general, there are three important components of tissue engineering: cell type, biomaterial type and the environment that will enable tissue formation (Arenas-Herrera et al., 2013). The success of tissue engineering depends on the management of these components in order to achieve a faithful substitute of the in vivo environment. The necessity of the use of three- dimensional (3D) scaffolds is derived from the inability of monolayer cell growth and low- complexity environments, which are characteristic features of the standard two- dimensional (2D) cell culture model, to produce an environment that allows for the construction of functional tissue (Evans et al., 2006).

It has been challenging to create an ideal scaffold that replicates the niche features to support the growth of a functional tissue. Different chemical and architectural design of various scaffold materials affect cell growth, adhesion, and differentiation, leading tissue engineers to examine myriad different synthetic and biological candidate materials (Londono & Badylak, 2015). A promising biomaterial option for use in scaffolding systems is decellularized biological tissues, as they provide the natural composition, structural microarchitecture and vasculature of the natural extracellular matrix (ECM) (Londono & Badylak, 2015). It is important that the chosen material provide high levels of host tissue integrability, load-bearing capacity, and remodeling capacity, aside from the necessity of an appropriate size to fit the specific defect being treated (Bhumiratana & Vunjak-Novakovic, 2012). Decellularized bovine placenta has been shown to maintain its arrangement of

principal ECM proteins throughout the decellularization process (Barreto et al., 2018). In addition, placental scaffolds have a rich ECM and a well-developed vasculature, are readily available, can be harvested without harming the donor, and are simple to collect, thus representing a biomaterial with low harvest cost (Kakabadze & Karalashvil, 2014; Kakabadze & Kakabadze, 2015), making it an appropriate and feasible choice for large scale scaffold production.

The choice of cell source is equally critical for tissue engineering applications, as it must be a cell that is sufficiently differentiated to fill the desired role, but flexible enough to manage the tissue microenvironment as a whole. As discussed above, MSC are easy to isolate, non-tumorigenic, reduce levels of apoptosis and formation of scar tissue, support the regeneration of functionality of intrinsic progenitor cells, increase angiogenesis, and have high differentiation potential (Meirelles et al., 2009; Peroni & Borjesson, 2011; Kim & Park, 2017; Samsonraj et al., 2017).

However, it still remains unknown whether placental scaffold can provide a matrix that is conducive for adipose-derived cell recellularization and differentiation into different lineages. As a whole, the results of the research presented demonstrate that placental scaffold has the ability to receive adipose-derived stem cells and support their osteogenic and chondrogenic lineage differentiation in a dynamic cell culture environment, contributing to fundamental, therapeutic and clinical applications in regenerative medicine. Likewise, this thesis engages multidisciplinary fields and furthers the primary goals of stem cell technologies and bioengineering sciences, using the bovine species as a model for engineering three-dimensional biomaterials.

HYPOTHESIS AND OBJETIVES

Finding new usable biomaterials is crucial for scientific progress in the field of tissue engineering. The fact that placental tissue can be decellularized while maintaining its primary fiber arrangement raises the question of whether it can be recellularized with MSC, a cell source that could potentially differentiate into multiple lineages, creating a high quality and low cost cell-scaffold complex.

The hypothesis of this work was that the placental scaffold would provide a conducive matrix for adipose-derived cell recellularization and differentiation into osteogenic and chondrogenic lineages, demonstrating its potential for use in tissue engineering therapies.

To test this hypothesis, the following objectives were established:

1.   To enzymatically isolate stem cells from adipose tissue based on their rapid adherence to the culture plastic.

2.   To study the potential of placental scaffolds to support bovine adipose-derived cell recellularization.

3.   To study the potential of placental scaffolds to support bovine adipose-derived cell differentiation into osteogenic and chondrogenic lineages.

CHAPTER 1: LITERATURE REVIEW

ARTICLE 1: Applications of mesenchymal stem cell technology in bovine species

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Abstract

Mesenchymal stem cells (MSCs) have received a great deal of attention over the past 20 years mainly because of the results that showed regeneration potential and plasticity that were much stronger than expected in prior decades. Recent findings in this field have contributed to progress in the establishment of cell differentiation methods, which have made stem cell therapy more clinically attractive. In addition, MSCs are easy to isolate and have anti-inflammatory and angiogenic capabilities. The use of stem cell therapy is currently supported by scientific literature in the treatment of several animal health conditions. MSC may be administered for autologous or allogenic therapy following either a fresh isolation or a thawing of a previously frozen culture. Despite the fact that MSCs have been widely used for the treatment of companion and sport animals, little is known about their clinical and biotechnological potential in the economically relevant livestock industry. This review focuses on describing the key characteristics of potential applications of MSC therapy in livestock production and explores the themes such as the concept, culture, and characterization of mesenchymal stem cells; bovine mesenchymal stem cell isolation; applications and perspectives on commercial interests and farm relevance of MSC in bovine species; and applications in translational research.

Keywords: Mesenchymal stem cells, Cell culture, Pluripotent, Livestock, Cow, Cattle, Biotechnology, Cellular therapy, Regenerative medicine, Translational research

Background

Stem cell biology has been a very active field over the past decade. The number of studies has increased significantly, and this has been accompanied by breakthroughs in several areas in the field. Stem cell therapy has rapidly advanced prospects for personalization of therapy, tissue engineering, and chronic and regenerative disease mitigation. In human and veterinary research, stem cells derived from adult tissues are promising candidates for disease treatment, specifically for their plasticity, their low immunogenicity, and their high antiinflammatory potential [1]. In addition, mesenchymal stem cells (MSCs) characteristically produce bioactive mediators and adhesion molecules that help to inhibit scar formation and apoptosis, increase angiogenesis, and stimulate intrinsic progenitor cells to regenerate their functionality [2, 3]. Stem cell therapy offers potential solutions for a variety of chronic diseases for which current pharmacologic therapy does not provide effective treatment [4] as well as for some surgical procedures. In addition, an exciting new step in cellular therapy is the use of MSC for immune modulation [5].

Veterinary regenerative medicine research has focused principally on companion and sport animals, but a critical reading of published findings, combined with select papers published in livestock species, allows us to generate valuable insights into the future of regenerative medicine applications in animal husbandry. Among all domesticated species, cows have crucial importance in the economics of the livestock industry, with 69.6 million tons of meat and 811 million tons of milk produced worldwide in 2017 [6, 7]. There are several medical conditions, such as mastitis, lameness, and fracture that can reflect negatively on meat and milk production as well as on reproductive efficiency in cattle. For cattle with high economic or genetic potential, these losses pose significant costs to the owner, who is therefore willing to employ expensive and effective treatments [8]. In this review, we discuss the importance of stem cell technology in bovine species in order to address disease and injury with both animal welfare and economic benefits.

The nature of mesenchymal stem cells

The term "stem cell" emerged in the nineteenth century, describing mitotically quiescent primordial germ cells capable of regeneration of a variety of tissues [9]. Stem

cells are defined by their ability to self-renew and by their potential to differentiate into functional cells under appropriate conditions [10]. In animals, two classes of stem cells have been identified: embryonic stem cells (ESC) and adult (somatic) stem cells (ASC) [11], which include mesenchymal stem cells, hematopoietic stem cells, and tissuespecific stem/progenitor cells [12].

MSCs are responsible for tissue turnover; therefore, when tissue repair is necessary, these cells can be stimulated to proliferate and differentiate, resulting in their presence in many [10], if not all [2], tissues. In addition, MSCs display important features that render them valuable for cell therapy and tissue engineering such as their low immunogenicity, high anti-inflammatory potential [1], ability to modulate innate immune responses [5], bioactive mediation and adhesion capacity to inhibit scar formation and apoptosis, increased angiogenesis, and stimulation of intrinsic progenitor cells to regenerate their functionality [3]. Due to their clinically relevant characteristics, MSCs have received more attention than the other ASC types.

During early embryogenesis, the trophectoderm differentiates into extraembryonic tissues, while the inner cell mass of the embryo, populated by embryonic stem cells, gives rise to the embryo itself, thus being able to differentiate into all cell types that form the body [11]. In contrast, it was a generally held belief that MSCs have restricted differentiation ability, being able to differentiate into mesenchymal lineages only. In the early 2000s, some discussion took place regarding the veracity of the definition of mesenchymal stem cells, concerning their potential to differentiate into non-mesenchymal lineages and whether the differences that seemed to exist between ESC and MSC had narrowed to a point that it was questionable whether they existed at all [13]. In 2002, it was shown that bone marrow-derived cells expressed some pluripotent markers, such as Oct-4, Rex-1, and SSEA; were able to differentiate into three germ layers in vitro; and when injected into an early blastocyst, were able to contribute to all organs [14]. The number of studies investigating the pluripotent ability of MSC has grown recently, and many researchers have reported cells derived from bone marrow [15, 16], adipose tissue [17], ovarian tissue [18, 19], placenta [20], and uterus [21] that express pluripotent markers. MSCs derived from several different species, including bovine, have been shown to differentiate into mesodermal, endodermal, and ectodermal lineages [16, 22]. A relevant clinical difference between ESC and MSC is that MSCs do not form teratomas when injected in vivo [14, 17], which is favorable for their clinical use.

Rigorous evaluation of the differentiation capacity of MSC is a critical step in the solidification of support for their redefinition as pluripotent. In order to study the

functionality of MSC, experiments were performed to evaluate the transdifferentiation of MSC in vivo. Studies have shown the ability of MSC to transdifferentiate into various types of skin cells, islet-like cell clusters, and renal epithelium cells [23–25]. These three studies are just a few examples of the considerable amount of data that has been collected over the past decade supporting the transdifferentiation potential of MSC when transplanted in vivo. Considering these results together, MSCs have been proven to functionally differentiate into three germ layers. If MSCs express pluripotent markers and have the ability to differentiate in vitro into three germ layers and transdifferentiate in vivo into three germ layers, perhaps there is a lack of precision concerning terminology in some papers when they are called multipotent.

Mesenchymal stem cell culture

Cell culture begins after mechanical or enzymatic disaggregation of the original tissue and can be performed under various conditions such as in an adhesive layer, a solid substrate, or in a suspension culture. It is well established that MSCs adhere to plastic substrate culture plates [26], a characteristic condition of MSC that arises after tissue disaggregation. Disaggregation is achieved by proteolytic enzyme digestion that is very effective at isolating cells from a tissue; however, it also has the potential to damage them. According to Gazit [10], MSC derived from adipose tissue can be easily isolated after enzymatic treatment with collagenase. This enzyme is the most frequently used for isolation of MSC due to its ability to cleave collagen connections [27]. The optimum concentration of the enzyme, the incubation time, and the temperature must be carefully monitored during isolation [28].

Different protocols have been used to isolate, expand, and characterize MSC. One common protocol, based on cell adherence to the plastic during the first 48–72 h of culture, is effective, though typically results in a heterogeneous population of cells [19, 29, 30]. To select a homogenous or a desirable population of MSC, more stringent isolation protocols have been proposed. These include the use of different cell culture media [31], cell sorting [15, 32, 33], and cell adherence to the plastic during the first 3 h of culture [18, 19].

MSCs have the capacity to expand several times in culture, maintaining their growth potential and plasticity, with a doubling time which is variable according to the tissue and initial plating density [34]. Each time that the cells fill the flask culture area, they need to be enzymatically removed from the flask for further re-cultivation, a

process defined as cell passage [35].

In order for the cells to become able to survive, proliferate, and differentiate in vitro, the culture system must emulate the in vivo conditions of the cells' original tissue [36]. The cells must be maintained in an incubator with 5% CO_2, which facilitates pH maintenance in the culture medium [37], at the physiological temperature optimal for the donor species.

Supplementation of the medium should be performed to mimic in vivo conditions in order to sustain cell growth. Fetal bovine serum (FBS) is used in the cell culture medium as a source of growth factors and a vital nutrient, which supports expansion and attachment of MSC to the culture plate [38]. The use of antibiotics is important to prevent contamination, and it is necessary to evaluate the type of contamination that cells may be exposed to and potential toxicity of the dose when choosing which antibiotic to use. The most commonly used antibiotics are penicillin and streptomycin, making an effective and relatively non-toxic combination at the concentrations of 100 U/mL and 100 mg/mL, respectively [28].

Mesenchymal stem cell characterization

Stem cells are defined by their ability to self-renew and by their potential to differentiate into functional cells under the right conditions [10]. Different protocols have previously been reported regarding the isolation, characterization, and expansion of MSCs. Generally, MSCs express CD105, CD73, and CD90 and lack the expression of hematopoietic markers such as CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR surface molecules [26]. However, MSCs from different species do not express all the same markers [39]. Additionally, it has been demonstrated that MSCs isolated from different tissues express different markers and have different plasticity [40]. A summary of MSC surface markers in different species can be found in Table 1.

Canine MSCs, for example, have been shown to be positive for the markers STRO-1 and CD44 and negative for CD73, a classic human MSC marker [41]. Later, when MSC molecular markers from canine adipose tissue and ovarian tissue were compared, it was found that both derived cell types expressed CD44, CD90, and CD105; however, ovarian MSC-derived cells expressed higher levels of OCT4 than adipose-derived cells [18].

In equine species, MSCs from bone marrow (BM-MSC), adipose tissue (AT-MSC), and umbilical cord (UC-MSC) were compared with respect to their

immunophenotypic characterization and differentiation potential. It was shown that all three sources of MSC expressed CD105, CD90, and CD44; however, UC-MSC had lower expression of CD90 than the other sources. Interestingly, BM-MSC and AT-MSCs showed faster in vitro differentiation than UC-MSC [42].

In humans, BM-MSC, AT-MSC, and UC-MSC were compared and demonstrated to express varying levels of certain MSC markers, including lower expression of CD90 and higher expression of CD105 by UC-MSC than the other sources [43], similar to the results found in equine species. Human adipose tissue-, bone marrow-, umbilical cord blood-, and nasal septum (NSP-MSC)- derived cells were compared with regard to their pluripotency markers. It was shown that AT-MSC had the highest expression of Sox2, Klf4, and Lin28 but the lowest of Oct4 and cMyc genes. Meanwhile, BM-MSC had more expression of Nanog and cMyc and the lowest expression of Rex1. UC-MSC and NSP-MSC had more expression of Rex-1 and Oct4, respectively [44].

Regarding bovine species, some characterization has taken place, as shown in Fig. 1. Bovine MSCs derived from different tissues have been shown to be positive for mesenchymal markers related to adhesion such as CD29, CD166, CD105, surface enzymes such as CD73, receptors such as CD44, and glycoproteins such as CD90 [1, 16, 22, 45–48]. Interestingly, bovine MSC also expressed pluripotency markers such as OCT4, SOX2, and NANOG [1, 16, 21, 45, 46], supporting the idea that MSCs have the potential to be pluripotent and differentiate into three germ layers, which was previously shown by the successful differentiation of bovine MSC into osteoblasts, lipoblasts, hepatocytes, islet cells, and neurocytes [22].

Regardless of the cell source or isolation procedure, MSC should express CD105, CD73, and CD90 and lack the expression of hematopoietic markers such as CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR surface molecules, as established by the International Society for Cellular Therapy as the minimum criteria for MSC characterization in humans [26]. These are used as de facto criteria in other species as well. Currently, there are no specific criteria for mesenchymal stem cell characterization in cattle. Future challenges include defining a standard characterization protocol of MSC in this species. Despite the lack of commercial antibody availability for cattle, PCR can be used for the study of MSC molecular profile. For translational medicine, a complete evaluation of different sources of MSC needs to be performed, in order to evaluate similarities between human and bovine MSC.

Sources of bovine mesenchymal cells

Bone marrow

Bone marrow was the first tissue described as a source of plastic-adherent, fibroblast-like cells that develop fibroblastic colony-forming units (CFU-F) when seeded on tissue culture plates. MSCs derived from bone marrow were first isolated and identified in mice and were described as non-hematopoietic cells with the potential to differentiate into mesodermal tissues, such as adipocytes, osteoblasts, chondrocytes, and skeletal muscle cells [49]. In cattle, bone marrow has been the source for MSC in several studies [16, 46, 50–52]. In this procedure, marrow cells are aspirated from calves and isolated for further analysis. Many reports with bovine BM-MSC focused on chondrogenic differentiation [50–52]. Spontaneous chondrogenesis of bovine MSC in pellet culture occurred without the addition of any external bioactive stimulators, i.e., factors from the transforming growth factor (TGF)-β family, previously considered necessary [50]. The same group isolated bovine MSC from eight calves and induced them to undergo osteogenic, chondrogenic, and adipogenic differentiation [51]. One year later, the same group analyzed the MSC chondrogenic response during culture on different types of extracellular matrices (ECM). Bovine MSCs were cultured in monolayer as well as in alginate and collagen type I and II hydrogels, in both serum-free medium and medium supplemented with TGF-β1. Differentiation was most prominent in cells cultured in collagen type II hydrogel, and it increased in a time-dependent manner. TGF-β1 treatment in the presence of collagen type II provided more favorable conditions for the expression of the chondrogenic phenotype. It was concluded that collagen type II has the potential to induce and maintain MSC chondrogenesis, but in the presence of TGF-β1, the cells expressed higher transcript levels of genes associated with differentiation, suggesting a higher fidelity differentiation [52]. The presence of BM-derived MSC with a pluripotent profile was demonstrated in later experiments. The cells were adherent to plastic surfaces and exhibited fibroblast-like morphology. In addition, the cells expressed pluripotent markers, such as OCT4, SOX2, and NANOG, as well as typical MSC markers, including CD29, CD90, and CD105. When the cells were isolated from fetal BM, they exhibited fibroblast-like morphology and were able to differentiate into hepatogenic and neurogenic lineages. The cells were not only positive for MSC markers CD29 and CD73 but also for the pluripotency markers, whereas they were negative for hematopoietic markers CD34 and CD45 [16].

Adipose tissue

Currently, bone marrow and adipose tissue are the main sources of MSC in veterinary medicine [53]. However, AT-MSCs have some advantages over BM-MSC, including faster development in vitro [53], easier isolation, and higher density stromal cells [54]. To date, there are only two studies in bovine species with MSC isolated from adipose tissue [47, 55]. In both studies, cells exhibited fibroblast-like morphology and were able to differentiate into osteogenic, chondrogenic, and adipogenic lineages; they expressed different MSC markers in each of the studies. In one study, cells were positive for CD105, CD73, CD29, CD90, and H2A markers and negative for CD45, CD34, and CD44 markers [55], while in the other study, cells were positive for CD90, CD105, and CD79 and the negative for CD45, CD34, and CD73 [47]. MSCs are known to demonstrate considerable variability between populations in their proliferation, differentiation, and molecular phenotype [39, 40, 56].

Umbilical cord

The umbilical cord has two sources of MSC. One is the cord blood, from which the cells are isolated by density gradient, and the other is the cord tissue, from which the cells can be disaggregated by enzymatic action. The cord blood is collected non-invasively and represents an alternative source of stem cells when compared to adipose tissue and bone marrow. In addition, the high availability and lower immunogenicity of umbilical cord blood cells compared to other sources of stem cells such as bone marrow have made them a viable and valuable source for cell therapy [45].

It was reported that cells isolated from the umbilical cord blood of humans have more MSC volume and greater plasticity, are genetically more flexible than bone marrow MSC, and also, as noted above, produce a less prominent immune response [57, 58]. While MSCs derived from umbilical cords in human, murine, and avian species have been the subject of many investigations, little is known about these cells in livestock species [22]. The first study that isolated bovine MSC from the umbilical cord blood observed that the cells grew into monolayer cell sheets and could be expanded into high passages. In addition, the cells expressed OCT4 and CD73 and were able to differentiate into osteogenic, chondrogenic, and adipogenic lineages [45]. In another study, isolated cells were sub-cultured to passage 32 and expressed CD29, CD44, CD73, CD90, and CD166 [22]. Moreover, those cells were able to differentiate into osteoblasts, lipoblasts, hepatocytes, islet cells, and neurocytes, indicating their potential

use for experimental and clinical applications for bovine, and very importantly showing evidence that MSCs have the potential to differentiate into non-mesodermal lineages [22].

Placenta and fetal fluids

The placenta performs a number of very important roles during pregnancy, including being responsible for the supply of nutrients, production of hormones, elimination of waste, and facilitation of gas exchange [59]. The placenta can be isolated easily by non-invasive harvest after delivery without any ethical or moral concern [20]. Only one study with bovine placenta-derived mesenchymal stem cells has been published, in which the authors successfully differentiated islet-like cells from the placental stem cells. The isolated cells expressed typical mesenchymal stem cell markers, including CD73 and CD166, and a pluripotent marker, OCT4, but not hematopoietic markers, such as CD45 [20].

Regarding fetal fluids, it has been reported that the amnion and amniotic fluid are abundant sources of mesenchymal stem cells that can be harvested at low cost and without ethical conflict [1]. The authors isolated MSC from amniotic fluid, and the cells exhibited fibroblast-like morphology only starting from the fourth passage, being heterogeneous during the primary culture. Immunofluorescence results showed that amniotic fluid MSCs were positive for CD44, CD73, and CD166 but negative for CD34 and CD45. In addition, the cells expressed OCT4 and, when appropriately induced, were able to differentiate into ectodermal and mesodermal lineages [1].

Uterus

The endometrial stromal cells are dynamic, growing, and differentiating throughout the estrous cycle and pregnancy [60]. In addition, these cells are known to modulate the immune system and could have clinical applications for human and animal health [48]. Some studies have isolated and characterized bovine mesenchymal stem cells in the endometrium [21, 48, 60]. The cells had fibroblast-like morphology, and when cultured in a specific osteogenic medium, they rapidly developed the characteristics of mineralized bone [60]. The endometrium-derived cells were found to express MSC markers such as CD29 and CD44 [31] and pluripotent markers such as OCT4, SOX2, and c-KIT [21]. Moreover, the cells demonstrated excellent clonicity, differentiation potential in mesodermal lineages, and excellent maintenance of quality

after the cryopreservation process [48]. A recent report showed the ability of endometrial cells to adhere to the plastic culture dishes, displaying fibroblast-like morphology, high proliferative capacity, and the ability to differentiate into chondrogenic, osteogenic, and adipogenic lineages.

Therapeutic delivery of mesenchymal stem cells

To achieve the best response after cell therapy, the general health of the patient, time of cell application, cell type, delivery route, and number of applications must be considered [35]. Following stem cell derivation, cell expansion is needed for subsequent transplantation into the patient [61]. In addition, cryopreservation of these cells can provide a ready source of abundant autologous stem cells [62]. Cryopreservation of bovine MSC may be achieved successfully with no change in the characteristics between fresh and thawed cells [48]. The delivery of the cell preparation should take place rapidly in order to avoid changes in cell viability and to prevent biological contamination of the cells [61]. Moreover, it has been suggested that early administration of stem cells is presumed to be more advantageous than attempting treatment when fibrous scar tissue has already been formed [63].

The most effective delivery method depends on the condition that is being treated. Intravenous administration is possible due to the ability of MSC to migrate across the endothelium and home to injured tissues [10, 39]. However, cells can become trapped in the lungs [39]. Thus, direct injection to the injured tissue provides a more convenient method [64], aiming a high concentration of MSC at the injury site without the risk of cell migration to other sites in the body [10]. In cases in which relevant structural defects are present, such as segmental bone, articular cartilage, and soft tissue defects, the cells need to be delivered by a carrier in order to have a substrate to control cell adhesion as well as the location of the cells in vivo, and to form a template for the formation of new tissue [64]. Recently, decellularized tissue has proven to be a promising option for scaffold construction [65]. The bovine model in particular has an advantage when compared to smaller animal models such as mice, due to the larger quantity of tissue to be decellularized, providing a much closer analogy to human conditions for eventual translational applications in organ construction and tissue engineering [66].

Bovine mesenchymal stem cell therapy

Mastitis

The dairy industry is a multi-billion dollar industry, with 811 million tons of milk produced in 2017 [7]. Clinical mastitis significantly reduces milk production and animal value. It has a severe impact on udder tissue and is also an animal welfare issue. Very importantly, the damage caused by mastitis cannot be mediated or reversed with current therapeutic strategies. Bovine mammary stem cell therapy offers significant potential for the regeneration of the udder tissues such that they could be replaced/repaired with minimal side effects [67]. Furthermore, the anti-inflammatory properties of the MSC [1] could potentially reduce the severity of the disease. Stem cells modified with therapeutic agents may also be employed to combat mastitis. It has been reported that cloning the bovine lactoferricin (LfcinB) gene into the PiggyBac transposon vector is a feasible means of creating MSCs with heterologous expression of the hybrid antibacterial peptide LfcinB [68]. These cells would then confer their high antibacterial properties against bovine mastitis origin Staphylococcus aureus and Escherichia coli directly into the mammary gland, providing strong innate udder immunity to fight against intramammary infections [68]. This study represents a template for cost-effective expression of other antimicrobial peptides in genetic engineering. In addition to the therapeutic advantage of this approach, because of the high milk production ability, bovine mammary glands can be used as bioreactors for the production of proteins on a large scale for the pharmaceutical industry [68].

Biotechnology applied in animal reproduction

Nuclear transfer was successfully performed in amphibians in the 1950s and in mammals some 30 years later. Dolly the sheep was the first mammal to be cloned by somatic nuclear transfer [69]. The goal of nuclear transfer research was to introduce precise genetic modifications in livestock species by making the desirable modifications in cells used as nuclear donors [70]. MSC could be used to produce transgenic animals for the improvement of the animal's health as well as for biomedical interest, for example, to produce cows resistant to mastitis [71] and to recover proteins, such as human α-lactalbumin, from milk [72].

Another interesting possibility that arose from the development of nuclear transfer was that of cloned human embryos produced with the purpose of further establishment

of patient-specific ES cells for regenerative medicine [70]. However, bioethical issues and related regulations hampered the attempts at production of human embryonic stem cells. To overcome that issue, in 2006 [73], somatic cells were reprogrammed to a pluripotent state by introducing transcription factors (OCT3/4, SOX2, KLF4, and C-MYC) into their genome. These cells were called induced pluripotent stem cells (iPS) and had similar characteristics to ESC, including the ability to originate tissues from the three germ layers both in vitro and in vivo [73]. Despite the advantages of iPS, there are still several ethical issues related to their application, such as genetic instability, tumorigenicity, and differentiation. Also, efficient methods for cell transplantation need to be investigated further [74]. The low tumorigenicity and high differentiation potential have made MSC a very promising source of cells for the treatment of degenerative and inherited diseases [14].

Nuclear transfer technique is based on the transfer of the nucleus from a donor cell into an oocyte or early embryo from which the chromosomes have been removed [70]. The most important drawback of this technique is the inability of the ooplasm to eliminate epigenetic markers and restore the genetic material of the donor nucleus to the embryonic totipotent state [75]. Many studies have focused on resolving this inability, due to the importance of chromatin structure in the cell reprogramming process [76]. One of the areas that have been explored by these studies is the use of mesenchymal stem cells for somatic nuclear transfer, which has been suggested in bovine species [47, 55, 76]. For example, it was shown that the epigenetic status of bovine adipose-derived MSC was variable during culture. Of the cell passages examined in this study, passage 5 seemed to be the most efficient in the performance of nuclear transfer due to its high level of stemness, multipotency, and the low level of chromatin compaction [76]. The embryo production rate was also shown to improve when embryos were co-cultured with MSC [77], representing in yet another way the importance of MSC in addressing commercial goals.

Bone injuries

Although some bone fractures and small defects can regenerate, there are conditions in which tissue loss is too extensive, as well as cases of non-union fractures and other critical-size defects where osteogenesis does not physiologically occur [10]. This represents another opportunity in which the application of MSC could upregulate the body's regenerative process to improve patient recuperation.

The events associated with bone healing have been chronicled reviewed [78].

When a bone fracture occurs, the inflammatory response increases the blood supply to the region. Cellular recruitment initially leads to the replacement of the fracture hematoma with fibrous tissues and, progressively, cartilaginous matrix, which is subsequently replaced by bone through endochondral ossification in both the periosteal and endosteal callus. MSCs reside in the bone marrow in low densities, and the recruitment of MSC to the fracture site is critical. This recruitment occurs by way of a chemotactic stimulus and results in the homing of circulating stem cells to the site of injury. Once these cells arrive, they begin participating in repair mechanisms [78].

The reconstruction of large bone segments is a relevant clinical problem. Preclinical and clinical data are accumulating to support the use of MSC to enhance bone repair and regeneration [79]. There are no clinical data on the use of mesenchymal stem cells for bone repair in cattle, although the ability of MSC to differentiate into the osteogenic lineage has been shown [1, 21, 22, 46, 51, 56, 60, 80–83].

Attitudes in the livestock industry have shifted towards the preservation of the commercial viability of individual animals with high genetic value, leading in turn to an increase in medical expenditure to keep those animals healthy. Owners are frequently willing to elect expensive treatments, even when the prognosis is poor, when cattle have high economic or genetic potential [8]. This notwithstanding, a number of criteria should be carefully analyzed when deciding the best treatment for a bone fracture, such as cost and success rates of the treatment, the value of the animal, and the location and type of fracture. Unlike horses, only rodeo livestock cattle need to perform athletically; thus, musculoskeletal integrity is less of an issue. However, fractures can result in a loss of meat and milk production and interfere with reproductive efficiency, including nefarious effects on natural breeding and impairment of embryo and semen production as well [8]. Thus, MSC could represent an important auxiliary source in the treatment of bone fracture for cattle for multiple reasons, including their anti-inflammatory potential [1], their ability to increase angiogenesis, and their ability to stimulate intrinsic progenitor cells to regenerate tissue functionality [3]. MSC treatment has the potential to reduce animal recovery time and reduce economic loss associated with bone injury, reducing the time for repair that can negatively influence milk and meat production and interfere with natural breeding, as mentioned above. In addition, the reduction of the recovery period can improve the outcomes for cattle with aggressive behavior, in which conventional treatment would be impractical due to the necessary motion constraints and temperament issues.

Joint injuries

In cattle, chronic osteoarthritis (OA) has been reported to be a significant cause of infertility in bulls [84], leading to economic loss and decrease in animal value. OA is a degenerative disease of the articular cartilage, which causes the release of pro-inflammatory cytokines [85]. The molecules involved in the OA process include growth factors, transforming growth factor β (TGF-β1), and cytokines and chemokines such as IL-8 [86, 87]. These molecules influence a wide range of biological processes that include cell proliferation, differentiation, migration, and apoptosis [88]. In horses, the efficacy of stem cells for the treatment of OA has been evaluated in the form of experimental and clinical studies, with more favorable results for bone marrow-derived cells than adipose-derived cells. The fact that MSCs secrete paracrine signaling molecules and trophic factors that influence cell response to injury and modulate the innate immune response [5] demonstrates the potential use of those cells for OA treatment in cows. In this species, there are no current clinical data, although some studies have demonstrated the isolation of MSC and their potential to differentiate into the chondrogenic lineage [21, 45, 50–52, 55, 81, 83, 89–91]. Methods are evolving to achieve this goal. To induce MSC to undergo chondrogenic differentiation, factors that support strong cell-cell interaction, growth factors, and an environment which maintains spherical morphology such as polymer gels have been shown to be required [52]. It has further been reported that the age of the cell donor and the biochemical microenvironment are the major determinants of both bovine chondrocyte and MSC functional capacity [90].

Diabetes mellitus

Currently, experimental and clinical data have provided support for the use of MSC for the treatment of diabetes mellitus [92]. Diabetes mellitus occurs in cattle and is similar to juvenile onset diabetes mellitus in humans, in that it is often immunomediated [93]. In cattle, no genetic background for diabetes has yet been confirmed [94]. Other etiologic factors have been implicated. Cases of diabetes have been reported in cattle infected with bovine diarrhea virus [94–96], and with foot and mouth disease [97]. Two mechanisms have been proposed to explain how the virus causes diabetes mellitus: (1) the beta cells in the pancreas are directly destroyed by the virus or (2) the immune response against the virus infection could induce an autoimmune response in the host [96]. The lack of insulin in animals with diabetes mellitus results in elevated glucose levels in the blood and urine. In addition, fatty acid synthesis in the liver is impaired in

the diabetic animal and this leads to acid-base balance impairment, ketoacidosis, and dehydration, resulting in collapse, coma, and death [98]. MSCs were shown to transdifferentiate into islet-like clusters expressing insulin and glucagon [24]. At present, there are no clinical data available to validate MSC treatment for diabetes in bovine species. However, recent and promising evidence demonstrates that bovine MSCs have been successfully differentiated into islet-cells [20, 22]. More studies need to be done in order to prove the functionality of those cells for eventual use in preclinical trials and pharmaceutical studies.

Potential of the bovine model for improvements to human health

The use of domestic animals as models has an essential role in narrowing the gap between translational research and clinical practice [99]. In regenerative medicine, the greatest advantage of using these models is to answer questions regarding the benefits and potential risks of stem cell treatments [100]. Each treatment needs to be tested in animal models, outlining human phenotypes, such as the size of the organs and more similar physiology [100]. Once the safety and efficacy of the treatment are proven, it can be applied in human therapy [100, 101]. The traditional model used for stem cell biology is the mouse, mainly because of its low cost, rapid reproduction, and ease of genetic modification [100]. Despite these advantages, the mouse model fails to precisely reproduce certain human diseases [100, 102]. Additionally, mice have a short lifespan, small body size, and different physiology when compared to humans [102]. Moreover, it is difficult to mimic the complexity of genetically heterogeneous human populations when studies are done with small groups of inbred mice [103]. To effectively study regenerative medicine and make the jump from the laboratory to human health applications, different animal models need to be used, allowing for better and more complete evaluations of cell-based therapies. In order to achieve this goal, it is important to select the most appropriate animal model, considering both size and experimental tractability, for example, ease of surgical manipulation, abundance of blood and tissues, efficiency of cloning, and feasibility of xenotransplantation [99]. Generally, larger animals are a better choice of model than mice for this purpose, specifically because they have a longer life span, which enables longitudinal studies, and because their physiological parameters are closer to those of humans [100]. Moreover, large animal species are more appropriate for mimicking human clinical settings due to their anatomy and physiology [99, 104].

The increase of genetic information can lead to new and more effective

methodologies for the elimination or treatment of factors that negatively impact human health, such as cancer, cardiovascular disease, low birth weight, and infertility [99]. An important advantage of using cattle as a model is the possibility to study genetic and environmental influences on animal production and human disease [105]. The cattle genome contains a minimum of 22,000 genes, of which approximately 80% are shared with humans [106]. Due to these advantages over the mouse model, it is clear that more widespread adoption of the bovine model would have positive consequences for human health. In the field of tissue engineering, large animal models represent a promising tool that allows for the translation of novel experimental scaffolds into clinical practice [107].

An important advantage of large animals in tissue engineering is the fact that they provide large amounts of tissue that, after decellularization, can be used as scaffolds with similar organ size to that of humans as proven, for example, with the bovine placenta [66]. In order to elucidate physiological processes important to human health, the bovine model can be used for the study of reproduction regarding aging, physiology, gametogenesis, and infertility, as well as for bone structure formation, fat deposition, altitude and heat tolerance, hematopoiesis, leukemia, tuberculosis, xenotransplantation, gene therapy, and stem cells [99].

Although the use of a large animal model confers considerable advantages for translational applications, there are also some drawbacks that are important to consider when making a choice of model for an experiment. The major disadvantages of bovine models include the expenses of animal care, facility maintenance, necessity of veterinary support, and lesser availability of antibodies, probes, and reagents. However, due to the fact that they are more appropriate to mimic human scenarios than rodent models, these studies are essential to justify the risks and costs of clinical trials [108]. Research done in less translatable models such as mice necessitates repetition in more applicable organisms, leading to additional costs and delays developing critically needed therapies.

One example is stroke, which affects more than 795,000 people every year in the USA, costing $34 billion each year, frequently leaving victims permanently and severely disabled [109]. Current drug therapies are unable to regenerate lost tissue functionality, merely ameliorating the symptoms of the disease. Over the past 20 years, a number of promising studies have been published demonstrating the potential of MSC therapy to achieve recovery of the injured tissue, as reviewed in 2016 [110]. However, the vast majority of these studies have not been in translatable models, leading to a lack of progress towards new human therapies. With this in mind, future studies should focus

on large animal models in order to evaluate the responses and safety of MSC therapy and advance the progress towards translational results. The ability to regenerate the damaged tissue suggests superior results to traditional therapies, and likely at a lower cost. The profound improvement in patient outcomes suggested by a potential switch to regenerative therapies for stroke victims provides just a single advantageous example of the many diseases in which cell therapies would vastly improve standards of care. This improvement would also, importantly, be accompanied by a significant reduction in the cost of treatment. On average, a stem cell treatment costs $5000 [111], so to treat 795,000 people per year would cost approximately $4 billion per year, resulting in massive savings in healthcare spending when compared to current therapies. Also noteworthy here is that cell therapies are still in their nascency and will likely continue to become less expensive as protocols are more completely developed and refined.

One potential area for cost reduction is an improved culture and transplantation methods. For example, the recently developed capacity to select a homogenous population of MSC without the necessity of cell sorting, accomplished through the selection of only the most adherent cells, can reduce the cost of cell production, not only because there is no need for expensive equipment and antibodies but also because in the first passage, a population is already selected, thereby reducing the cost of cell culture [19]. Additionally, it is known that bovine cells, when cultured at a higher density, can lead to less time and cost before transplantation [112].

It has been suggested that the National Institutes of Health could provide a national consortium of core laboratories with large animal models, facilitating the scientific community's use of the models and furthering efforts to develop cell therapy and translation into human therapies [108]. This would provide a great improvement over the current, over-the-counter system, in which individual researchers are required to connect with individual livestock owners to arrange experiments. The opportunity to use cattle for regenerative medicine purposes may increase the efficiency of human therapy and reduce costs and work around the ethical issues of human clinical trials. Additionally, cell therapy in cattle creates the opportunity for producers to improve their production by applying cell-based therapy to their own animals, as previously discussed.

As discussed in this review, bovine mesenchymal stem cells have the potential to be differentiated into all three germ layers and can contribute to a large amount of studies in different areas of medicine that can be implemented in translational medicine, including bone and joint injuries, immunomediated diseases, type 1 diabetes,

musculoskeletal disorders, infertility, and mastitis. Regenerative medicine and translational research need to interact in order to achieve an interdisciplinary perspective, investigating new insights into traditional clinical therapy and benefiting human and animal health.

Conclusion

The fact that stem cell technology has developed significantly in non-bovine species creates both interest and background knowledge for the advancement of similar techniques in livestock. Mesenchymal stem cells are considered a promising source of cells for regenerative medicine. Initial interest in MSC was sparked decades ago due to both their inherent ethical appeal versus ESC and their suitability for laboratory work, resulting from the rapid cell culture and expansion that can take place after enzymatic disaggregation of tissue. This initial interest was compounded by revelations of diverse and medically relevant physiological effects such as their ability to proliferate in situ, modulate immune responses, and promote angiogenesis. Their potential clinical applicability and the scientific effort subsequently directed towards them were later expanded greatly when experiments proved that MSC could differentiate into cell types from all three germ layers, a typical minimum criterion for a cell type to be considered pluripotent. A potential reclassification of MSC as pluripotent is supported by results observed in bovine studies, which demonstrated again the ability of MSC to differentiate into all three germ layers and also showed them to display a gene profile consistent with pluripotency. The use of the bovine model for translational medicine has been shown to be advantageous, especially due to its abundance of biological material and similar size, anatomy, and physiology when compared to the traditional model. Isolation of bovine MSC has been performed from different tissues; however, the cells seem to express different markers according to the isolated tissue. More studies are needed to clarify species-specific protocols for bovine applications, in particular, because of the lack of availability of specific commercial antibodies. Additionally, their differentiation potential and clinical response need to be further investigated. It is clear from the information presented in the preceding articles that the ongoing development of bovine cell therapy shows promise for both veterinary clinicians and the livestock industry, especially for conditions that can result in loss of production from animals, such as mastitis and musculoskeletal disorders. The use of mesenchymal stem cells is an important tool both in the treatment of degenerative diseases and the improvement of functional recovery from traumatic injury. In addition, MSCs have the potential to be used to manipulate productivity in the cattle industry and to be used in nuclear transfer

and also represent a tool for the preservation of valuable genetic resources. The lack of published studies and available clinical data in cows indicates both a deficiency and an opportunity of economic interest in this field of research. The next step will be to apply bovine MSC in clinical trials and evaluate the response of the animals as well as the economic impact of the techniques.

Availability of data and materials

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

Authors' contributions

ABTH contributed to the conception and design of the manuscript, analyzed and interpreted the available data, and drafted the manuscript. FFB, BDM, and JMG contributed to the design of the manuscript and revised it for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Competing interests

 The authors declare that they have no competing interests.

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Figure 1.

CFU

PDT

Osteoblasts

Chondrocytes

Adipocytes

Isolation and expansion

POSITIVE MARKERS

CD29, CD166, CD105, CD73, CD44,CD79, CD90, OCT4, SOX2, NANOG, H2A

NEGATIVE MARKERS

CD34, CD45

Bone marrow
Adipose tissue
Uterus
Umbilical cord
Placenta and fetal fluids

Manipulation of productivity
Biotechnologies applied in
animal reproduction
Mastitis
Genetic resources
Joint injuries
Bone injuries
Diabetes

Fig. 1 Isolation, characterization, and potential applications of bovine mesenchymal stem cells. Bovine mesenchymal stem cells have been isolated from the uterus, umbilical cord, bone marrow, adipose tissue, placenta, and fetal fluids. After isolation, the cells are expanded and characterized to prove their mesenchymal nature. The ability to self-renew is an important feature to be characterized in vitro and can be done by analysis of colony unit formation (CFU) and population doubling time (PDT). The cells need to show the ability to differentiate into osteogenic, chondrogenic, and adipogenic lineages. The bovine-isolated cells have already been shown to be positive for some mesenchymal and pluripotent markers and negative for hematopoietic markers. After characterization, the cells can be injected into the animal for therapeutic applications. Uses of bovine MSC for treatment of joint injuries, mastitis, and bone injuries; preservation of genetic resources; manipulation of productivity; and use in biotechnology applied in animal reproduction have all been suggested.

Table 1. Cell surface markers in different species.

Cell Surface Markers		Species	References
Positive	Negative		
CD105, CD73, CD90	CD45, CD34, CD14, CD19, HLA-DR	Human	26
STRO-1, CD44, CD90, CD105	CD73, CD45, CD34	Canine	18, 41
CD105, CD90, CD44	CD34, MHC II	Equine	42
CD29, CD166, CD105, CD73, CD44, CD90	CD45, CD34	Bovine	1, 16, 22, 45, 46, 47, 48

CHAPTER 2: PLACENTAL SCAFFOLDS HAVE THE ABILITY TO SUPPORT ADIPOSE-DERIVED CELLS DIFFERENTIATION INTO OSTEOGENIC AND CHONDROGENIC LINEAGES

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Abstract

Prudent choices of cell sources and biomaterials, as well as meticulous cultivation of the tissue microenvironment, are essential to improving outcomes of tissue engineering treatments. With the goal of providing a high-quality alternative for bone and cartilage tissue engineering, we investigated the capability of bovine placental scaffolds to support adipose-derived cell differentiation into osteogenic and chondrogenic lineages. Decellularized bovine placenta, a high-quality scaffold with practical scalability, was chosen as the biomaterial due to its rich ECM, well-developed vasculature, high availability, low cost, and simplicity of collection. Adipose-derived cells were chosen as the cell source as they are easy to isolate, non-tumorigenic, and flexibly differentiable. The bovine model was chosen for its advantages in translational medicine over the mouse model. When seeded onto the scaffolds, the isolated cells adhered to the scaffolds with cell projections, established cell-scaffold communication and proliferated while maintaining cell-cell communication. Throughout a 21-day culture period, osteogenically differentiated cells secreted mineralized matrix, and calcium deposits were observed throughout the scaffold. Under chondrogenic specific differentiation conditions, the cells modified their morphology from fibroblast-like to round cells and cartilage lacunas were observed as well as the deposit of cartilaginous matrix on the placental scaffolds. This experiment provides evidence, for the first time, that placental scaffolds have the potential to support mesenchymal stem cell adherence and differentiation into osteogenic and chondrogenic lineages. Therefore, the constructed material could be used for bone and cartilage tissue engineering.

Key words: cartilage tissue engineering; bone tissue engineering; placenta; mesenchymal stem cells; decellularization; recellularization; cows; translational medicine

Introduction

In adult mammals, most tissues manifest an inflammatory response and scar tissue formation after injury, which, although having beneficial effects, is often associated with loss of tissue functionality (Londono & Badylak, 2015). When an injury to osteogenic tissue is extensive, surpassing the tissue's healing capacity, the injury site is not properly regenerated, and, consequently, fibrous connective tissue becomes the dominant tissue type in the injured area (Liu et al., 2010). The orthopedic surgical community, which carries out more than 1 million surgical procedures involving fracture repair, partial excision of bone, and bone grafting each year in the United States of America alone (U.S. Census Bureau, 2006), typically employs autograft and allograft procedures to mitigate this phenomenon. Though well established, these approaches entail significant postoperative complications and limited availability (Reichert et al., 2011). In this context, the field of bone tissue engineering aims to develop a more effective, less traumatic, easily scalable treatment through the in vitro production of biological structures (Shafiee & Atala, 2017) that restore or establish tissue functionality, regenerating defects or even curing diseases (Evans et al., 2006).

Cartilaginous tissue, like osteogenic tissue, has poor capacity for healing and is prone to accidental damage and age-related abnormalities in many species (Agrawal et al., 2018), leading to a similarly inadequate state of current treatments for common diseases. This is particularly significant in articular cartilage, which consists of avascular connective tissue and chondrocytes within the extracellular matrix (ECM), and has poor self-repair capacity (Risbud & Sittinger, 2002). The most prevalent of all musculoskeletal system disorders is osteoarthritis (OA), which is a degenerative disease of the articular cartilage that has a negative impact on the quality of the patient's life (Risbud & Sittinger, 2002). In humans, OA affects approximately 10% of the population older than 60 years (Peat, et al., 2011). In companion animals, the disease is often triggered early in life (Brown et al., 2010). In cattle, OA may impact reproduction and cause infertility (Wolfe, 2018), leading to economic loss and decrease in animal value. Performance horses are particularly prone to injuries of the musculoskeletal system and prognoses are generally poor (Brehm, 2012). Due to its limited ability to regenerate, the repair of articular cartilage remains one of the most challenging problems in musculoskeletal medicine (Huang et al., 2018). Currently, treatments for OA include pain mediators, nonsteroidal anti-inflammatory drugs (NSAIDs), lubricating supplements, and surgical interventions (Altman et al, 2009). In the United States of America, US$600 billion are spent annually on pain medication, the majority by patients with arthritis and other musculoskeletal pain (Simon, 2012). The biggest concern is that

those treatments are based on temporarily alleviating symptoms, rather than correcting the pathogenesis of the disease or reversing the process of OA (Mei et al., 2017). Attempts to regenerate cartilage began in the 1990's with the use of autologous chondrocyte transplantation (Brittberg et al., 2001). This technique consists of cartilage tissue harvesting and the isolation, in vitro expansion, and implantation of chondrocytes into the injury site. The unsolved challenges in this approach are the damage to the donor cartilage tissue at collection, low cell density at isolation, dedifferentiation of the chondrocytes during cell expansion and, even, the risk of inducing fibrocartilage formation (Schnabel et al., 2002).

Considering these issues, tissue engineering approaches for cartilage aim to restore the injured area through the transplantation of cells on a supportive matrix, a combination that mimics natural cellular environments more faithfully than conventional cell cultures (Risbud & Sittinger, 2002). The most important components of tissue engineering are the characteristics of the cells used, the support of an adequate biomaterial (scaffold) for cell adherence, and the creation of an appropriate environment for the promotion of tissue formation (Evans et al., 2006; Shafiee & Atala, 2017). In fact, creating a scaffold that replicates that environment sufficiently enough to support the development of functional tissue has proved challenging. In bone and cartilage engineering, currently used biomaterials can be divided into synthetics, including polycaprolactone (Jeong et al., 2012) and polylactic acid (Oshima et al., 2009); inorganic materials including calcium phosphates and bioactive glasses (Jones et al., 2006), and natural biomaterials, such as collagen (Tohyama et al., 2009). Although inorganic scaffolds can easily bond to bone, they tend to be too fragile, week and brittle for load-bearing applications. Typically, synthetic materials offer biomechanical strength, but can also trigger immune responses; they also present difficulty in achieving satisfactory tissue differentiation and integration (Benders et al., 2013). The flaws mentioned in synthetic and inorganic scaffolds are well-documented and have persisted over several decades of dedicated research, begging the question of whether a more suitable class of material exists. With this question in mind, studies have focused on biological materials, whose natural biocompatibility overcomes the challenge of host rejection, and whose biodegradability overcomes the challenge of host integration. Critically, the inherent ability of biological scaffolds to provide the composition and structure of a natural extracellular matrix (Londono & Badylak, 2015), including, importantly, the tissue microarchitecture and vascular structures, suggests an ability to support cell adherence and differentiation to a much higher degree than could materials that lack such detailed and uniquely suitable features. Thus, for a successful culture, it is very important to find the ideal scaffold to act as the ECM as well as the ideal cell to interact with the scaffold in order to generate a new tissue.

Considering these criteria, placenta avails itself as a promising organ for biological scaffold production. It has a rich ECM and a well-developed vasculature, which can facilitate anastomosis with the host tissue (Kakabadze et al., 2014). An examination of bovine placenta decellularization found that it is also able to maintain its arrangement of principal ECM proteins throughout the decellularization process (Barreto et al., 2018) which in conjunction with its large size and other previously mentioned desirable characteristics, suggests that bovine placenta constitutes an appropriate choice of material for the development of large-scale scaffolds with complex vascular architecture. Additionally, the placental scaffold has been found to retain a number of beneficial biomolecules, including collagen, elastin, glycosaminoglycan, fibronectin and laminin, which are known to play an important role in cell adhesion (Barreto et al., 2018); cytokines, and growth factors such as GCP, SDF-1 and VEGF, HGF, EGF, FGFs, PDGF, and TGF-beta that favor chemotaxis and angiogenesis (Choi et al., 2013).

Mesenchymal stem cells (MSC) have become increasingly popular as an alternative for clinical use due to their low immunogenicity, high anti-inflammatory potential, ability to produce bioactive mediators and adhesion molecules that play a role in apoptosis and scar formation inhibition, angiogenic properties, and stimulation of intrinsic progenitor cells to regenerate their functionality (Kim & Park, 2017; Samsonraj et al., 2017). Due to the various advantages that MSC hold over embryonic stem cells, adipose derived mesenchymal stem cells were chosen as the cell source for recellularization of the present experiment.

Taken together, these advantages clearly imply the relevance and utility of answering the question of whether placental scaffolds would support MSC adherence and differentiation into osteogenic and chondrogenic lineages. The use of the bovine species is further supported by its value in translational research, due to its abundance of biological material and similar size, lifespan, anatomy and physiology to humans when compared to the traditional model, the mouse (Hill et al., 2019), potentially enabling breakthroughs in human tissue engineering research.

Materials and Methods

Scaffold production

The placentas were rapidly frozen after collection in preparation for the subsequent decellularization procedure, which was performed by perfusion of sodium dodecyl sulfate by umbilical vessels and isolation of cotyledons and validated as previously described (Barreto et al., 2017). The cotyledons' major vessels were cannulated by a urinary catheter and

perfused with phosphate buffer solution (PBS). Next, the decellularization process began with perfusion of sodium dodecyl sulfate (SDS), first at 0.01% for two days and then at 0.1%, 0.25% and 1% for 2 additional days each. In order to remove debris, the cotyledons were washed with PBS under agitation 3 times (Barreto et al., 2017).

Cell derivation and culture

Cell isolation procedure was approved by the Ethics Committee on the use of animals of UNESP‑FCAV (CEUA; protocol no. 018806/17). Therefore, all methods were performed in accordance with the relevant guidelines and regulations. Adipose tissue was collected from the base of the cow tail of six adult cows (*Bos taurus indicus*). Before the beginning of the procedure, the base of the tail was trimmed and carefully cleaned. Next, epidural anaesthesia was administered with 2% lidocaine chlorhydrate. A 2-centimeter incision was made at the base of the tail to retrieve approximately 1 g of adipose tissue. The isolation procedure was performed as previously described (Hill et al., 2017; Hill et al. 2018). The adipose tissue was placed in a 50 mL Falcon tube filled with phosphate buffered saline solution (PBS) on ice until arrival at the lab. In the laminar flow cabinet, the tissue was washed three times with PBS and minced with sterile scissors and tweezers for further digestion into a dish with collagenase type I. After three hours in the incubator at 38.5°C, the dish contents were washed with maintenance medium composed of Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Grand Island, USA) supplemented with 10% fetal bovine serum (FBS, Cripion, Brazil) and 2.5mg/mL of amikacin (NovaFarma, Brazil). The tissue was centrifuged at 300g for 10 min and the pellet was transferred into a T25 culture flask (Corning, New York, USA), constituting a two-dimensional culture (2D culture). The first medium change was made three hours after the beginning of the culture, as previously shown to be a method effective in generating a homogenous population of mesenchymal stem cells at the first passage through the selection of only the most adherent population of cells (Hill et al., 2017; Hill et al. 2018). When the cells reached 75-80% confluence, they were trypsinized (TrypLE, Invitrogen, Grand Island, USA) and plated on news dishes. The cells were cultured at 38.5°C in a humidified atmosphere containing 5% of CO_2.

Cell differentiation

Cell differentiation analysis was performed by seeding the passage two cells at a density of 1.0×10^4 cells per well, in biological triplicate, in a 4 well culture dish. Differentiation

medium kits were used in assays for osteogenic and chondrogenic differentiation (StemPro; Thermo Fisher Scientific, Invitrogen, Grand Island, USA) over the course of 21 days. The medium was replaced every 4 days. For cytochemical staining, differentiated cells were washed with PBS, fixed with 4% formalin for 10 minutes, and washed again with PBS for further staining. The cells were exposed to tissue-specific cytochemical staining protocols according to the manufacturer's guidelines, where the cells were stained by Von Kossa for osteogenic differentiation, and Alcian blue for chondrogenic differentiation.

Quantitative reverse-transcription analysis

Gene expression quantification was evaluated by quantitative real time polymerase chain reaction (qPCR). This assay was performed in the six isolated cell lines and in biological triplicate of cell-scaffold complexes. The RNA from the cell-scaffold complex was extracted with Trizol (Carlsbad, USA). In both cases, mRNA were treated with DNase I (Qiagen, Hilden, Germany) and 500 ng of total RNA was reverse-transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). For each qPCR reaction, 12.5 µL of SyBr Green PCR master mix, 1.0 mM of each primer, 2.0 µL of diluted cDNA (63.0 µL of ultrapure water and 1.0 µL of cDNA) and 5.5 µL ultrapure water were used. The default cycle program from the 7500 real-time PCR system was used (qPCR, 7500, Applied Biosystem). Data were analyzed according to the dCt method from Livak et al.(2001). The expression of each gene was calculated as a ratio with the geometrical mean of two housekeeping genes. Primers were designed using the NCBI primer design tool (Table 1). The statistical analyses were performed using a T-test in GraphPad Prism, $P < .05$ was considered to be statistically significant.

Scaffold cell seeding

Before cell seeding, the scaffolds were minced, in order to have approximately 0.5 cm in size, sterilized by UV light for 30 min and placed in the incubator for 5 days with only culture medium in order to observe potential microorganism contamination. The cell seeding on the scaffolds was performed in 96-well culture plates at a density of 1×10^4 cells/well, in biological triplicates of four cell lines, using a 2D rocker system that completed 24 cycles per minute. The cells seeded onto the scaffold constitute a three-dimensional culture (3D culture). Five experimental groups were maintained: (1) cells seeded without the scaffold with maintenance medium, (2) cells seeded without the scaffold in osteogenic or chondrogenic induction medium, (3), cells seeded onto the scaffolds and cultured with

maintenance medium, (4) cells seeded onto the scaffolds and cultured in osteogenic or chondrogenic induction medium, (5) scaffolds with no cells culture with differentiation medium. The experiment was performed for 21 days; differentiation and maintenance medium were changed every four days.

Histochemical staining

Recellularized placental scaffolds were fixed in 4% buffered paraformaldehyde (PFA) solution for 48 h, dehydrated in graduated concentrations of ethanol, diaphanized in xylene, paraffin embedded, sectioned in 5 μm by a microtome, and placed onto glass slides. Hematoxylin-eosin (HE) and Von Kossa (VK), Safranin O and Fast green stains were performed to evaluate cell population density, calcium deposits and cartilage deposition, respectively. Von Kossa solution was prepared using 5g of silver nitrate in 100 mL of distilled water. The samples were exposed to UV light for 60 minutes while in this solution. After the washing steps, the samples were incubated with 5g of sodium thiosulfate in 100 mL of distilled water for 3 minutes, followed by staining with fast red solution for 5 minutes. Fast green solution was prepared using 0.1g of Fast green in 100 mL of 1% acetic acid solution. The samples were incubated with Weigert's iron hematoxylin staining solution for 5 min, washed in running tap water for 5 min, incubated with 0.1% Fast Green solution for 4 min, and rinsed in 1% acetic acid solution, followed by incubation in 0.2% Acid Fuchsin for 10 min and finally washing in 96% ethanol for 1 min and then 100% ethanol for 1 min. For HE stain analysis, the samples were incubated with Hematoxylin for 4 minutes, washed and incubated with eosin for 2 minutes, after which the over-staining was removed by washing in running water. For Safranin O stain analysis, the samples were incubated with 0,1% Safranine O for 15 minutes and the over-staining was removed by washing in running water. The results were visualized by light microscopy (Eclipse 80i, Nikon).

Immunohistochemical analysis

The isolated cells and the recellularized placental scaffolds were fixed in 4% PFA for 48h. To inhibit nonspecific antibody binding, the scaffold-cell complexes were incubated with the blocking solution, 2% bovine serum albumin in PBS, at room temperature for 1h. After blocking, the material was incubated with primary antibodies, at a 1:100 dilution, against CD90 (ab225, Abcam), CD105 (MA5-11854, Invitrogen), CD34 (ab81289, Abcam), CD45 (ab10558, Abcam), CD73 (ab202122, Abcam) and Collagen type 1 (COL1; 600-401-103S, Rockland), that were diluted in 0.1% Triton X-100 in PBS, at room temperature for 1h.

After washing steps, the cell-scaffold complexes were incubated with secondary antibody at a 1:100 dilution for 1 h. Negative controls were performed by incubation with the secondary antibody only. After several washing steps, the scaffolds were incubated with 4′,6-diamidino-2-phenylindole (DAPI, 1:10.000, 62248, Thermo Scientific) for nuclear staining, which stains in blue. The results were obtained using confocal microscope (FV1000, Olympus).

Scanning Electron Microscopy (SEM)

Scaffolds were fixed in Karnovsky solution for 48 h, dehydrated in graduated concentrations of ethanol, dried using a critical point machine and covered in gold for further evaluation using a scanning electron microscope (Zeiss EVO MA-10).

Results

Scaffold production

The cotyledonary component of bovine placenta was successfully decellularized and its integrity was validated as previously described (Barreto et al., 2017). Fig. 1a shows the native placenta and fig. 1b shows the absence of cells on placental scaffolds after the decellularization procedure, evidenced by nuclear staining (DAPI). SEM analysis confirmed the absence of cells and highlighted the maintenance of the vili (Fig. 1c) and major fibers (Fig. 1d) after the decellularization. Additionally, histological sections stained with HE showed the absence of cells after decellularization, which can be observed in Fig. 2 g.

Mesenchymal Stem Cell Characterization

In order to select the most adherent cells, a previously developed protocol based on cell adherence in the first three hours of culture, at which point the first medium change was performed, was employed (Hill et al., 2017; Hill et al., 2018). Cells from all digested tissues were isolated and demonstrated their ability to rapidly adhere to the plastic surface and proliferate in a homogeneous monolayer of cells. The isolated cells, group 1, displayed fibroblast-like morphology (Fig. 2a) and proliferated for more than 10 passages. Moreover, the cells were positive for the MSC markers CD73, CD90 and CD105, and were negative for the hematopoietic markers CD34 and CD45, as evidenced by immunocytochemistry analysis (Fig. 3a). In addition, group 3, cell-scaffold complexes cultured in maintenance

medium, not only continued to express the MSC markers CD73, CD90 and CD105, but also increased the expression of CD105 (p= 0.0065) and CD90 (p=0.0027) when compared to the group 1, cells cultured without the scaffold in maintenance medium (Fig. 3b).

Next, we verified the ability of the derived cells to differentiate into osteogenic and chondrogenic lineages in the absence of the scaffold in experimental group 2. During 21 days of culture, cells were exposed to specific differentiation conditions. Calcium deposits were observed by Von Kossa staining, confirming osteogenic differentiation (Fig. 2b). To verify chondrogenic commitment, proteoglycan accumulations were observed by Alcian blue staining (Fig. 2c).

Cell-scaffold interactions

After characterization of the isolated cells as MSC, they were seeded onto the placental scaffold and cultured using a dynamic 2D rocker system. In group 3, cells seeded onto the scaffolds and cultured for 21 days in maintenance medium were able to adhere and proliferate throughout the scaffold, which can be observed by HE histology stain (Fig. 2d). The cells maintained their fibroblast-like morphology and grew in layers. Communication by cell-cell contact was observed, and cell projections interacted with the scaffold (Fig. 4a).

Cell differentiation on the scaffold

The last step was to verify the ability of the scaffolds to support osteogenic and chondrogenic lineage differentiation of the isolated cells, a process carried out in experimental group 4. Regarding osteogenic differentiation, after one week of culture with differentiation medium only a few cells could be observed throughout the scaffold (Fig. 5a) and the COLLAGEN TYPE 1 (COL1) stain was weak. In the second week of differentiation (Fig 5b), COL1 stain increases, as does cell number, evidenced by nuclear staining (Fig. 5b), a pattern which continued in the third week of differentiation (Fig. 5c). By histological analysis, it was possible to observe calcium deposits throughout the scaffold (Fig. 5d) in the first week of differentiation, which progressively increased in the second (Fig. 5e) and the third week of differentiation (Fig. 5f), as evidenced by Von Kossa stains. HE stain was used to observe the presence of cells in the third week of differentiation (Fig. 5g). Fig. 5h shows the enlargement of panel (f) for better observation of calcium deposits throughout the scaffold. Moreover, cell secretion could be observed not only by histological analysis but also as evidenced by scanning electron microscopy (Fig. 4c and d). In addition, cell-scaffold

complexes expressed COL1, an osteogenic marker, when analyzed by qRT-PCR (Fig. 5i), whereas group 3, cultured in maintenance medium, had no amplification of this gene and but had amplification of MSC markers (Fig. 3b), providing evidence that the cells in this group preserved their mesenchymal nature. Additionally, by histological analysis of group 3, no signs of differentiation were observed when the cells were seeded onto the scaffold cultured with maintenance medium, which is evidenced by a lack of calcium deposits and lack of osteogenic matrix formation as showed by Von Kossa stain (Fig. 2e). Histological analysis of group 5, where the scaffolds were cultured in the absence of cells with differentiation medium, also showed no signs of calcium deposits and no osteogenic matrix formation, as can be observed by Von Kossa stain (Fig. 2h).

In regard to the chondrogenic lineage differentiation of group 4, the cells displayed changes in morphology from fibroblast-like (Fig. 4a) to round cells (Fig. 4b) after 21 days. After one week of culture with differentiation medium cells were able to adhere to the scaffold (Fig. 6a and d). After 21 days of differentiation, Safranin O staining revealed the accumulation of cartilage matrix and chondrocyte-like cells (Fig. 6b), as well as the formation of cartilage lacunas (Fig. 6c). The same cartilage matrix deposition and chondrocyte-like cells were observed by Fast Green staining (Fig. 6e and f). HE stain was used to observe the presence of cells in the third week of differentiation (Fig. 6g). In addition, transcripts for a well-known cartilage-specific gene, COLLAGEN TYPE 2 (COL2), were found to be expressed by the cell-scaffold complexes after 21 days of differentiation by qPCR analysis (Fig. 6h). Moreover, no signs of differentiation were observed in the control groups; group 3, cells seeded onto the scaffolds and cultured with maintenance medium continued to express mesenchymal markers (Fig. 3b), preserved their fibroblast-like morphology (Fig. 2d and Fig. 6a), and had no amplification of differentiation markers. Additionally, when this group was stained with Safranin O, neither cartilaginous deposits nor chondrocyte-like cells were observed (Fig. 2f). Lastly, group 5, the scaffolds cultured with no cells and chondrogenic medium for 21 days was unable to form cartilaginous matrix (Fig. 2i).

Taken together, all these findings provide evidence that when the cells are seeded onto the scaffolds and cultured with osteogenic or chondrogenic medium they are able to differentiate and to induce osteogenic matrix and cartilaginous matrix formation, respectively; however, the scaffolds without the cells do not have the ability to respond to the chemicals present in the differentiation medium, having no ability to produce new ECM; the cells seeded onto the scaffolds and cultured with maintenance medium maintained their mesenchymal nature.

Discussion

This is the first experiment to show that bovine placental scaffolds support osteogenic and chondrogenic differentiation of bovine MSC selected by a rapid adherence protocol. The distinct qualities of the scaffold material and cell type chosen demonstrated the synergies that were hypothesized on the cellular level, with the placental scaffolds clearly supporting the attempted differentiations. The bovine model was used due to its potential for translational research. As discussed in Hill et al. (2019), human MSC and bovine MSC have shown various similarities, including the presence of markers, such as CD105, CD73, CD90 and the lack of CD34 and CD45. In general MSC profiles are similar across species, increasing their suitability for translational work.

The use of adipose-derived mesenchymal stem cells for this experiment was similarly informed by the goal of investigating the most practical and effective factors available. Adipose-derived cells are considered to be ideal for applications in regenerative therapy due to their ease of isolation and maintenance in culture, great differentiation potential, low immunogenicity, and ability to secrete trophic factors that enforce therapeutic and regenerative outcomes in a wide range of applications (Frese et al., 2016; Samsonraj et al., 2017). Moreover, it has been previously reported that, when compared to bone marrow-derived cells, adipose-derived cells are preferable, due to their higher rates of survival, proliferation and their ability to promote bone matrix formation (Koroleva et al., 2015).

Adipose tissue is heterogeneous in its composition, due to its endocrine and metabolic roles. This heterogeneous nature dictated the use of a previously developed protocol to select a more homogenous population of MSC, based on the rapid adherence of the cells to the plastic during the first three hours of culture (Hill et al., 2017; Hill et al. 2018). This method is advantageous because of its ability to select cells with a MSC profile at initial passages, representing a significant reduction in costs of cell culture and cell sorting compared to other isolation techniques (Hill et al. 2018). The isolated cells displayed fibroblast-like morphology and grew in a monolayer in both the presence and absence of the scaffold, as represented by groups 1 and 3, respectively, and group 3 was able to form layers with cell-cell communication on the scaffolds. Additionally, the cells of group 3 expressed mesenchymal markers after 21 days of culture, including CD90, CD105, and CD73. The fact that the cells maintained their mesenchymal nature suggests the possibility of transplanting the scaffolds with undifferentiated cells for therapeutic purposes. This approach would take advantage of their angiogenic and anti-inflammatory potential, as well as their ability to stimulate intrinsic progenitor cells to regenerate their functionality, potentially providing a novel method for the delivery of biologic factors in the field of tissue

engineering, applicable to a wide variety of approaches.

It has been proposed that an ideal scaffold should have the ability to support cell viability, attachment, growth, differentiation, new matrix production, vascularization and host integration (Amini et al., 2012). In accordance with this ideal, the cells used in this experiment for recellularization displayed adherence and growth, as well as clear cell-scaffold interactions through cellular projections, which facilitates cell-cell communication, and the ability to successfully differentiate. The other aspects of the ideal scaffold were not directly observed in this experiment, providing clear direction for further investigation.

Another important consideration when using biological scaffolds is the potential transfer of hazardous biological materials from the donor to the host. In order to minimize these risks, a protocol was adapted for this experiment that rigorously removes all antigens before seeding of new cells begins (Barreto et al., 2017). Our findings suggest success in the creation of a nurturing environment with the chosen scaffold material and do not indicate the presence of toxic effects that could potentially be caused by residues of the chemicals used during the decellularization procedure.

The osteogenic and chondrogenic lineage differentiations performed in this study were successful, as supported by histology, staining assays, and SEM. In previous reports on osteogenic differentiation, focus has been placed on scaffold composition, which has been shown to influence the results of differentiation. For example, the ratio of hydroxyapatite to tri-calcium phosphate may influence MSC osteogenic differentiation (Arinzeh et al., 2005). Another approach that has been used to support cell differentiation is the application of mechanical force to a collagen-gel scaffold, which has been shown to facilitate the differentiation of MSC into ligament tissue (Altman et al., 2002). The fact that the placental scaffold was able to support osteogenic and chondrogenic lineage differentiation without the influence of such factors represents both an advancement and a simplification in bone and cartilage tissue engineering, removing the necessity of expensive techniques and complex scaffold production protocols while still favouring the desired lineage differentiations.

The chondrogenic lineage differentiation protocol was supported by analogous assays. Previous reports have shown that the matrix composition of the scaffold may influence cell behavior, affecting the eventual cell differentiation, including chondrogenic differentiation (Li et al., 2015). Cartilage ECM can be divided into two major categories: collagen and proteoglycans, which are composed of aggrecan and glycosaminoglycans (Risbud & Sittinger, 2002). The decellularized placenta employed in this study contains collagens I, III and IV, fibronectin, and laminin (Barreto et al., 2017), making placental scaffolds suitable for chondrogenic differentiation under appropriate stimuli. Safranin O

stains proteoglycans, chondrocytes and type II collagen in varying shades of red. In this study, Safranin O staining identified characteristic cartilage lacunas with round chondrocyte-like cells inside.

Considering the preponderance of support for the ability of decellularized placental scaffolds to facilitate osteogenic and chondrogenic differentiation of adipose-derived cells, the results of this study are strongly indicative of the assertion that this material has excellent potential to advance bone and cartilage tissue engineering, warranting further examination of its qualities with respect to transplantation, host integration, and clinical applications of the constructed material.

Acknowledgements

Data Availability

The authors declare that the data supporting the findings of this study are available within the article or are available upon request to the corresponding author.

Conflict of Interest Statement

The authors declare no conflict of interests.

Authors' contributions

ABTH contributed to the conception and design of the experiment, data production, analysis and interpretation, and manuscript drafting. AASA contributed to placental scaffold production and validation of the decellularization process. RBN contributed to placental scaffold production, validation of the decellularization process, data production, and revision for important intellectual content. FFB and MAM contributed to data collection and revision for important intellectual content. JMG contributed to the design of the manuscript and revision for important intellectual content. All authors reviewed the manuscript.

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Figures

Figure 1.
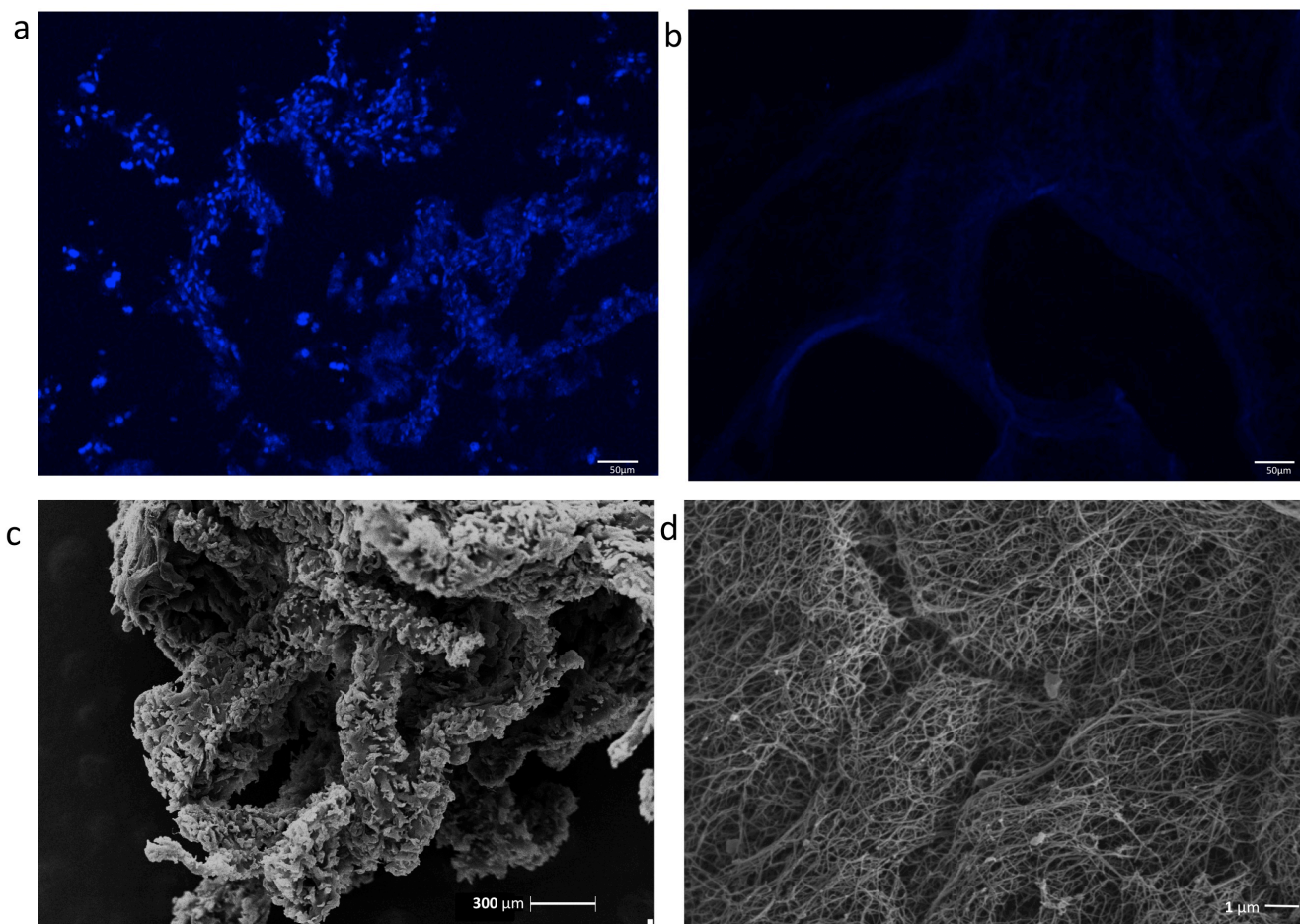
Figure 1. Decellularized placenta. (a) The native placental tissue and the decellularized placental tissue. (b) No cells are observed on the placental scaffo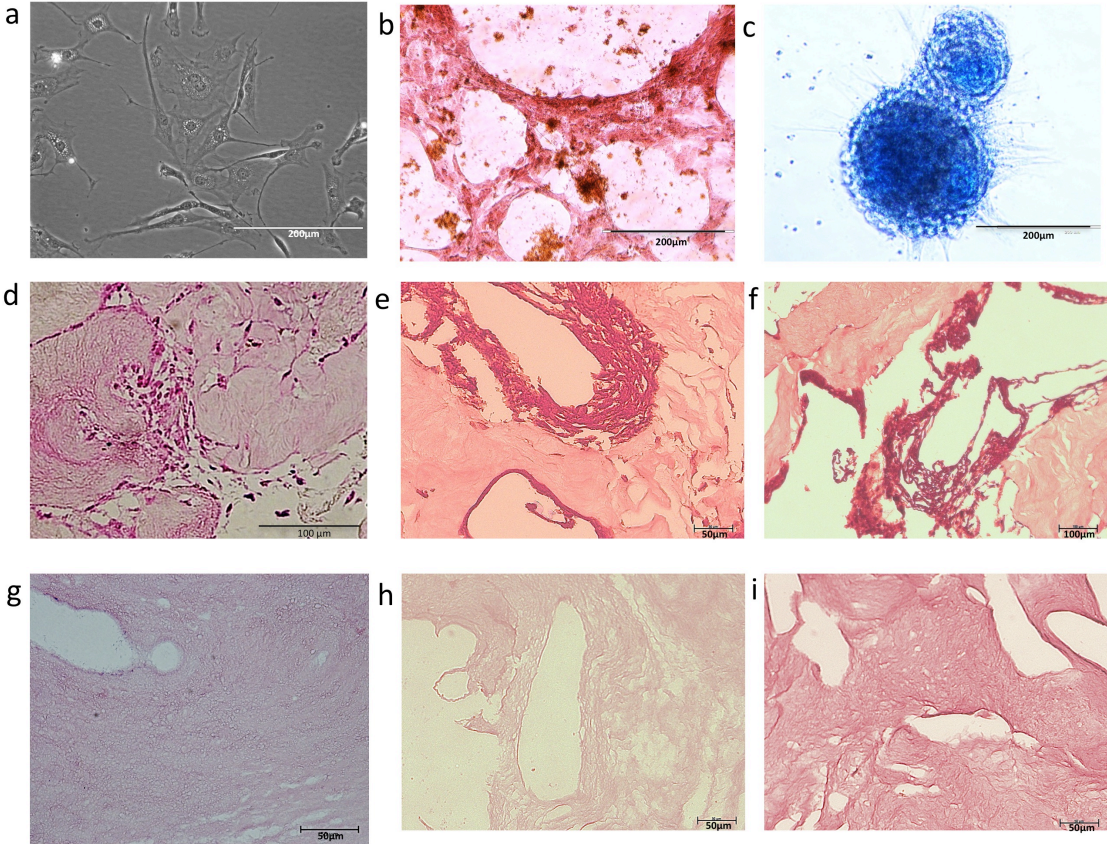ld after decellularization procedure. (c) The villi preserved its physical structure. (d) The fibers, mainly fibrous proteins from villi, are organized in a circular manner with large spaces` between them.

Figure 2.

Figure 2. Cell morphology, differentiation and negative controls. (a) The isolated cells adhered to the plastic surface of the culture dish and exhibited fibroblast-like morphology, experimental group 1. (b) Osteogenic differentiation of group 2, cells seeded in the absence of placental scaffolds, as stained by Von Kossa after 21 days of culture. (c) Chondrogenic differentiation of group 2, cells seeded in the absence of placental scaffolds, as evidenced by proteoglycans stained by Alcian Blue after 21 days of culture. (d) Experimental group 3, cells seeded onto the placental scaffolds cultured with maintenance medium, showed adherence to and growth on the biomaterial. Additionally, group 3, when stained with Von Kossa (e) and Safranin O (f) showed no signs of differentiation and no deposits of calcium or cartilaginous matrix. Experimental group 5, decellularized placenta cultured in the absence of cells and with differentiation medium, was stained with HE (g), evidencing the absence of cells, with Von Kossa (h) which shows the absence of mineralized matrix, and was also stained with Safranin O (i), showing the absence of cartilaginous matrix.

Figure 3.
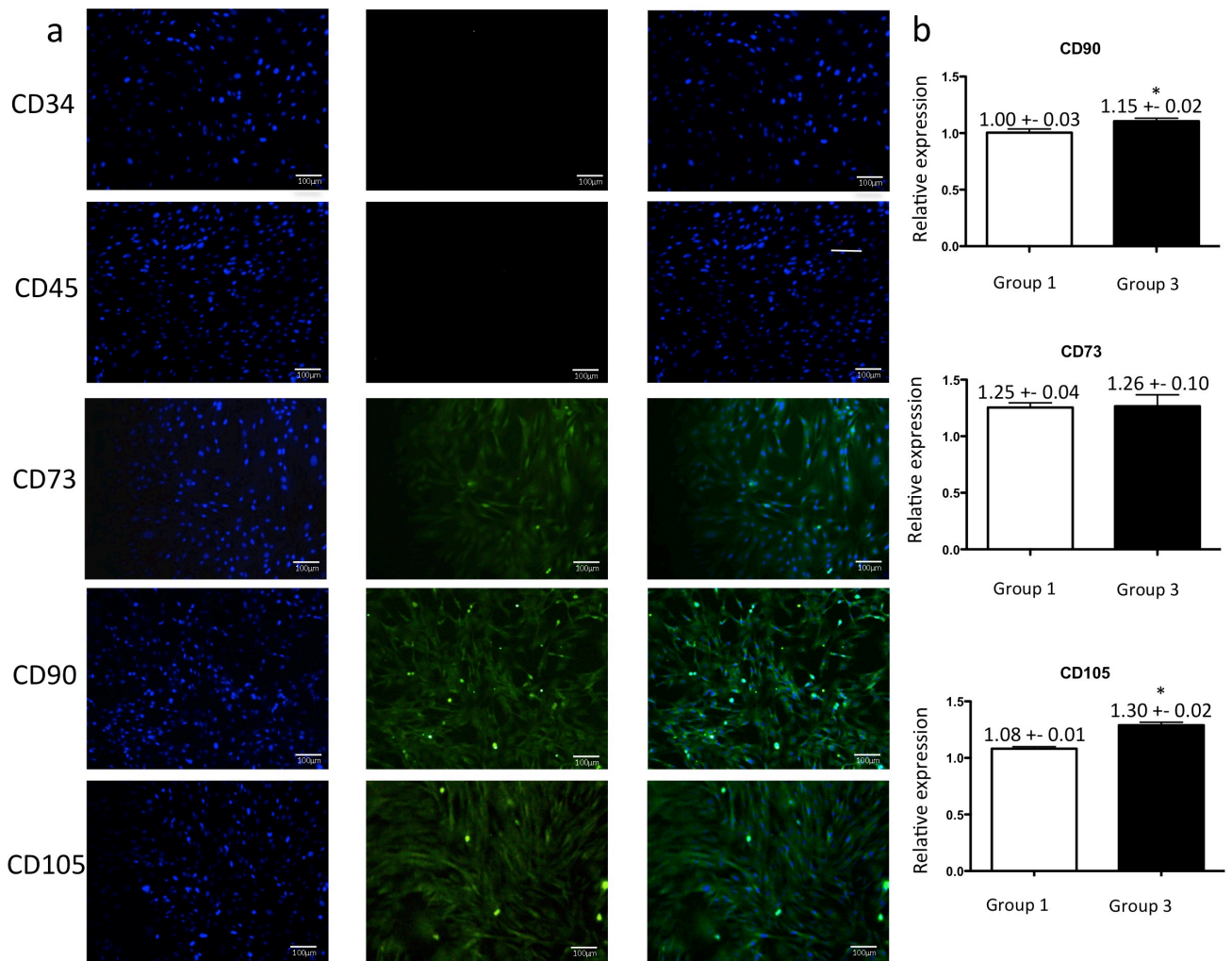
Figure 3. Mesenchymal stem cell characterization. (a) Immunocytochemical analysis of group 1, cells cultured with maintenance medium in the absence of the scaffold, in which the nuclei of the cells can be observed in blue by DAPI stain, the surface markers in green, and the merge of both. (b) qPCR analysis of group 3 showed not only continued expression of CD90, CD73 and CD105 after 21 days of culture on the scaffold, but also an increase in the expression of CD105 and CD90 when compared to group 1.

Figure 4.
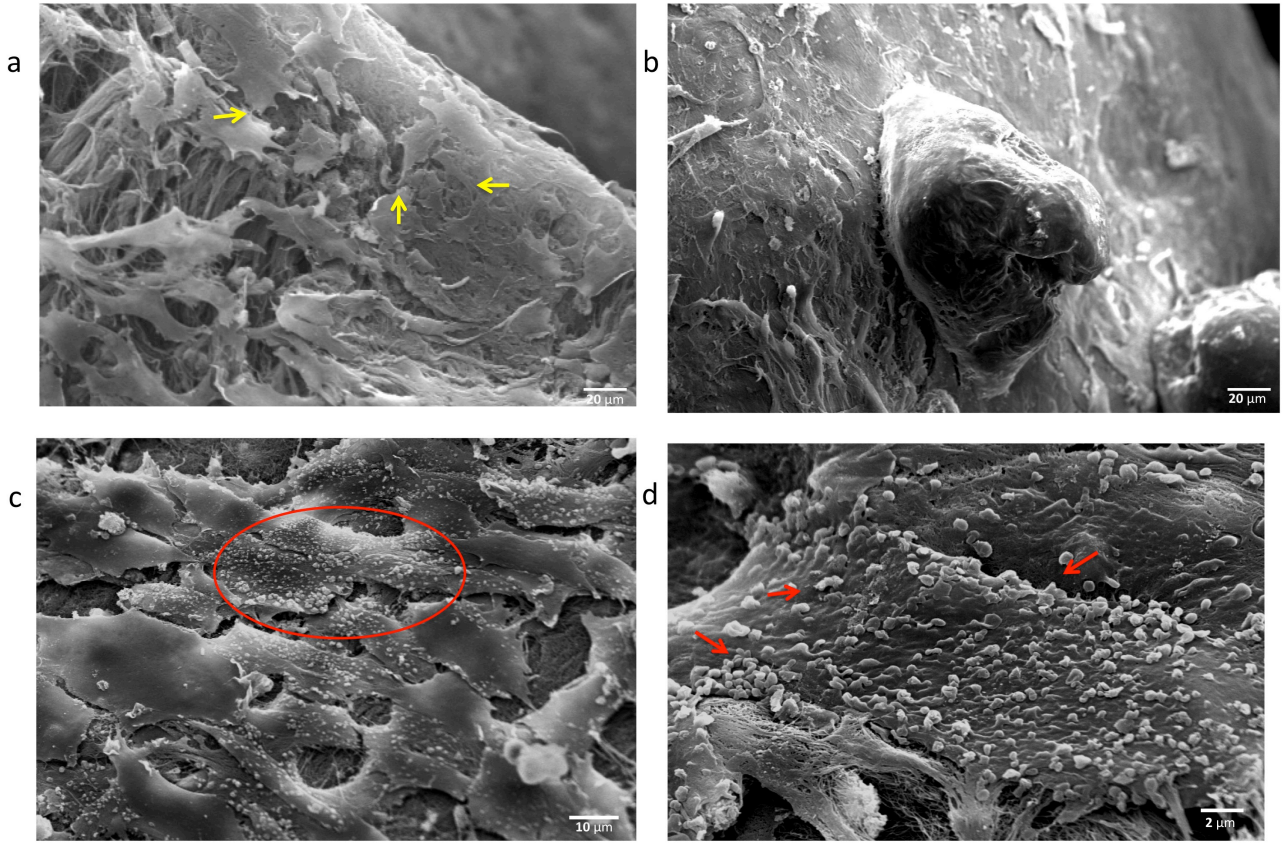
Figure 4. Scanning electron microscopy. (a) Group 3, cells seeded onto placental scaffolds cultured in maintenance medium showed monolayer growth with fibroblast-like morphology. The yellow arrows point out cell projections that may facilitate cell-cell communication and adhesion to the scaffold. (b) Group 4, after 21 days of culture in chondrogenic differentiation induction conditions, changed morphology when compared to group 3 and assumed a more a globular/spherical morphology that is consistent with chondrocyte differentiation. (c) Group 4, when seeded under osteogenic induction conditions for 21 days, showed globular/round structures compatible with mineralized matrix secretions, several of which are indicated by the red circle. (d) Higher magnification of panel c for better secreted structures visualization, which are pointed by the red arrows.

Figure 5.
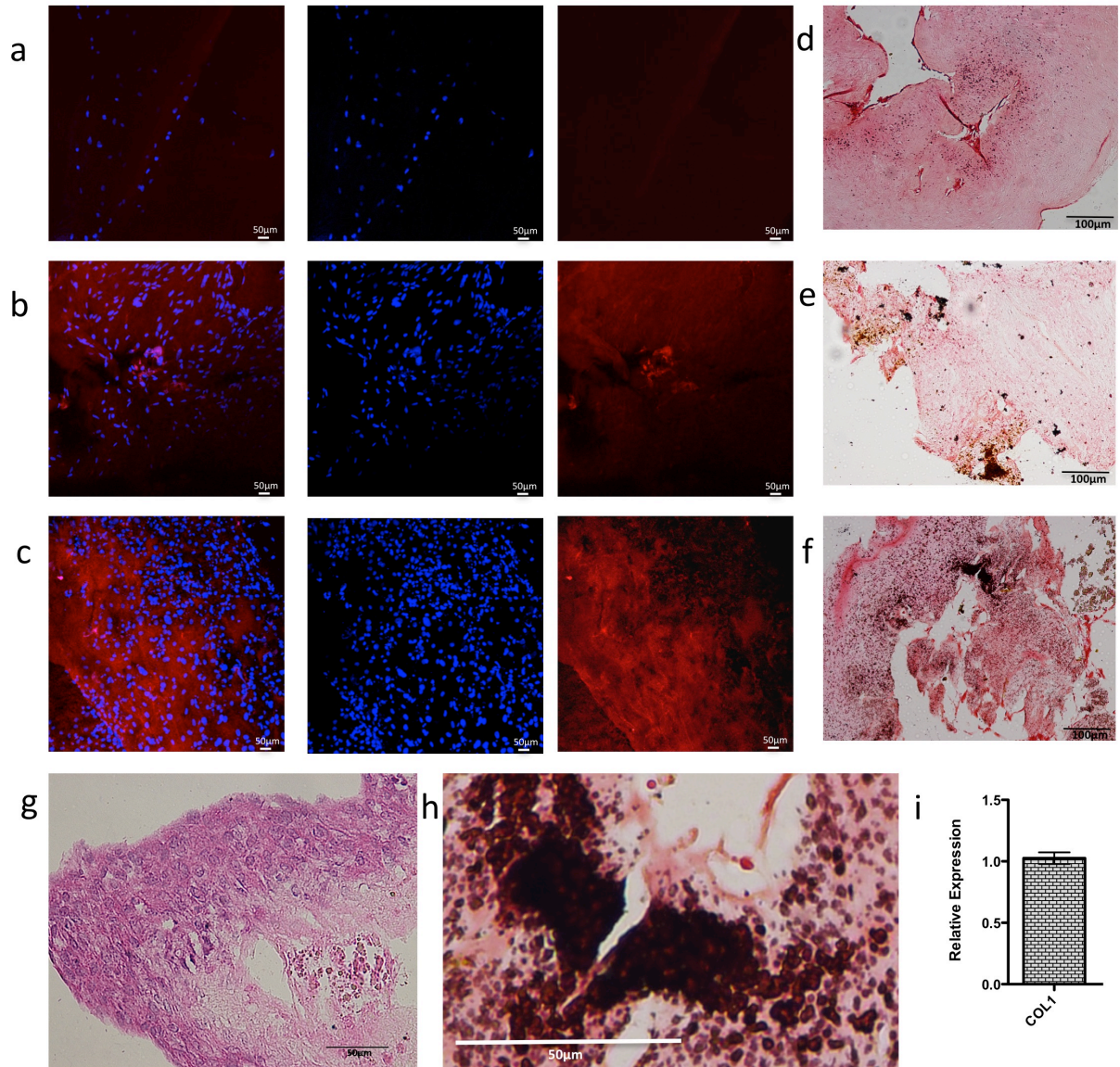
Figure 5. Osteogenic differentiation of cells seeded onto the scaffolds. Cells exhibited increased growth and differentiation in a time-dependent manner, which can be observed by the greater abundance of cells, evidenced by cell nuclei staining in blue, and COL1 in red, respectively, at week 3 (c) when compared to weeks 1 (a) and 2 (b). The same pattern was observed by histological analysis. Widespread calcium accumulations were also observed on the scaffolds by Von Kossa staining as time progressed. In the first week of differentiation (d) fewer cells and less calcium accumulation were observed in the scaffold when compared to the second (e) and third week of differentiation (f). (g) The histological HE staining showed the presence of cells throughout the scaffold in the third week of osteogenic lineage differentiation. (h) Higher magnification of panel (f) for better observation of calcium deposits throughout the scaffold. (i) Gene expression of COL1.

Figure 6.
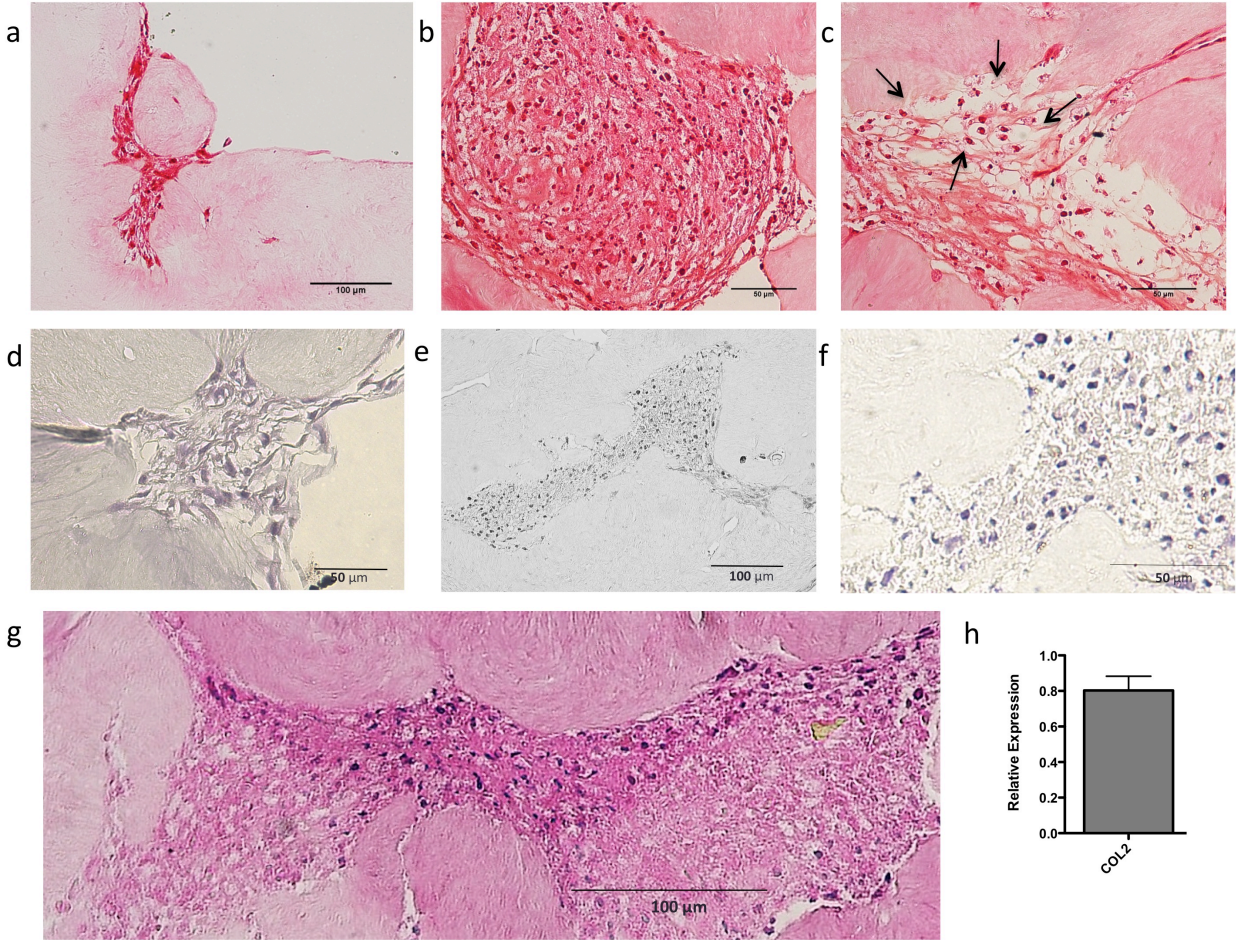
Figure 6. Chondrogenic differentiation on the placental scaffold. (a) After one week of culture with differentiation medium, cells were able to adhere to the scaffold. The pink Safranin O staining shows the accumulation of proteoglycans. (b) After 21 days of culture with differentiation medium, the cells had increased in number, and Safranin O staining revealed the accumulation of cartilage matrix and chondrocyte-like cells, as well as the formation of cartilage lacunas, indicated by the arrows (c). A similar pattern can be observed by Fast Green staining. In the first week, fewer cells were observed (d), but, as differentiation continued, the number of cells increased, as did their semblance of chondrocyte-like morphology and the amount of cartilage matrix (e and f). (g) Panel (e) was also stained with HE for better cell morphology and nuclei observation. (h) Gene expression of COL2.

Table 1. Sequences of primers used for gene expression analysis

Gene Symbol	Gene ID	Sequence (5'-3')	Tm (ºC)	Amplicon
GAPDH	281181	F: TCTGGCAAAGTGGACATCG R: GACCATGTAGTGAAGGTCAATGAA	60.0	60
ACTB	280979	F: GCGGACAGGATGCAGAAA R: ACGGAGTACTTGCGCTCAG	60.0	89
CD34	281051	F: GGGCATCGAGGACATCTCTG R: AATAAGGGTCTTCGCCCAGC	62.0	179
CD45	407152	F: TTGCCTTTCAGAAGGACGCA R: GGTCGTGGTCTGCTCATCTT	60.0	171
CD73	281363	F: GCAGCATCCCAGAAGATCCA R: CATCCGGGTGTCTCAGGTTG	62.0	198
CD105	615844	F: ACTCCAGCTGCCAAGCTAAG R: AGAGGCTGTCCGTGTTGATG	61.5	174
COL2	407142	F: GGTGAGCTATGATCCGCCTC R: GGGCTGAGGCAGTCTTTCAT	62.0	241
COL1	282187	F: ACATGCCGAGACTTGAGACTCA R: GCATCCATAGTACATCCTTGGTTAGG	61.5	86

CHAPTER 3: GENERAL DISCUSSION

Currently, the scientific community supports the use of stem cell therapy for a number of animal health conditions. The development and improvement of new cell culture conditions and differentiation methods led to progress in this area, which has made stem cell technologies more attractive for clinical use (Hill et al., 2019). Studies are still scarce in bovine species, even though bovine MSC have the potential to differentiate into all three germ layers (Xiong et al., 2014; Dueñas et al., 2014) and can contribute to a large number of studies in different areas of veterinary medicine that could be implemented in translational medicine. These include bone and joint injuries (Raoufi et al., 2011; Lara et al., 2017; Bosnakovski et al., 2005; Sun et al., 2014), immunomediated diseases, such as type 1 diabetes (Xiong et al., 2014; Peng et al., 2017) and mastitis (Sharma et al., 2013; Sharma et al., 2019). When compared to the traditional model, the mouse, bovine species hold advantages for use in translational medicine studies, including their abundance of biological material and greater similarity to humans in size, anatomy, lifespan and physiology (Bähr & Wolf, 2012; Ireland et al, 2008; Cibelli et al., 2013; Sirard, 2017).

This is the first experiment to show evidence that bovine placental scaffolds have the potential to be used for a variety of tissue engineering approaches when seeded with bovine adipose derived cells selected by a rapid adherence protocol. The isolated cells, when seeded onto placental scaffolds, demonstrated the ability to adhere, grow and differentiate into different lineages, providing evidence that placental scaffolds is able to support osteogenic and chondrogenic lineages differentiation.

Generally, in mammals, most tissues respond to an injury with extensive inflammation and, consequently, scar tissue formation, which can often lead to loss of tissue functionality (Londono & Badylak, 2015). In particular, when the injury is so extensive that it surpasses the tissue's healing capacity, the injury site is not properly regenerated, and fibrous connective tissue is formed as a consequence (Liu et al., 2010). In order to restore tissue functionality, new tissue engineering techniques have been extensively investigated and explored, probing for solutions to the underlying practical necessity of finding a cell-scaffold complex that has the potential to be easily fabricated at low cost for clinical dissemination. In this context, we have shown that bovine placental scaffolds seeded with bovine adipose-derived cells, both of which fit the necessary criteria for these goals, have the potential to be used for tissue engineering techniques.

Stem cell niches are dynamic environments composed of various elements, including the extracellular matrix, growth factors, the cells themselves and their secreted

factors (Li et al., 2015). The ECM plays a critical role in tissue engineering; being involved in various biological functions, including physical support, regulation of intercellular communication and growth factor storage (Gao et al., 2014). Thus, it is extremely important to find a scaffolding system that provides the right ECM, as well as the right cell type to interact with that scaffold. Many beneficial biomolecules can be found in decellularized placenta, including molecules that are conducive to cell adhesion such as collagen, elastin, glycosaminoglycan (Choi et al., 2013; Barreto et al., 2017), fibronectin and laminin (Barreto et al., 2017; Brigido et al., 2018) as well as cytokines, chemokines, and growth factors that play an important role in both chemotaxis, such as GCP and SDF-1, and angiogenesis or vasculogenesis, such as VEGF, HGF, EGF, FGFs, PDGF, TGF-beta (Choi et al., 2013). In addition, placentas have anti-inflammatory, antibacterial and antiscarring properties (Lobo et al., 2016). All of these properties of placental scaffolding's native ECM harmonize very well with the properties of MSC, which, when seeded onto the scaffolds, have been shown to adhere and proliferate. It has been proposed that an ideal scaffold should have the ability to support cell viability, attachment, growth, differentiation, new matrix production, vascularization and host integration (Amini et al., 2012). In accordance with these ideals, we have found that the placental scaffold used in this work supported cell attachment, proliferation and maintenance of the cells in their undifferentiated state, when culture with expansion medium. Therefore, these results are strongly indicative of the assertion that this material has excellent potential, warranting further examination of its qualities with respect to transplantation, host integration, and clinical applications of the constructed material.

In this study, we attempted to ensure that MSC surface markers would mediate cell adhesion, proliferation, and communication with the scaffold, so we therefore selected cells based on rapid adherence to the culture plate. Due to the fact that adipose tissue is heterogeneous in its cellular composition (Berry et al., 2015), it is important to isolate the right cell type. The protocol used for cell isolation in these experiments was previous reported to efficiently isolate homogeneous MSC at initial culture (Hill et 2017; 2018). Besides the advantage of efficient cell selection, this methodology decreases cell culture costs when compared to other cell selection techniques such as cell sorting or even cell adherence to the plastic during the first 48-72h, in which cell passaging is necessary to obtain a homogenous population (Hill et al., 2017; Hill et al., 2018). The fact that the cells cultured with expansion medium adhered and were uniformly distributed throughout the scaffold shows that the isolation method was effective in this regard. In addition, the cells showed extensions similar to fillipodic

structures, which have been suggested to play an important role in cell attachment to the scaffold and in cell communication (Barlian et al., 2018).

Another important finding is that the cells, after 21 days of culture with the scaffolds, maintained their fibroblast-like morphology and expressed MSC markers, such as CD90 and CD105. The fact that the cells were able to maintain their mesenchymal nature suggests the possibility of scaffold transplantation with undifferentiated cells for therapeutic purposes. This approach would have the advantage of the cells' potential to increase angiogenisis and anti-inflammatory modulation, in addition to their ability to foster the regeneration of intrinsic progenitor cell functionality, potentially providing the field of tissue engineering with a widely applicable and novel method for the delivery of biologic factors. The presence of growth factors that modulate angiogenesis (Choi et al., 2013) in the scaffolds, as well as the anti-inflammatory, antibacterial and antiscarring properties of the placenta (Lobo et al., 2016) lead us to suggest that the scaffolds and the cells could work together synergistically, with the potential to offer all the benefits listed above to the injured tissue, providing a high quality biomaterial for tissue engineering approaches.

In regard to osteogenic differentiation, the fact that calcium deposits were observed throughout the scaffold suggests that the placental scaffold supports osteogenic differentiation and the correct matrix deposition. In corroboration, the cell-scaffold complexes expressed osteogenic markers, such as COL1, RUNX and OSTEOCALCIN, which were not observed in the group cultured in expansion medium. These findings suggest that the placental scaffold supports MSC differentiation towards the osteogenic lineage, contributing to bone the tissue-engineering field and to transplantation techniques that could potentially regenerate defects and restore tissue functionality.

Due to the limited ability of cartilaginous tissue to regenerate, the repair of articular cartilage remains one of the most challenging problems in musculoskeletal medicine (Huang et al., 2018). In this context, we developed a biomaterial that could successfully differentiate into chondrogenic lineages, which was evidenced by changes in cell morphology, morphing from fibroblast-like to round cells. In addition, Fast Green and Safranin-O stained proteoglycans, chondrocyte-like cells, cartilage lacunas, and type II collagen, showing the deposition of cartilaginous matrix provided by cell differentiation towards cartilage lineages. Moreover, well-known cartilage-specific genes, including AGGREGRAN and COL2 were found to be expressed by the cell-

scaffold complexes. In regard to cartilaginous tissue, this scaffolding system provides the possibility of locally delivered cells that would support growth and differentiation, overcoming the perennial challenge of the regeneration in these tissues.

Here, we provide evidence that placental scaffolds support not only mesenchymal stem cell survival, growth, and cell communication, but also osteogenic and chondrogenic lineage differentiation. The potential therapeutic implementation of undifferentiated cells on transplanted scaffolds, benefitting from their stimulation of intrinsic progenitor cells and anti-inflammatory properties, represents a significant advancement in the search for new treatments in the field of tissue engineering in the form of a versatile and novel biologic factor delivery method. Specifically, this advancement allows for the potential eventual circumvention of autologous graft surgeries and, from a broader perspective, a step towards being able to truly regenerate lost functionality in cases of extensive bone and/or cartilage injury and chronic disease instead of palliative care or tissue substitution. It also represents an advancement in the practicality and implementability of eventual therapies due to the repurposing of a widely available and low cost biological waste product for the construction of high quality scaffolds.

In order to capitalize on the potential revealed by this experiment for advances in human and veterinary medicine, further experiments should be conducted with the aim of specifying and clarifying certain important details. Chief among these is the in vivo evaluation of transplanted MSC-scaffold complexes, which will provide data regarding the ability of the complexes to restore bone and cartilage function. Another important matter to be investigated is whether a transplant conducted with MSC that have already differentiated into osteogenic-like and chondrogenic-like cells would evoke a more efficient outcome than undifferentiated MSC with regard to restoring tissue function. These proposed experiments would build the foundation for the clinical use of these techniques and clear the way for novel regenerative therapies to treat musculoskeletal dysfunction.

Final Conclusions and Perspectives

The development of bovine cell therapy holds promise not only in the veterinary clinics and the livestock industry but also for translational medicine. Numerous studies have reported the ability of mesenchymal stem cells to undergo chondrogenic and osteogenic lineage differentiation, however, tissue regeneration using scaffolding systems has been challenging, especially due to the difficulties in finding the appropriate choice. Prudent choices of cell sources and biomaterials, as well as meticulous cultivation of the tissue microenvironment, are essential to improving outcomes of tissue engineering treatments. The findings in this manuscript provide evidence that mesenchymal stem cells can recellularized the placental scaffolds. In addition, the placental scaffold supported osteogenic and chondrogenic lineage differentiation, potentiating a number of promising and possibly breakthrough- level ideas in regard to the application of those cells in tissue engineering applications. Tissue engineering treatments hold the potential to greatly improve patient outcomes in a number of areas in which current treatments remain highly invasive and unable to completely restore the lack of functionality. Bovine placenta represents an attractive choice of scaffold material due to its high availability, large size, low cost, ability to be harvested without damaging the donor, rich ECM and well-developed vasculature. These investigations would allow the potential of this biomaterial, which possesses so many beneficial qualities and, just as importantly, could be mass-produced and made widely available, to make groundbreaking changes in myriad areas of medicine. Future work should focus on defining the in vitro and in vivo characteristics of the differentiated cells, including the presence and prevalence of specific cell types, their behavior when transplanted, and their ability to integrate with the host. We speculated that, potentially, the transplanted scaffold cells would have the ability to stimulate and support cell growth and differentiation, as well as ensure the deposition of the correct cellular matrix.

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