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Canine placenta: A promising potential source of highly proliferative and immunomodulatory mesenchymal stromal cells?

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ABSTRACT

In veterinary medicine, therapeutic mesenchymal stromal cells (MSC) have been traditionally isolated from adult bone marrow or adipose tissue. Neonatal tissues, normally discarded at birth from all species have become an alternative source of cells for regenerative medicine in the human clinic. These cells have been described as being more primitive, proliferative and immunosuppressive than their adult counterparts. Our objective was to examine if this phenomena holds true in dogs. Little information exists regarding canine neonatal MSC characterisation. In this study, we were able to both isolate, phenotype and assess the differentiation and immunomodulatory properties of MSC from canine foetal adnexa allowing us to compare their characteristics to their more well-known bone marrow (BM) cousins. Neonatal tissues, including amnion (AM), placenta (PL), and umbilical cord matrix (UCM) were collected from 6 canine caesarean sections. Primary cells were expanded *in vitro* for 5 consecutive passages and their proliferation measured. BM-MSC were isolated from 5 control dogs euthanised from other studies and grown *in vitro* using an identical protocol. All MSC lines were systematically evaluated for their ability to differentiate into 3 mesodermal lineages (adipocyte, osteocyte and chondrocyte) and phenotyped by cytometry and qPCR. In addition, the enzymatic activity of the key immunomodulatory marker indoleamine 2,3-dioxygenase (IDO) was evaluated for each MSC line.

MSC displaying a fibroblastic appearance were successfully grown from all neonatal tissues. PL-MSC exhibited significantly higher proliferation rates than AM- and UCM–MSC ($p = 0.05$). Cytometric analysis showed that all MSC express CD90, CD29, and CD44, while no expression of CD45, CD34 and MHC2 was detected. Molecular profiling showed expression of CD105 and CD73 in all MSC. Low levels of SOX2 mRNA was observed in all MSC, while neither NANOG, nor OCT4 were detected. All MSC differentiate into 3 mesodermal lineages. Following inflammatory stimulation, the activity of the immunomodulatory enzyme IDO was significantly higher in neonatal MSC compared to BM-MSC ($p = 0.009$).

Our results show that canine foetal adnexa cells share very similar properties to their adult equivalents but upon stimulation show significantly higher IDO immunomodulatory activity. Further studies will be needed to confirm the potential therapeutic benefits of these cells.

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Abbreviations: AM, amnion; AT, adipose tissue; BM, bone marrow; BMP-2, bone morphogenetic protein 2; BSA, bovine serum albumin; D, day; DMEM, Dulbecco's Modified Eagles Medium; EDTA, ethylenediaminetetraacetic acid; FCS, foetal calf serum; FGF-β, Basic fibroblast growth factor; HGF, hepatocyte growth factor; HK, housekeeping gene; HPRT1, hypoxanthinephosphoribosyl-transferase 1; IDO, indoleamine 2,3-dioxygenase; IFN-γ, interferon gamma; ISCT, International Society for Cellular Therapy; MSC, mesenchymal stromal cells; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; NaCl, sodium chloride; NO, nitric oxide; P1, passage 1; PGE2, prostaglandin E2; PL, placenta; qPCR, quantitative polymerase chain reaction; UCM, umbilical cord matrix; PBS, phosphate buffered saline; PDT, population doubling time; SD, standard deviation; TGF-β, transforming growth factor beta.

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1. Introduction

Mesenchymal stromal cells (MSC) have garnered a lot of attention in the past decade due to their successful use in regenerative medicine (Maumus et al., 2011). Traditionally, MSC were isolated from bone marrow (BM) or adipose tissue (AT) and used for autologous transplantation. More recently, neonatal tissues, discarded for a long time as medical waste, have emerged as a valuable alternative source of cells. MSC from neonatal tissues have been described as more primitive, proliferative and immunosuppressive than their adult counterparts (Cremonesi et al., 2011; El Omar et al., 2014). Use of neonatal material offers many other advantages, including tissue availability and a non-invasive ethical correct collection procedure. One recent report suggests that neonatal MSC are more "immune evasive than immune privileged" (Ankrum et al., 2014). In addition, several studies showed them to be less immunogenic than adult cells (Deuse et al., 2011; Prasanna et al., 2010), which potentially could allow their allogeneic use as an "off the shelf product". These properties could help to alleviate some of the limits found during autologous use of adult cells, including patient-to-patient variability, decline of MSC capabilities in adult tissue with age (Caplan, 2007) and delayed treatment times.

MSC use in veterinary regenerative therapy is an emerging field, (Fortier and Travis, 2011). Investigations using AT-derived MSC for the treatment of canine orthopaedic injuries have reported positive results (Black et al., 2007; Guercio et al., 2012; Vilar et al., 2013). Trials on larger animals may serve as an attractive preclinical models for the hospital clinic.

Canine MSC isolated from adult bone marrow and adipose tissue have been extensively described (Screven et al., 2014; Takemitsu et al., 2012; Vieira et al., 2010) while data about MSC derived from canine neonatal tissues, including umbilical cord blood (Seo et al., 2009), umbilical cord matrix (UCM) (Zucconi et al., 2010), amniotic membrane (AM) (Filioli Uranio et al., 2011; Lee et al., 2013a,b; Park et al., 2012) and amniotic fluid (Filioli Uranio et al., 2011) are more inconsistent. In particular, significant variation has been noted as to their proliferation potential, immunophenotype and molecular profiles. These differences may be due to diverse isolation and culture methods coupled to the scarcity of canine specific reagents along with a lack of appropriate experimental controls. Such confusion warrants further precise investigation of neonatal MSC from different sources.

Recent evidence has shown that clinical benefits of MSC transplantations rely mainly on their ability to modulate the local environment by secreting trophic and immunomodulatory factors, rather than their previously postulated *in vitro* differentiation potential (Lee et al., 2009; Murphy et al., 2013). MSC also interact with many effector cells involved in innate or adaptive immunity (Bernardo and Fibbe, 2013; Nauta and Fibbe, 2007). Molecular mechanisms by which MSC exert their immunosuppressive properties have been extensively described in human and rodent models. In particular, human MSC suppress T cell proliferation through the production of high levels of indoleamine 2,3-dioxygenase (IDO), while murine MSC produce high levels of nitric oxide (NO) (Ren et al., 2009). Currently, little information related to the immunomodulatory properties of MSC isolated from veterinary species is available. Soluble bio-factors produced by MSC can vary among species and by tissue sources (Carrade and Borjesson, 2013). For example, equine MSC isolated from bone marrow and umbilical cord blood produce NO whilst MSC derived from adipose tissue or umbilical cord matrix do not (Carrade et al., 2012). In dogs, only Kang and colleagues have reported the immunomodulatory properties of adipose tissue- (AT) MSC (Kang et al., 2008). They showed that AT–MSCsecrete soluble factors, such as transforming growth factor beta (TGF-β), hepatocyte growth factor (HGF), prostaglandin E2 (PGE2) and IDO that exert their effects on immune cells through a paracrine mechanism. To date, no information has been published about the immunomodulatory properties of canine neonatal MSC.

The aim of this study was to characterise the MSC isolated from 3 canine neonatal tissues: Amniotic membrane, placenta and the umbilical cord matrix, according to the guidelines designated by the International Society for Cellular Therapy (ISCT) (Dominici et al., 2006). This minimal set of criteria include 1-plastic adherence of the cells under standard culture conditions; 2-the expression of a panel of cell surface markers (CD105, CD73, CD90) and the lack of hematopoietic, endothelial and MHC2 markers; 3-the tri-lineage differentiation potential (adipocyte; osteocyte; chondrocyte). Stem cell-associated genes expression was also analysed. In addition, the proliferative potential of each MSC was compared over 5 passages. Finally, in order to fulfil the last proposal of the ISCT (Krampera et al., 2013), the immunological characterization of the MSC was carried out by measuring the IDO activity after inflammatory stimulation. For each assay, BM–MSC was used as a reference point.

2. Materials and methods

2.1. Tissue collection–MSC isolation and expansion

Canine neonatal tissues were obtained from full-term pregnant bitches following caesarean section performed at the Department of Theriogenology (VetAgro Sup, Marcy l'Etoile Lyon). All animal procedures were carried out in accordance with guidelines put in place by the Committee of Ethics of the University. Amniotic membrane ($n = 6$), placenta ($n = 6$) and umbilical cord matrix ($n = 5$) were carefully dissected and processed individually.

AM were washed extensively in 0.9% NaCl, then minced into small pieces and incubated for 30 min at 37 °C with 0.05% trypsin-EDTA to remove epithelial cells (Miki et al., 2010). The trypsin was inactivated by adding low glucose Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% foetal calf serum (FCS; Hyclone). AM was further digested for approximately 1 h with collagenase type I (Sigma–Aldrich) at 37 °C. Digested tissue was filtered through a 100 μm cell strainer (BD Biosciences) and centrifuged for 10 min at 250 G. The cell pellet was suspended in growth medium, consisting of DMEM supplemented with 100 U/ml Penicillin/Streptomycin, 2 mM L-glutamine, 10% FCS and 2.5 ng/ml basic fibroblast growth factor (FGF-β; R&D Systems). After 2 days, the cell culture medium was replenished with fresh medium and the cells were grown until sub-confluence. Placental tissue was exposed to the same procedure for cell isolation but the first trypsin digestion step was omitted and collagenase digestion was reduced to 30 min.

Umbilical cord matrix was processed using an explant technique. Briefly, umbilical cord was cut into small slices using a surgical blade and placed into cell culture dishes. Tissue pieces were covered with growth medium to allow cell migration from the explants. After 7 days, tissue pieces were carefully removed and cells were grown for an additional week before treatment by trypsin.

To validate the protocols used to phenotype the neonatal canine MSC, all the procedures have been concomitantly carried out using bone marrow (BM)-derived MSC. Femoral bone marrow was collected straight after the euthanasia of 5 dogs enrolled in an approved clinical protocol of the University (Beagles, 2–4 years old). Briefly, the bone was cracked under aseptic conditions and the marrow was scraped into a petri dish containing DMEM. Tissue was then mechanically minced, washed, and filtered through a 100 μm cell strainer. Following centrifugation, the cells were suspended in growth medium then seeded at 2.10^5 cells/cm^2. Non adherent cells were removed after 2 days and fresh medium added.

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