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**Disease Modifying Osteoarthritic Drug, Pentosan
Polysulfate Sodium Modulates Cytokine-Induced
Osteoarthritic Changes and Promotes Articular Cartilage
Tissue Regeneration *in vitro***

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A Dissertation submitted at Hokkaido University in fulfillment of the requirements for doctor of
philosophy (PhD) in veterinary Surgery

Hokkaido University

Sapporo

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Dedication

I dedicate this work to my lovely wife, Chanda Chitala and our two beautiful children; Jennifer Maambo Bwalya and Taizya Jacob Bwalya for their unconditional love, support and sacrifice throughout the four (4) years (October 2013 - September 2017) of the study program that we had been intermittently apart.

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List of publications related to the dissertation

The contents of this dissertation are based on the following original publications and submitted manuscripts under review listed below by their Roman numeral I-VI of the research conducted during the period October 2013 - September 2017.

- I **Bwalya, E. C.**, Kim, S., Fang, J., Wijekoon, H. M. S., Hosoya, K. and Okumura, M.
2017. Pentosan polysulfate inhibits IL-1 β -induced iNOS, c-Jun and HIF-1 α upregulation in canine articular chondrocytes. *PloS One* **12**: 1-17.
- II **Bwalya, E. C.**, Kim, S., Fang, J., Wijekoon, H. M. S., Hosoya, K. and Okumura, M.
2017. Effects of pentosan polysulfate and polysulfated glycosaminoglycan on chondrogenesis of canine bone marrow-derived mesenchymal stem cells in alginate and micromass culture. *J Vet Med Sci* In publication.
- III **Bwalya, E. C.**, Wijekoon, H. M. S., Fang, J., Kim, S., Hosoya, K. and Okumura, M.
2017. Independent chondrogenic potential of canine bone marrow-derived mesenchymal stem cells in monolayer expansion cultures decreases in a passage-dependent pattern. *BMC Vet Res* Under review.
- IV **Bwalya, E. C.**, Wijekoon, H. M. S., Fang, J., Kim, S., Hosoya, K. and Okumura, M.
2017. Pentosan polysulfate sodium restores the phenotype of dedifferentiated monolayer canine articular chondrocytes cultured in alginate beads. *Stem cell Res Ther* Under review

List of abbreviations

3D	three-dimensional;
ACT	autologous chondrocyte transplantation
ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
ANOVA	analysis of variance
AP-1	activation protein-1
BMP	bone morphogenetic protein
BSA	bovine serum albumin
CACs	canine articular chondrocytes
cBMSCs	canine bone marrow-derived mesenchymal stem cells
CCLR	cranial cruciate ligament rupture
CDM	chondrogenic differentiation medium
CFU-F	colony forming unit-fibroblasts
COX-2	cyclooxygenase-2
DAPI	4',6-diamidino-2-phenylindole
DJD	degenerative joint disease
DMEM	dulbecco's modified eagle medium
DMOADs	disease modifying osteoarthritic drugs
ECM	extracellular matrix
ERK	extracellular regulated kinase
FBS	fetal bovine serum
FCS	fetal calf serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
GAGs	glycosaminoglycans
GlcN	glucosamine
HACs	human articular chondrocytes
HIF-α	hypoxia inducible factor-alpha

ICC	immunocytochemistry
ICE	interleukin 1-beta-converting enzyme
Ihh	indian hedgehog
IL	interleukin
IL-1Ra	interleukin-1 receptor antagonist
iNOS	inducible nitric oxide synthase
ITS	insulin-transferrin-sodium selenite medium supplement
JNK	c-Jun N-terminus Kinase
LCPD	legg-calvè-perthes disease
LIF	leukaemia inhibitory factor
LPS	lipopolysaccharide
LSD	fisher's least significant difference
MAPK	mitogen-activated protein kinases
MMC	micromass cultures
MMPs	matrix metalloproteinases
MPCs	mesenchymal precursor cells
MSCs	mesenchymal stem cells
NCBI	national center for biotechnology information
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
OA	osteoarthritis
PBS	phosphate buffered saline
PBST	phosphate buffered saline-tween buffer
PDGF	platelet-derived growth factor
PG	proteoglycan
PGE₂	prostaglandin E2
PHDs	prolyl hydroxylases
PPS	pentosan polysulfate
PSGAG	polysulfated glycosaminoglycan
PTHrP	parathyroid hormone related protein

PTK	protein tyrosine kinases
pVHL	von Hippel-Lindau-tumour suppressor protein
qPCR	quantitative real-time polymerase chain reaction
rhIL-1	recombinant human interleukin-1
RT	room temperature
RT-PCR	reverse transcriptase-polymerase chain reaction
Runx2	runt-related transcription factor-2
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel
SF	synovial fluid
Sox-9	SRY-box 9
TBS-T	tris-buffered saline-Tween buffer
TGF-β	transforming growth factor-beta
TNF-α	tumor necrosis factor-alpha
TRITC-PPS	tetramethyl-rhodamine isothiocyanate labeled-PPS

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1 General Introduction

Osteoarthritis (OA) or degenerative joint disease (DJD) is a degenerative joint disease that progressively causes loss of joint function (Krasnokutsky *et al.*, 2008) and is the most common and costly form of arthritis (Hellio Le Graverand-Gastineau, 2009; Athanasiou *et al.*, 2013). In the United States (US) alone, the estimated clinical OA among the human adults was previously reported to be 27 million with the prevalence projected to increase with the aging population (Athanasiou *et al.*, 2013; Lawrence *et al.*, 2008). With regard to OA in companion animals, approximately 20% of the dogs over 1 year of age have significant arthritis with OA as the commonly encountered condition (Fossum *et al.*, 2007; Vaughan-Scott and Taylor, 1997). While cases of OA in dogs are much more commonly encountered, older cats have been reported to have a high incidence of radiographic changes suggesting joint disease with the use of more in-depth owner-perceived signs or behavioral changes proposed to show a high prevalence of OA than previously realized especially that abnormalities found by physical examination may not be detected in cats (Klinck *et al.*, 2012; Perry, 2016; Slingerland *et al.*, 2011). In Great Britain (GB), the prevalence of OA in horses was previously reported to be 13.9% and was the most prevalent among long-term/recurrent conditions (Ireland *et al.*, 2013).

Osteoarthritis (OA) can be a primary disease of joint cartilage, but is more often secondary to abnormal stresses on joints. This disease does not only affect articular cartilage but also involves the entire joint including the subchondral bone, ligaments, capsule, synovial membrane and menisci although a common feature in all OA and related disorders is loss of articular cartilage in association with bone features such as osteophyte and subchondral bone

sclerosis (Dequeker and Luyten, 2008; Hellio Le Graverand-Gastineau, 2009; MacPhail, 2000). While the mechanisms responsible for OA progression remain complex and poorly understood, cartilage tissue degeneration leading to OA occurs when the equilibrium between breakdown and repair of the joint tissues becomes unbalanced (Alcaraz *et al.*, 2010). This imbalance is predominantly driven by cytokine cascades and the production of inflammatory mediators. A number of cytokines including interleukin-1 (IL-1 α , IL-1 β), IL-4, IL-6, IL-8, IL-11, IL-17, IL-18, leukaemia inhibitory factor (LIF) and tumor necrosis factor-alpha (TNF- α) and recently IL-36 α have been identified and implicated in the pathogenesis of OA (Conde *et al.*, 2015; Fernandes *et al.*, 2002; Goldring and Goldring, 2004). However, there is substantial evidence that IL-1 and TNF- α are the crucial inflammatory cytokines that play pivotal roles in the pathogenesis of OA. Accordingly, IL-1 is the pivotal cytokine at early and late stages, while TNF- α is involved primarily in the onset of OA (Fernandes *et al.*, 2002; Goldring, 1999; Hosseinzadeh *et al.*, 2016; Wang *et al.*, 2006). Notably, OA chondrocytes in affected joints produce increased levels of IL-1 β and TNF- α , which in turn decrease anabolic collagen synthesis and increase catabolic including matrix metalloproteinases (MMPs) and other inflammatory mediators such as IL-8, IL-6, prostaglandin E₂ (PGE₂) and nitric oxide (NO) (Krasnokutsky *et al.*, 2008; Murrell *et al.*, 1995; Zhong *et al.*, 2013). IL-1 β , TNF- α and IL-6 have been detected in synovial fluid (SF) of canine stifle joints with OA secondary to cranial cruciate ligament rupture (CCLR) and with rheumatoid arthritis (Carter *et al.*, 1999; Hay *et al.*, 1997). Of particular interest, OA chondrocytes express inducible nitric oxide synthase (iNOS) which in turn produces high concentrations of NO, especially upon stimulation by proinflammatory cytokines IL-1 and TNF- α (Järvinen *et al.*, 1995; Murrell *et al.*, 1995; Stichtenoth and Frölich, 1998). The synthesis of NO by iNOS is considered to play an

important catabolic role in OA cartilage degradation and disease perpetuation. The stimulation of chondrocytes by NO is partly responsible for the upregulation of interleukin 1-beta-converting enzyme (ICE) and IL-18 synthesis while decreasing the level of the ICE inhibitor PI-9 (Boileau *et al.*, 2002). Evidence also exists which shows that NO plays a regulatory role in the activation of metalloproteinases in articular chondrocytes (Mendes *et al.*, 2002a; Mendes *et al.*, 2002b; Murrell *et al.*, 1995) and a relative deficit in the production of natural antagonists of the IL-1 receptor (IL-1Ra) has been reported in OA synovium and this has been associated to an excess production of NO. The excess production of NO coupled with an upregulation in the IL-1 receptor level has been shown to be an additional enhancer of the catabolic effects of IL-1 β in OA (Boileau *et al.*, 2002; Fernandes *et al.*, 2002). Therefore, the selective inhibition of pathologically enhanced NO synthesis by iNOS has been identified as a novel therapeutic target for the prevention and treatment of inflammatory joint diseases (Balaganur *et al.*, 2014; Pelletier *et al.*, 2000, 1999; Stichtenoth and Frölich, 1998; Tung *et al.*, 2002).

The primary cause of articular cartilage insult leading to the development of OA may sometimes be difficult to identify but it is associated with multiple risk factors, most notably age, joint trauma, altered biomechanics, and obesity (Sokolove and Lepus, 2013; Vaughan-Scott and Taylor, 1997), and after a period of degeneration, the process becomes self-perpetuating (Sokolove and Lepus, 2013). In some cases, the initial process may be a mild injury to a collateral ligament and these tend to recover fully, but the associated cartilage damage results in clinical disease of the joint years later. This highlights the fact that, although direct injury to cartilage may be the cause of damage, in most cases, the joint degenerates due to continuing insult from the inflammatory process. OA should therefore, not be thought of as a single disease, but rather as the clinical endpoint of numerous disorders leading to the eventual

failure of one or more joints of the body (Sokolove and Lepus, 2013; Vaughan-Scott and Taylor, 1997), a situation which is exacerbated by the limited ability of articular cartilage to self-renew and repair itself with currently no available effective treatment options to repair and restore OA damaged cartilage (Darling and Athanasiou, 2005; Foldager, 2013).

Treatment options for OA and cartilage damage depend on both the level of progression and the nature of the injury (Athanasiou *et al.*, 2013). The current non-surgical treatment options for OA mostly focus on alleviating discomfort, preventing the occurrence of further degenerative changes, pain relief and improvement of joint function with the use of analgesics such as non-steroidal anti-inflammatory drugs (NSAIDs) as the mainstream pharmacological intervention. Other available non-surgical treatment options include use of intraarticular injections of steroids and hyaluronans (Qvist *et al.*, 2008). However, these interventions do not alter the underlying pathophysiological process on the structural degradation of joint tissue but merely control signs of pain and inflammation (Krasnokutsky *et al.*, 2008; Qvist *et al.*, 2008; Vaughan-Scott and Taylor, 1997). Over the past decades, the increased understanding and knowledge on the molecular mechanisms driving the progression of the disease has led to the use of alternative treatments designated as disease modifying osteoarthritic drugs (DMOADs). These agents are anticipated to target the pathophysiological processes of OA thereby preventing, retarding progression of, or reversing morphologic changes associated with OA cartilage joints (Clouet *et al.*, 2009; Loeser, 2005). Among the prospective DMOADs include polysulfated glycosaminoglycan (PSGAG), glucosamine (GlcN), chondroitin, pentosan polysulfate (PPS), diacerein, doxycycline and hyaluronan etc. Some DMOADs such as PSGAG and PPS are already available in certain parts of the world for management of canine and equine OA/lameness whereas GlcN and chondroitin are available as over-the-counter

dietary supplements for human OA and off-label use in companion animals. Despite their use, there is no concrete clinical evidence to support their use as disease modifying agents in both humans and animals as no single prospective DMOAD when administered alone has demonstrated clinical benefit and capable of restoring the original structure and function of damaged cartilage and other synovial tissues. Furthermore, the mechanism of action of most prospective DMOADs and effects on novel therapeutic targets for OA remains to be fully elucidated. Surgical treatment involving partial or total joint replacement with synthetic prostheses or arthroplasty is considered as the last treatment option of end-point OA (Athanasίου *et al.*, 2013). Among other available surgical treatment options, autologous chondrocyte transplantation (ACT) has long been widely regarded as a promising treatment option for the repair of isolated OA cartilage lesions (Darling and Athanasίου, 2005; Athanasίου *et al.*, 2013). ACT procedure requires harvesting a small biopsy of articular cartilage from non-loading areas of the joint which is then enzymatically digested to release the chondrocytes. The released chondrocytes are then expanded in monolayer cultures *in vitro* prior to implantation either as scaffold or scaffold-less implants. Unfortunately, when cultured *in vitro*, articular chondrocytes lose their phenotype and dedifferentiate to fibroblastic-like cells synthesizing fibrocartilage proteins instead of hyaline cartilage proteins (Darling and Athanasίου, 2005; Freshney, 2010; Hamada *et al.*, 2013; Singh *et al.*, 2011). Fibrocartilage does not possess the biochemical composition or structural organization to provide suitable mechanical function within the load demanding joint environment and therefore degrades with time due to insufficient load-bearing capacity (Buckwalter and Mankin, 1998; Darling and Athanasίου, 2005; Athanasίου *et al.*, 2013).

Previously, a number of attractive catabolic mediators of OA have been identified as

novel potential targets to inhibit cartilage tissue degeneration and disease progression. Furthermore, efforts to successfully regenerate cartilage tissue and repair of small OA defects have been proposed to focus on defining novel *in vitro* culture conditions capable of restoring the phenotype of dedifferentiated monolayer expanded chondrocytes from fibrocartilage to hyaline cartilage synthesizing cells prior to implantation. On the other hand, PPS has been shown to be involved in prevention of inflammatory intracellular responses induced by IL-1 β via inhibiting nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B p65) and inhibition of phosphorylation of, p38 and extracellular regulated kinase (ERK) mitogen-activated protein kinases (MAPKs) (Sunaga *et al.* 2012) and preservation of cartilage integrity by supporting chondrocyte anabolic activities, including biosynthesis of aggrecan and suppression of catabolic MMPs (Collier and Ghosh, 1989; Costeseque *et al.* 1986; Ghosh 1999; Munteanu *et al.*, 2000; Rogachefsky *et al.*, 1993). However, its effects on novel potential targets for OA treatment and its chondroinductive potential in articular chondrocytes for cartilage tissue engineering purpose are yet to be verified. Therefore, the purpose of the present study was to investigate the effects of PPS on some novel therapeutic targets for OA treatment as well as evaluate its chondroinductive potential to restore the phenotype of dedifferentiated monolayer articular chondrocytes and promote chondrogenesis of canine bone marrow-derived mesenchymal stem cells (cBMSCs) for the purpose of cartilage tissue engineering and repair of isolated OA cartilage lesions.

2 Study 1 PPS inhibits IL-1 β -induced iNOS, c-Jun and HIF-1 α upregulation in canine articular chondrocytes

2.1 Summary

Osteoarthritic (OA) chondrocytes are shown to express iNOS which produces high concentrations of NO, particularly when stimulated with proinflammatory cytokines. NO is involved in OA cartilage degradation. On the other hand, c-Jun N-terminal Kinase (JNK) pathway mediates the activation and transcription of c-Jun, which is required for IL-1-induced of MMP-13 in OA pathogenesis. Therefore, the selective inhibition of pathologically enhanced NO synthesis by iNOS and c-Jun in OA joints are a novel therapeutic target for the treatment and prevention of OA. The purpose of the study was to investigate the inhibitory effects of pentosan polysulfate (PPS) on IL-1 β -induced iNOS, c-Jun and hypoxia inducible factor-alpha (HIF- α) isoforms upregulation in canine articular chondrocytes (CACs). Primary (P0) chondrocytes were isolated and cultured from femoral head cartilages of three dogs. First passage (P1) chondrocytes were preincubated with 0, 1, 5, 15 and 40 μ g/mL of PPS for 4 hr before treatment with 10 ng/mL recombinant human IL-1 β (rhIL-1 β) for a further 8 hr. In addition, the effects of single and multiple cytokine with or without LPS on iNOS protein induction were evaluated. PPS significantly inhibited ($P < 0.05$) rhIL-1 β -induced iNOS, c-Jun and HIF-1 α mRNA upregulation in a dose-dependent pattern. iNOS mRNA was significantly inhibited at 15 and 40 μ g/mL whereas c-Jun and HIF-1 α were significantly downregulated at 5, 15 and 40 μ g/mL of PPS compared to chondrocytes treated with only rhIL-1 β . Intriguingly, CACs were recalcitrant to single rhIL-1 β , recombinant canine IL-1 β (rcIL-1 β), recombinant

canine TNF- α (rcTNF- α) or lipopolysaccharide (LPS)-induction of iNOS protein including to a combination of rcIL-1 β + rcTNF- α , rcIL-1 β + LPS except for rcTNF- α + LPS and rcIL-1 β + rcTNF- α + LPS suggestive of a protective mechanism from iNOS detrimental effects on perpetuating OA. rcIL-1 β + rcTNF- α + LPS-induced iNOS protein expression was significantly abrogated by PPS. The findings of this study demonstrated for the first time that PPS is a novel inhibitor of IL-1 β -induced iNOS, c-Jun, and HIF-1 α mRNA upregulation and iNOS protein induction which may be beneficial for prevention and treatment OA.

2.2 Introduction

In dogs, OA is clinically characterized by lameness, which may have a gradual onset but can flare up acutely after exercise (Vaughan-Scott and Taylor, 1997). Affected dogs have limited mobility in one or multiple joints and chronic intermittent lameness that is exacerbated by exercise, extended rest or cold weather. Palpation of affected joints may reveal pain, swelling, poor range of motion, capsular thickening and crepitus. The degree to which dogs with OA are affected ranges from an occasional mild lameness to complete disability (MacPhail, 2000). Damage to the articular cartilage and stiffening of the subchondral bone may be initiating events or consequences of OA. Regardless, it is now apparent that in both humans and animals, a combination of biochemical and biomechanical events are involved in the pathophysiology of OA which centers on an imbalance of cytokine-driven cartilage degradation and synthesis resulting in progressive degradation of the articular cartilage extracellular matrix (ECM), despite the increased biosynthetic activity of the chondrocytes (Abramson, 2008; Fernandes *et al.*, 2002; Piscoya *et al.*, 2005; Sandell and Aigner, 2001; MacPhail, 2000). Cytokines, particularly IL-1 β and TNF- α , degradative enzymes MMPs and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) and other catabolic mediators like excess NO released from chondrocytes and synoviocytes mediate these events.

Particularly, OA chondrocytes express iNOS and produce high concentrations of NO, especially upon stimulation by proinflammatory cytokines (IL-1 β , TNF- α , IL-18, IL-36,) (Conde *et al.*, 2015; Järvinen *et al.*, 1995; Murrell *et al.*, 1995; Olee *et al.*, 1999; Stichtenoth and Frölich, 1998; Zhong *et al.*, 2013). This pathologically increased NO production plays an important catabolic role in OA cartilage degradation. The synthesis of excess NO by iNOS

plays an important regulatory catabolic factor in OA cartilage degradation with the stimulation of chondrocytes by NO being partly responsible for the upregulation of ICE and IL-18 synthesis while decreasing the level of the ICE inhibitor PI-9 (Boileau *et al.*, 2002). The upregulated IL-18 has been shown to regulate chondrocyte responses and contributes to cartilage degradation by stimulating the expression of several genes including iNOS, inducible cyclooxygenase-2 (COX-2), IL-6, and MMP-3 as well as enhance NO production (Olee *et al.*, 1999). There is also evidence indicating that NO plays a regulatory role in the activation of MMPs (collagenase and stromelysin) in bovine and human articular chondrocytes (HACs) (Mendes *et al.*, 2002a; Mendes *et al.*, 2002b; Murrell *et al.*, 1995). Moreover, mere excessive mechanical stress on articular cartilage has been reported to decrease synthesis of matrix components in chondrocytes through a NO-regulated pathway (Iimoto *et al.*, 2005) and promote *in vivo* cartilage damage (Ko *et al.*, 2013). Furthermore, a relative deficit in the production of natural antagonists of the IL-1 receptor (IL-1Ra) has been reported in OA synovium and this has been associated to an excess production of NO. The excess production of NO combined with an upregulated IL-1 receptor level has been shown to be an additional enhancer of the catabolic effects of IL-1 β in OA (Boileau *et al.*, 2002; Fernandes *et al.*, 2002). Furthermore, OA cartilage has been shown to produce a larger amount of NO than the normal cartilage, both in spontaneous and IL-1 β and TNF- α -stimulated conditions. In addition, a high level of nitrites/nitrates has been found in the SF and serum of arthritis human patients (Farrell *et al.*, 1992). Additionally, *in vivo*, OA chondrocytes stimulated by inflammatory cytokines IL-1 β and TNF- α in turn induces the expression of iNOS, and the subsequent synthesis of excess NO. Importantly, the elevation of NO production appears to be an important factor in the pathophysiology of OA as it is able to reduce IL-1Ra synthesis by chondrocytes and increase

the level of IL-1 β , associated with a decreased IL-1Ra level (Pelletier *et al.*, 1996). Therefore, the selective inhibition of pathologically enhanced NO synthesis by iNOS in OA joints has been identified as an attractive novel therapeutic target for the treatment and prevention of OA (Balaganur *et al.*, 2014; Mendes *et al.*, 2002a; Stichtenoth and Frölich, 1998). Inhibition of iNOS with selective or competitive inhibitors has been demonstrated to profoundly reduce NO production, synovial inflammation and tissue damage in various *in vivo* and *in vitro* models of OA (McCartney-Francis *et al.*, 1993; Taskiran *et al.*, 1994; Pelletier *et al.*, 2000, 1999; Balaganur *et al.*, 2014). As part of the signaling pathway, HIF-2 α has also been proposed as a catabolic factor that directly targets MMP-13 and iNOS through specific binding to the respective hypoxia-responsive elements (Saito *et al.*, 2010; Saito and Kawaguchi, 2010; Yang *et al.*, 2010). However, the role of HIF- α isoforms (HIF-1 α and HIF-2 α) in OA pathogenesis is currently controversial and has led to species-dependent roles being proposed especially between murine and large mammals (Murphy, 2010). For example, HIF-2 α has been shown by others to be an anabolic factor responsible for hypoxic induction of cartilage matrix genes (Bohensky *et al.*, 2009; Lafont *et al.*, 2008, 2007; Murphy, 2010) and to be a potent regulator of autophagy in maturing mouse and HACs by acting as a brake to the autophagy accelerator function of HIF-1 α (Bohensky *et al.*, 2009).

Pentosan polysulfate (PPS), a semi-synthetic sulfated polysaccharide derived from wood of beech plant, *Fagus sylvatica* has been shown to preserve cartilage integrity by supporting chondrocyte anabolic activities, including biosynthesis of aggrecan and suppression of catabolic MMPs (Collier and Ghosh, 1989; Costeseque *et al.*, 1986; Ghosh, 1999; Munteanu *et al.*, 2000; Rogachefsky *et al.*, 1993). PPS has been shown to alleviate mouse arthritogenic alphavirus-induced arthritis model resulting in reduced cartilage destruction with

corresponding significant reduction in immune infiltrates, significant increase in anti-inflammatory cytokine IL-10, and reduced proinflammatory cytokines (Herrero *et al.*, 2015). Additionally, it has been revealed as a multifaceted exosite inhibitor of the aggrecanases (ADAMTS-4 and -5) and protects cartilage against aggrecan degradation (Troeberg *et al.*, 2008). It has also been shown to be involved in prevention of IL-1 β -induced nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B p65), p38 and ERK pathways activation but with no demonstrable effect on c-Jun N-terminus Kinase (JNK) pathway phosphorylation and activation (Sunaga *et al.*, 2012). However, the JNK pathway has been shown to mediate the activation and transcription of c-Jun, which is required for IL-1-induction of MMP-13, an enzyme considered as a central mediator of type II collagen degradation in OA cartilage (Mengshol *et al.*, 2000). Therefore, the inhibition of c-Jun is also a potential therapeutic target for the prevention and treatment of OA joints. Currently, the mechanism of action of PPS remains to be fully elucidated, and its effects on iNOS, c-Jun and HIF- α isoforms in IL-1-stimulated articular chondrocytes have not been clarified. Therefore, the objective of the present study was to investigate the effects of PPS on IL-1-induced iNOS, c-Jun and HIF- α isoforms upregulation in CACs. It was hypothesized that PPS is a novel inhibitor of IL-1-induced iNOS and c-Jun upregulation in CACs.

2.3 Material and Methods

2.3.1 Chondrocytes culture

Canine articular cartilage samples were obtained with owners' formal consent from femoral head cartilages of three dogs; a 1-year-old and 10-months-old that underwent femoral head and neck ostectomy due to Legg-Calvè-Perthes Disease (LCPD) and a 9-year-old that underwent hind limb amputation due to osteosarcoma affecting the distal femoral bone at Veterinary Teaching Hospital, Hokkaido University. Briefly, chondrocytes were released from cartilage samples by dissection and digestion for 24 hr in 0.3% Collagenase Type I (Wako Pure Chemicals Industries Ltd, Osaka, Japan) based on a protocol previously described elsewhere (Freshney, 2010) with minor modifications. The cartilage was sliced into small pieces in a 100 mm polystyrene culture plate (Corning, Lowell, MA, USA) containing 10 mL of 0.05% trypsin solution and incubated with moderate magnetic agitation for 25 minutes at room temperature (RT). After rinsing, cartilage slices were digested in 20 mL 0.3% collagenase (Wako Pure Chemical Industries Ltd) by incubating for 24 hr at 37°C in an incubator with 5% CO₂. Cell suspension was mixed homogeneously by repeated pipetting and sieved through a 70 µm nylon filter. The filtrate was centrifuged at 400 x g for 10 minutes and resulting cell pellet was resuspended in 20 mL Dulbecco's Modified Eagle Medium (DMEM; GIBCO BRL, Grand Island, NY, USA) supplemented with of 10% fetal bovine serum (FBS) (Nichirei Biosciences Inc, Tokyo, Japan, Batch #: 83300104), 100 IU/mL of penicillin and 0.1 mg/mL of streptomycin. Cells were counted manually and viability assessed by 0.5% Trypan blue exclusion test with haemocytometer. Chondrocytes were plated at 1×10^4 cells/cm² and cultured to ~85-90% confluence in 10% DMEM in 100 mm polystyrene culture plates at 37°C

in 5% CO₂. Medium was replenished every 72 hr and at confluence, P0 monolayer cells were detached using TryPle™ Select Enzyme (1X) (GIBCO BRL) according to manufacturer's recommended protocol. First passage (P1) chondrocytes were used in all experiments in this study.

2.3.2 Chondrocytes treatment, preparation of cell extracts and analysis

Chondrocytes (P1) (7.0×10^5 cells/well) were seeded in 12-well polystyrene culture plates (Corning) containing serum free DMEM and incubated at 37°C in 5% CO₂ for 24 hr. Medium was removed and cells were treated either with 0 (control, CTL), 1, 5, 10 and 20 ng/mL rhIL-1 β (Thermo Scientific, Life Technologies, Rockford, Illinois, USA) in serum free DMEM for 8 hr or preincubated first with 1, 5, 15 and 40 μ g/mL of PPS (Cartrophen Vet® injection, NaPPS - 100 mg/mL; Biopharm Australia, Bondi Junction, NSW, Australia) for 4 hr then treated with 10 ng/mL of rhIL-1 β for a further 8 hr (Table 1). The use of rhIL-1 β in CACs was based on a previous study that demonstrated that rhIL-1 β -induced catabolic mediators in CACs (Sunaga *et al.*, 2012).

Table 1 Treatment of first passage canine articular chondrocytes in monolayer cultures

Treatment number	Treatment group	PPS (µg/mL)	rhIL-1β (ng/mL)
1	CTL	0	0
2	-	0	1
3	-	0	5
4	PC	0	10
5	-	0	20
6	PPS + rhIL-1β	1	10
7	PPS + rhIL-1β	5	10
8	PPS + rhIL-1β	15	10
9	PPS + rhIL-1β	40	10

PPS; pentosan polysulfate, rhIL-1β: recombinant human interleukin-1β, CTL; control without treatment, PC; positive control

In addition, the temporal effect of rhIL-1 β on iNOS protein induction was evaluated by treating chondrocytes with 20 ng/mL of rhIL-1 β for 0, 3, 6, 9 and 24 hr. Furthermore, to investigate the effects of multiple cytokines with or without LPS (Wako Pure Chemicals Industries Ltd) on iNOS protein induction, chondrocytes were treated for 8 hr with single rcTNF- α (10 and 20 ng/mL) (Kingfisher Biotech, St Paul, Minnesota, USA) or LPS (50 and 75 μ g/mL) (Wako Pure Chemicals Industries Ltd), and a combination of rcTNF- α (20 ng/mL) + LPS (50 μ g/mL), LPS (25 μ g/mL) + rcIL-1 β (20 ng/mL) (rcIL-1 β , Kingfisher Biotech), rcTNF- α (20 ng/mL) + rcIL-1 β (20 ng/mL), and rcIL-1 β (20 ng/mL) + rcTNF- α (20 ng/mL) + LPS (50 μ g/mL). In this experiment, rcIL-1 β was used to verify and validate the results obtained initially with rhIL-1 β .

Total RNA and protein were extracted using TRIZol® reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacture's protocol. Total RNA was quantified by spectrophotometry at 260 nm using Biowave DNA - WPA, 7123 V1.8.0 (Biochrom, Cambridge, UK) while protein was quantified by Bradford protein assay using the Thermo Scientific NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific, NanoDrop products, Wilmington, Delaware, USA) and stored at -20°C until use.

Total of 500 ng RNA was reverse transcribed (RT) into cDNA using ReverTra Ace® qPCR RT Master Mix (Toyobo Co, Osaka, Japan) and amplified by PCR using TaKaRa Ex taq (TaKaRa Bio, Tokyo, Japan) according to manufacturer's recommended protocol. This technique was employed to amplify mRNAs specific for iNOS, c-Jun, HIF-1 α and HIF-2 α . Amplified products were analysed by electrophoresis on 2% agarose gel and visualized by ethidium bromide staining. PCR products were identified and verified based on the expected product size relative to the standard ladder. The PCR conditions were an initial denaturation of

94°C for 1 min followed by 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s and then a finishing step of 72°C for 1 min. Quantitative real-time PCR (qPCR) was performed with KAPA SYBR® FAST qPCR kit (KAPA biosystems, Woburn, MA, USA) to determine the relative mRNA expression by the two step method. The qPCR conditions were an initial denaturation of 95°C for 20 s followed by 40 cycles of 95°C for 3 s and 60°C for 20 s then a pre-melt condition of 60°C for 90 s followed by a final melt step. The standard curve method was used to determine the relative mRNA quantification. All PCR reactions were validated by the presence of a single peak in the melt curve analysis and single band on gel electrophoresis. All mRNA expressions were normalised against the reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the CTL group was used as the calibrator to determine the mRNA-fold changes. The sequences, product size and accession codes for each of primers used in the experiments are indicated in Table 2. Primer sequences for all genes were designed using data published on the National Center for Biotechnology Information (NCBI) website using NCBI's standard and pairwise BLAST programs. Due to non-specificity of primers initially used for iNOS, two primers targeting the upstream and downstream segments of iNOS mRNA were used validate the results. For Western blot analyses, the identity of the proteins was verified by the expected protein band size relative to the protein marker or against a standard purified protein loaded as a control. Because the antibodies used in the experiments were nonspecific to canine species although validated to react in dogs, to confirm immunogenic identity, homologous sequence comparison was performed using homology BLAST on NCBI website. Antibodies with immunogenic identity of $\geq 80\%$ with canine target proteins were considered to have significant cross reactivity. All antibodies used in all the experiments showed immunogenic identity of $\geq 80\%$.

Table 2 Sequence of primers used in the experiments

Name of gene	Domain	^a Primer	^b Fragment	Accession
GAPDH	664-683	5' -CTGAACGGGAAGCTCACTGG-3'	129 bp	NM_001003142.1
	773-792	5' -CGATGCCTGCTTCACTACCT-3'		
iNOS	13-32	5' -TGGCAGTTTCTGTTCAAGGC-3'	139 bp	XM_005624846.1
	132-151	5' -TGCTGAGGCTGTGACACTTG-3'		
iNOS	3681-3700	5' - AATGGAGAGTTGGGCCTTCC-3'	227 bp	NM_001313848.1
	3887-3907	5' -TGGCCCTTAAGAGAAGACTGG-3'		
HIF-1 α	861-880	5' -GTACTTCACTGCACAGGCCA-3'	102 bp	NM_001287163.1
	943-962	5' -ACAAATCAGCACCAAGCACG-3'		
HIF-2 α	1248-1267	5' -TGCAAAGCACGGGGGCTACG-3'	72 bp	XM_531807.3
	1300-1319	5' -GGCTGCAGGTTGCGAGGGTT-3'		
c-Jun	1144-1163	5' -TCTACGACGATGCCCTCAAC-3'	159 bp	XM_005620245.1
	1283-1302	5' -TGAGCAGGTCCGAGTTCTTG-3'		

^aPrimers for forward & reverse sense are presented in a 5' to 3' orientation, ^bThe expected fragment size, GAPDH; glyceraldehyde-3-phosphate dehydrogenase, iNOS; inducible nitric oxide synthase, HIF; Hypoxia inducible factor

2.3.3 Western blot analysis: iNOS, HIF-1 α and HIF-2 α protein detection

The following primary antibodies were used; Rabbit polyclonal iNOS antibody (Thermo Fisher, Rockford, USA; Cat. #: PA1-036) (1: 100 dilution), mouse monoclonal Anti-HIF-1 α Clone H1 α 67 (Sigma-Aldrich, St. Louis, Missouri, USA; H6536) (1.0 μ g/mL or 1: 1000 dilution) and EPAS-1 (HIF-2 α) (C-16) goat polyclonal antibody (Santa Cruz Biotechnology, Dallas, USA; Cat. #: SC-8712) (1: 200 dilution). Normally, HIF- α isoforms protein are not readily detectable in HACs cultured under normoxic conditions as they are rapidly degraded in the presence of sufficient oxygen (Coimbra *et al.*, 2004; Jaakkola *et al.*, 2001; Lafont *et al.*, 2007; Thoms *et al.*, 2013; Thoms and Murphy, 2010). To verify that this is consistent with monolayer CACs cultured under normoxic conditions, the expression of HIF-1 α and HIF-2 α proteins was evaluated. F-Actin rabbit polyclonal antibody (Bioss Antibodies, Massachusetts, USA; Cat. #: 1571R) (1: 1,000 dilution) was used as an internal control. Secondary antibodies were; Pierce[®] Goat anti-Rabbit Poly-HRP (Pierce Biotechnology, Rockford, Illinois, USA; Cat. #: 32260, Lot number – QG217308), Zymed[®] Rabbit anti-mouse IgG-HRP conjugate (Invitrogen Corporation, Zymed Laboratories, Inc California, USA, Cat. #: 81-6720) and Anti-IgG goat Rabbit-Poly-HRP (R & D Systems, Minneapolis, Minnesota, USA, Cat. #: HAF0017). Western Blot Ultra-Sensitive HRP substrate (Takara) was used for the signal generation. iNOS electrophoresis standard 130 kDa (Cayman Chemical, Michigan, USA, Cat. #: 360862) was used as a standard iNOS control. Briefly, 20 μ g of protein from each treatment was denatured and separated on 12% (w/v) sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) for 55 min at 180 V in Tris/Glycine/SDS buffer (25 mM Tris, 190 mM Glycine, 0.1% SDS, pH 8.3). Two SDS PAGEs were run simultaneously, one was silver stained using AE-1360 EzStain Silver (Atto Corporation, Tokyo, Japan) according to manufacturer's

procedure to evaluate the expression profile of separated proteins while the proteins on other gel were electroblotted onto nitrocellulose membranes (Whatman, Dassel, Germany) at 60 V for 2 hr 30 min in transfer buffer containing 25 mM Tris, 190 mM Glycine, 20% methanol and 0.1% SDS (pH 8.3). Nonspecific antibody binding was blocked with 5% (w/v) skim milk prepared in Tris-buffered saline-Tween buffer (pH 7.6, TBS-T, 20 mM Tris-HCl, 150 mM NaCl, 0.1% (w/v) Tween-20) for 1 hr at RT on a shaker. Membranes were then incubated in primary antibody at RT on a shaker for 1 hr then incubated overnight at 4°C in a refrigerator. Membranes were washed three times with adequate TBST buffer at 5 min interval then incubated in HRP-conjugated secondary antibody at a dilution of 1:5,000 for 1 hr on a shaker at RT. Membranes were washed as before then incubated in HRP substrate for 5 min at RT. The protein-antibody reaction was visualized for chemiluminescent signal using FUJIFILM Luminescent Image Analyzer LAS-3000 (Fujifilm Life Science, LTD, Tokyo, Japan) according to the instrument manual for the imaging system. Protein bands were analysed using Multi-Gauge V 3.0 software (Fujifilm Life Science).

2.3.4 Immunocytochemistry: Colocalization of PPS with c-Jun and NF-κB p65

Immunocytochemistry (ICC) was performed to investigate the colocalization of PPS with NF-κB p65 and c-Jun, and to clarify whether PPS could inhibit NF-κB p65 and c-Jun nuclear translocation. Chondrocytes (1×10^4 cells) were plated in 8-well Permanox® slides (Thermo scientific nunc, New York, USA) in 500 μL of 10% FBS supplemented DMEM and incubated at 37°C in 5% CO₂ for 24 hr. Cells were gently washed with 1 x phosphate buffered saline (PBS, pH 7.4) then preincubated in serum free DMEM with or without 40 μg/mL of tetramethyl-rhodamine isothiocyanate labeled-PPS (TRITC-PPS) for 4 hr. Thereafter, 10

ng/mL of rcIL-1 β was added to the medium with TRITC-PPS and cells incubated overnight at 37°C in 5% CO₂. Cells were gently washed three times with cold 1 x PBS (2-8°C), fixed in cold methanol (-20°C) for 5 min at RT then washed on a shaker three times at 5min interval with cold 1 x PBS containing 0.1% tween 20 (PBST). Non-specific antibody binding was blocked by incubation for 1 hr in 5% bovine serum albumin (BSA) (Sigma-Aldrich, Cat. #: A3095) prepared in PBST. Primary antibodies used were anti-human c-Jun (H-79) Rabbit polyclonal antibody (Santa Cruz Biotechnology, SC-1694) (1:100 dilution) and anti-human NF- κ B p65 (C-20) Rabbit polyclonal antibody (Santa Cruz Biotechnology, SC-372) (1:100 dilution) by incubation at 4°C overnight. Cells were washed as before then incubated for 1 hr with goat anti-rabbit IgG-fluorescein isothiocyanate (FITC)-conjugated (Santa Cruz Biotechnology, SC-2012) (1:1,000 dilution). Cells were washed as before and excess wash buffer removed. Prolong® Diamond Antifade Mountant with 4',6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific, Eugene, OR, USA) was used to stain the nucleus and mount the slides according to manufacturer's instructions. Cells stained with only the secondary antibody were used as negative controls. Cells were viewed using Zeiss LSM 700 confocal laser microscope (Zeiss, Urbana, USA).

2.4 Data analysis

Quantitative qPCR data was analysed using SPSS version 16.0. Analysis of variance (ANOVA) was used to compare the mean relative gene expression between the treatments. Where significant difference was observed, multiple comparisons of group means was performed using *Post Hoc* Bonferroni. Linear regression analysis was performed to model the relationship between iNOS and HIF- α isoforms mRNA expression in response to IL-1 and

PPS. Significance level was defined as $P < 0.05$. All quantitative results in this study unless specified are presented as mean \pm standard deviation (SD).

2.5 Results

2.5.1 PPS inhibits IL-1 β -induced iNOS, c-Jun and HIF-1 α mRNA upregulation in CACs

Due to nonspecific PCR products or dimer formation of iNOS primers designed earlier in the study, two sets of primers targeting two different regions of the 4.0 kbp iNOS mRNA were used to optimize the accuracy of the results. Treatment of chondrocytes with rhIL-1 β resulted in upregulation of iNOS, c-Jun, HIF-1 α and HIF-2 α mRNA expression. However, the preincubation of chondrocytes with PPS inhibited rhIL-1 β -induced iNOS, c-Jun and HIF-1 α mRNA upregulation (Figure 1A and 1B). A dose-dependent inhibitory effect of PPS on rhIL-1 β -induced iNOS, c-Jun and HIF-1 α mRNA upregulation was observed (Figure 1B). Significant inhibition ($P < 0.05$) of iNOS mRNA was observed at PPS concentrations of 15 and 40 $\mu\text{g/mL}$ compared to the PC (only rhIL-1 β -induced cells), with the mRNA levels reaching levels similar to untreated CTL chondrocytes. The expression of c-Jun and HIF-1 α mRNA were significantly inhibited ($P < 0.05$) at almost all PPS concentrations compared to the PC. However, HIF-2 α mRNA remained relatively upregulated at 1, 5 and 15 $\mu\text{g/mL}$ of PPS and was only significantly inhibited at 40 $\mu\text{g/mL}$ compared to the PC (Figure 1B).

2.5.2 iNOS mRNA is readily detectable in CACs

The readily detectable iNOS mRNA expression in CACs unstimulated with IL-1 was very surprising since the expression of this inducible iNOS is normally associated with cytokine stimulation of chondrocytes in other species. Therefore, to verify whether, iNOS mRNA is readily detectable in normal CACs, chondrocytes isolated from two other dogs were cultured in standard DMEM with 10% FBS and in 1% FBS DMEM, then probed for iNOS

mRNA expression by RT-PCR gel electrophoresis. The results, confirmed the detection of iNOS mRNA in normal passaged monolayer cultured CACs untreated with any cytokines (Figure 2).

2.5.3 iNOS mRNA expression correlates with HIF-1 α , and HIF-2 α mRNA expression in IL-1-induced CACs

Linear regression analysis was performed to model the relationship between iNOS mRNA expression and HIF- α isoforms mRNA expression in IL-1-stimulated CACs. As shown in Figure 3 both HIF-1 α (Pearson's correlation coefficient = 0.80, $P < 0.001$) and HIF-2 α (Pearson's correlation coefficient = 0.61, $P < 0.001$) had a positive significant correlation with iNOS mRNA expression. In addition, a positive significant correlation was observed between HIF-1 α and HIF-2 α mRNA expression (Pearson's Correlation = 0.58, $P < 0.001$).

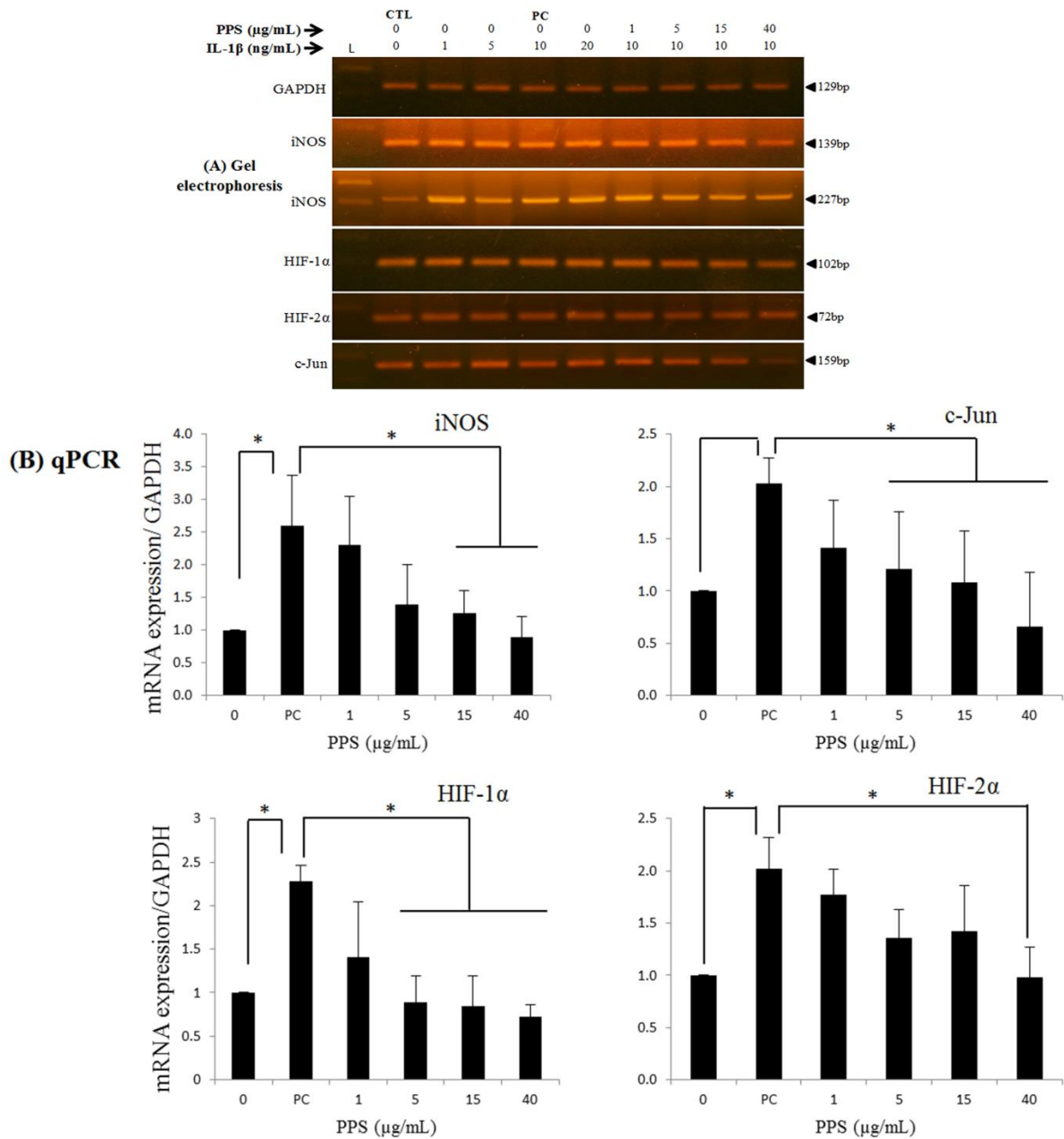


Figure 1 PPS inhibits rhIL-1β-induced iNOS, c-Jun and HIF-1α mRNA upregulation in CACs

(A) As demonstrated by RT-PCR gel electrophoresis and (B) qPCR results, preincubation of chondrocytes with PPS inhibits rhIL-1β-induced iNOS, c-Jun and HIF-1α mRNA upregulation compared to the positive control (PC – 10 ng/mL rhIL-1β without PPS). L; 100bp DNA ladder, CTL; Control (no treatment), gel electrophoresis results shown are from one representative experiment of three independent experiments, error bars represent the mean \pm SD mRNA. Significant difference was defined as $*P < 0.05$ (Bwalya *et al.*, 2017a).

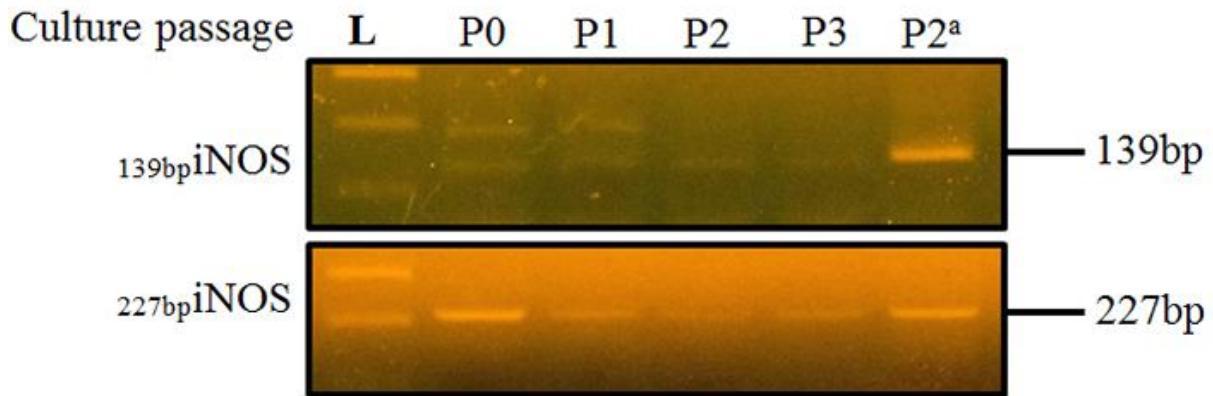


Figure 2 iNOS mRNA is detectable in monolayer passaged CACs

Primary (P0) canine articular chondrocytes (CACs) were cultured in 10 % DMEM and 1% DMEM (P2^a) and passaged up to third passage (P0 to P3). Cells were evaluated for iNOS mRNA expression by targeting the upstream 139bp and the downstream 227bp mRNA segments (Bwalya *et al.*, 2017a).

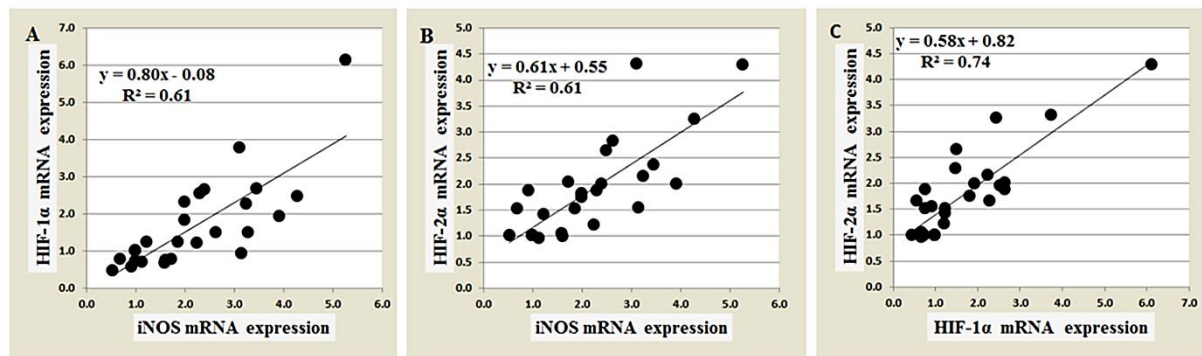


Figure 3 Regression line plot showing the correlation between HIF- α subunits and iNOS mRNA expression in CACs.

(A) HIF-1 α correlation with iNOS, (B) HIF-2 α correlation with iNOS, and (C) HIF-1 α correlation with HIF-2 α . 95% confidence interval (95% CI); 0.53 – 1.05 (HIF-1 α and iNOS), 0.41 – 0.81 (HIF-2 α and iNOS) and 0.43 – 0.72 (HIF-1 α and HIF-2 α). Significant correlation defined as * $P < 0.05$, ** $P < 0.01$. All the correlation analysis showed a strong significant correlation ($P < 0.001$) between the genes (Bwalya *et al.*, 2017a).

2.5.4 iNOS protein is recalcitrant to cytokine-induction in normal CACs; HIF- α proteins are undetectable under normoxia culture conditions

While iNOS mRNA levels were readily detectable and upregulated by 20 ng/mL of rhIL-1 β in cultured CACs, by Western blot, the active iNOS protein levels were undetectable in all the lysed cells protein extracts at all stimulation time points of 0, 3, 6, 9 and 24 hr (Figure 4A-4C).

Surprisingly, similar to observations made with single rhIL-1 β -stimulation, iNOS protein was still undetectable even in chondrocytes stimulated with single rcIL-1 β , rcTNF- α or LPS including to a combination of rcIL-1 β + rcTNF- α and rcIL-1 β + LPS (Figure 5). Interestingly, stimulation of chondrocytes with a combination of rcTNF- α + LPS and rcIL-1 β + rcTNF- α + LPS resulted in a weak and strong iNOS protein signal, respectively (Figure 5). In addition, while the HIF- α isoforms were readily detectable at the mRNA level, both isoforms were undetectable at the protein level (Figure 5). Treatment of chondrocytes with single or multiple cytokines with or without LPS did not result in stabilization and detection of the HIF- α isoforms at protein level, an indication of rapid post-translational proteosomal degradation of the proteins in the presence of sufficient oxygen (Figure 5).

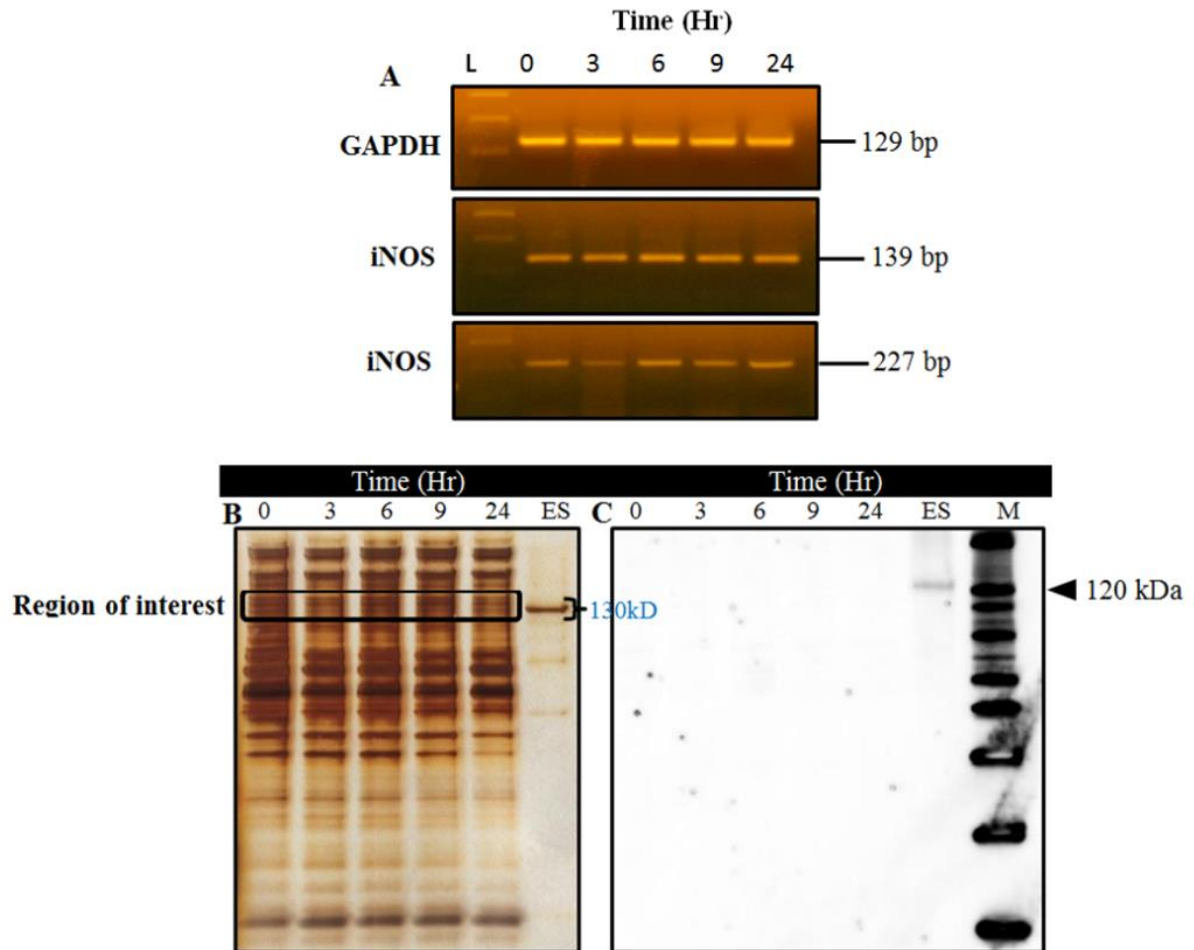


Figure 4 iNOS protein is recalcitrant to IL-1 β -induction in CACs.

(A) iNOS mRNA expression in chondrocytes (P1) stimulated with 20 ng/mL of rhIL-1 β for 0, 3, 6, 9 and 12 hr, (B) 20 μ g protein from each treatment time was denatured and separated on a 12% SDS page and silver stained to visualize the profile of separated proteins, (C) iNOS protein levels were undetectable by Western blot, an indication of resistance to cytokine induction. L; 100bp DNA ladder, ES; iNOS Electrophoresis Standard ~130 kDa (loaded at 100 ng). M; protein standards marker, results shown are from one representative experiment of three independent experiments (Bwalya *et al.*, 2017a).

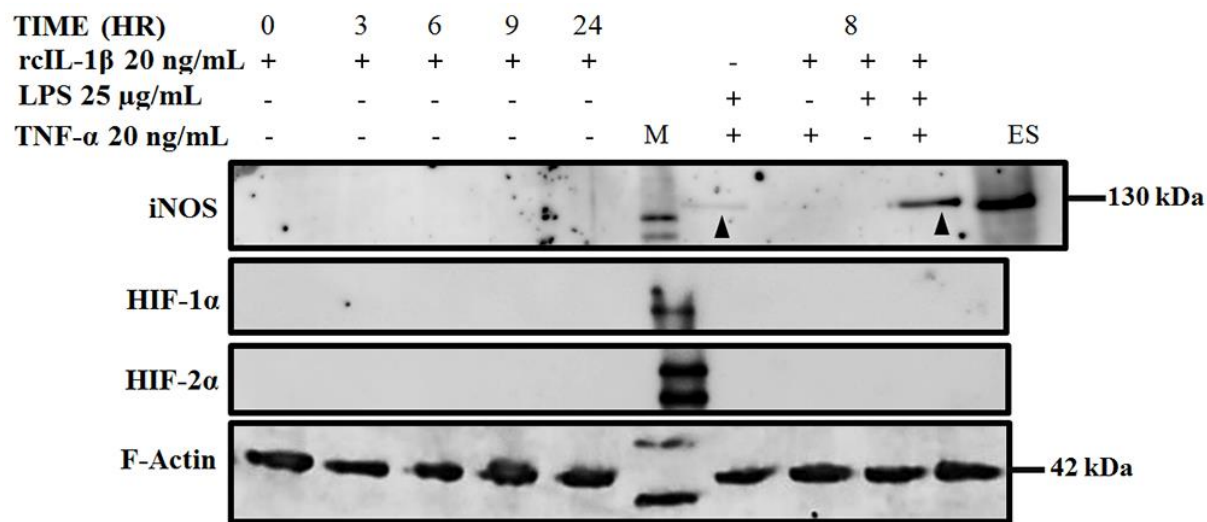


Figure 5 iNOS protein is recalcitrant to single or multiple cytokine induction in normal CACs.

iNOS protein levels were undetectable in chondrocytes stimulated with single rcIL-1 β , TNF- α or LPS. A combination of rcIL-1 β + TNF- α + LPS induced a strong iNOS protein expression while a combination of TNF- α + LPS induced a very weak iNOS protein band as indicated by black arrowheads. HIF-1 α and HIF-2 α proteins were undetectable. ES; iNOS Standard protein ~130 kDa (loaded at 500 ng), M; Protein Standards marker. F-Actin (Internal control): 42 kDa, iNOS: 130 kDa, HIF-1 α : 120 kDa and HIF-2 α : 115 kDa. Blots shown are from one representative experiment of three independent experiments (Bwalya *et al.*, 2017a).

2.5.5 PPS inhibits rcIL-1 β + rcTNF- α + LPS-induced iNOS protein expression in CACs

Because iNOS protein expression was only significantly induced in chondrocytes treated with a combination of rcIL-1 β + rcTNF- α + LPS, to confirm whether the observed inhibitory effects of PPS on iNOS mRNA were consistent with iNOS protein expression, 1.0×10^6 chondrocytes were incubated as previously described. Thereafter, chondrocytes were treated with serum free DMEM (CTL), 10 ng/mL of rcIL-1 β , a combination of rcIL-1 β (10 ng/mL) + rcTNF- α (10 ng/mL) + LPS (50 μ g/mL) or preincubated with 40 μ g/mL PPS for 4 hr then treated with a combination of rcIL-1 β (10 ng/mL) + rcTNF- α (10 ng/mL) + LPS (50 μ g/mL) for 8 hr. As consistently observed, a combination of rcIL-1 β + rcTNF- α + LPS significantly induced iNOS protein expression but preincubation of chondrocytes with PPS significantly abrogated iNOS protein induction hence proving the inhibitory effects of PPS on both iNOS mRNA and protein expression (Figure 6).

2.5.6 Immunocytochemistry: PPS colocalizes with NF- κ B and c-Jun in CACs

PPS (Ghosh, 1999; Ghosh *et al.*, 2010; Sadhukhan *et al.*, 2002; Sunaga *et al.*, 2012) and other active glycosaminoglycans (GaGs) (Campo *et al.*, 2009; Loeser *et al.*, 2009) have been proposed to exert their action through interaction with transcription factors and other intracellular proteins consequently blocking their downstream promoter activity. ICC was performed to investigate colocalization of PPS with NF- κ B p65 and c-Jun, and to clarify whether PPS could inhibit NF- κ B p65 and c-Jun nuclear translocation and localization. Chondrocytes treated with or without 10 ng/mL of rcIL-1 β predominantly showed nuclear localization of c-Jun (Figure 7A and 7B). However, when preincubated with 40 μ g/mL of TRITC-PPS then treated with 10 ng/mL of rcIL-1 β , chondrocytes demonstrated reduced c-Jun

nuclear translocation (Figure 7C) as indicated by increased perinuclear and cytoplasmic localization. The merged image (yellow/orange areas) in Figure 7C indicates that c-Jun colocalizes with PPS.

NF- κ B p65 predominantly accumulated in the cytoplasm of CTL chondrocytes without rcIL-1 β (Figure 8A). As expected, treatment of chondrocytes with 10 ng/mL of rcIL-1 β activated NF- κ B p65 as evidenced by increased nuclear translocation and localization (Figure 8B). However, preincubation of chondrocytes with 40 μ g/mL of TRITC-PPS decreased rcIL-1 β -induced NF- κ B p65 nuclear translocation in many chondrocytes as indicated by the accumulation and localization of the transcriptional factor in the perinuclear and cytoplasmic area (Figure 8C). The merged image demonstrates that NF- κ B p65 colocalizes with PPS (yellow/orange areas) (Figure 8C).

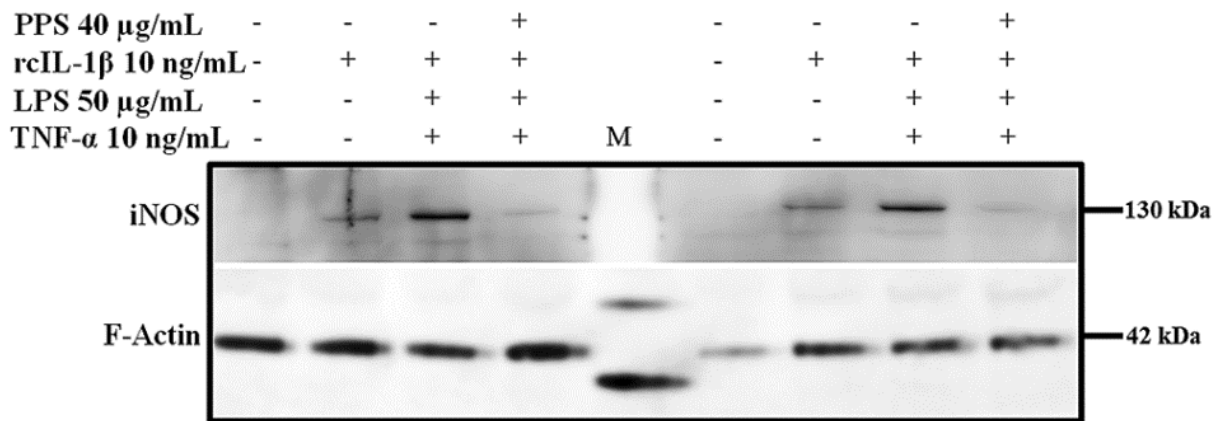


Figure 6 PPS inhibits rcIL-1 β + TNF- α + LPS-induced iNOS protein expression in CACs.

Single rcIL-1 β (10 ng/mL) induced a weak iNOS protein signal compared to a combination of rcIL-1 β (10 ng/mL) + rcTNF- α (10 ng/mL) + LPS (50 $\mu\text{g/mL}$) which significantly induced iNOS protein expression in canine chondrocytes. Pre-incubation of canine chondrocytes with 40 $\mu\text{g/mL}$ of PPS significantly inhibited rcIL-1 β + rcTNF- α + LPS-induced iNOS protein expression. M; Protein Standards marker. F-Actin (Internal control): 42 kDa, iNOS: 130 kDa. Blots shown are from two representative experiments of three experiments (Bwalya *et al.*, 2017a).

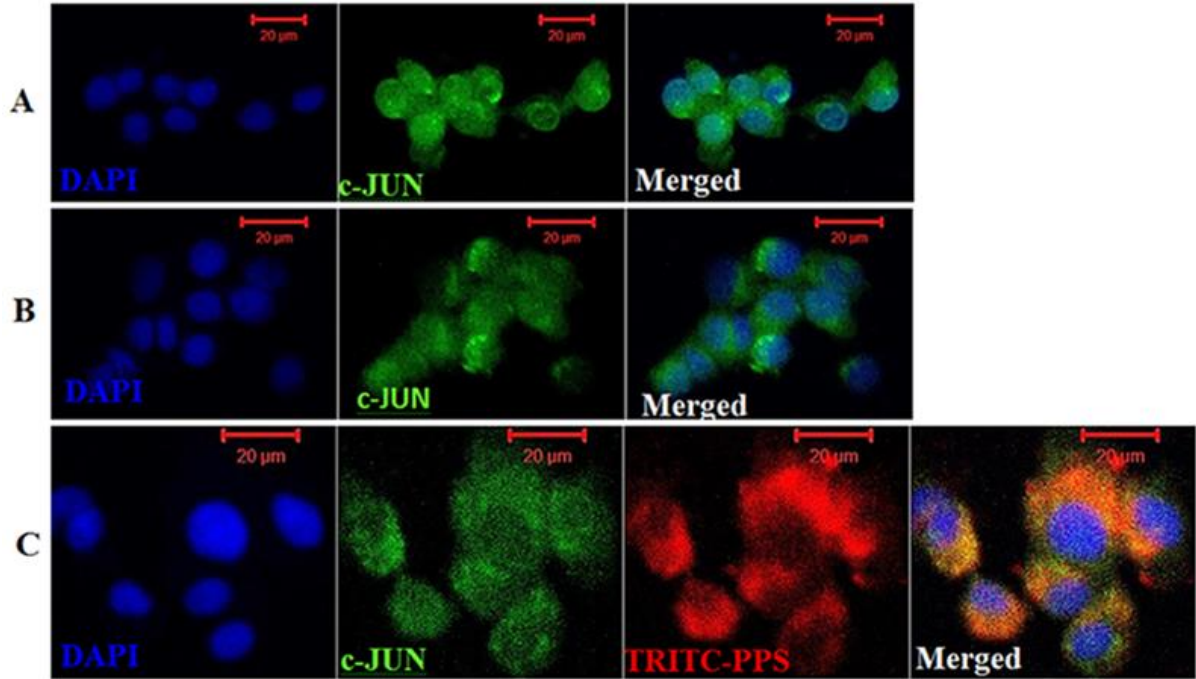


Figure 7 c-Jun colocalizes with PPS in CACs.

(A) Immunofluorescence staining of methanol-fixed chondrocytes showing diffuse nuclear accumulation of c-Jun (green) in control cells without rcIL-1 β , (B) shows increased focal nuclear localization of c-Jun in chondrocytes stimulated with 10 ng/mL rcIL-1 β (positive control, PC), and (C) shows chondrocytes preincubated with 40 μ g/mL TRITC-PPS (red) for 4 hr then stimulated with 10 ng/mL of rcIL-1 β . Chondrocytes preincubated with TRITC-PPS show reduced nuclear localization of c-Jun (green) indicated by increased perinuclear and cytoplasmic accumulation with TRITC-PPS (red) also accumulating in the perinuclear and cytoplasmic area. The yellow/orange areas in the merged image demonstrate that c-Jun colocalizes with PPS. **Scale Bars: 20 μ M** (Bwalya *et al.*, 2017a).

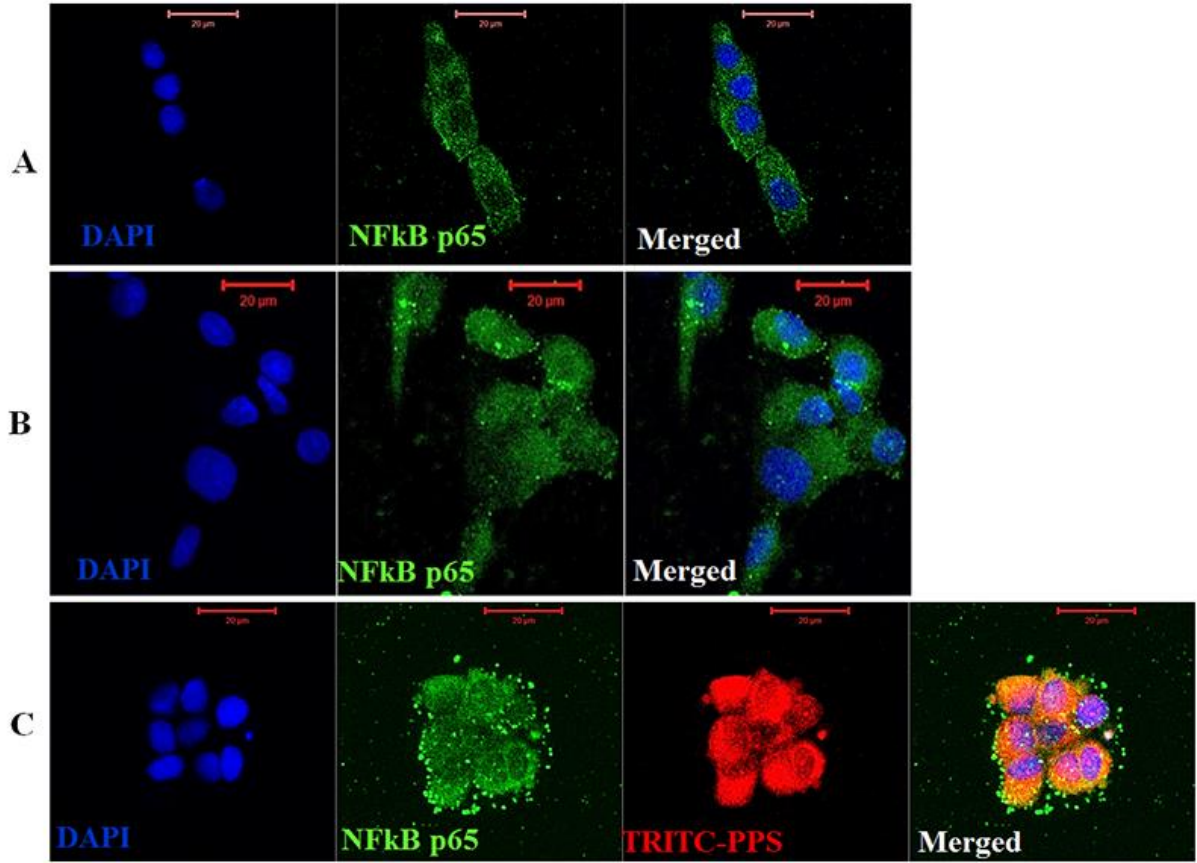


Figure 8 NF-κB p65 colocalizes with PPS in CACs.

(A) Immunofluorescence staining of methanol-fixed chondrocytes without rcIL-1 β (negative control) showing diffuse cytoplasmic localization of NF-κB p65 (green), (B) shows increased nuclear localization and accumulation of NF-κB p65 (green) in chondrocytes treated with 10 ng/mL of rcIL-1 β (positive control, PC) and, (C) demonstrates that chondrocytes preincubated with 40 μ g/mL of TRITC-PPS (red) for 4 hr then treated with 10 ng/mL of rcIL-1 β have reduced NF-κB p65 nuclear translocation activity as evidenced by increased cytoplasmic accumulation. The yellow/orange areas in the merged image show that NF-κB p65 colocalizes with PPS. **Scale Bars: 20 μ M** (Bwalya *et al.*, 2017a).

2.6 Discussion

The selective inhibition of pathologically enhanced NO synthesis by iNOS in OA joints is a novel therapeutic target for the treatment of OA (Balaganur *et al.*, 2014; Mendes *et al.*, 2002a; Stichtenoth and Frölich, 1998). This study demonstrated for the first time that PPS is a novel inhibitor of IL-1 β -induced iNOS, c-Jun and HIF-1 α mRNA upregulation with limited inhibitory effect on IL-1 β -induced HIF-2 α upregulation. Similar to observations with normal HACs cultures (de Andrés *et al.*, 2013; Korhonen *et al.*, 2005), the finding of this study demonstrates that normal CACs may be recalcitrant to iNOS protein production by single or multiple cytokines. While single rcIL-1 β or rhIL-1 β did not induce iNOS protein expression, treatment of CACs with a combination of rcIL-1 β + rcTNF- α + LPS significantly induced iNOS protein expression but this effect was significantly abrogated by PPS hence proving its inhibitory effects on induced iNOS mRNA and protein expression. The detection of iNOS mRNA in monolayer cultured chondrocytes treated with or without IL-1 β without active iNOS protein suggests that iNOS in CACs may be negatively regulated post-transcriptionally. Although more studies are required to fully elucidate the mechanism of action of PPS, ICC colocalization analysis results demonstrated that PPS colocalizes with NF- κ B p65 and c-Jun, and inhibits their nuclear translocation activity. This observation may partially support the suggestion that PPS may exert its inhibitory effects on OA by direct interaction with NF- κ B p65 and c-Jun, consequently repressing the downstream target genes like iNOS and MMP-13 which are implicated in the progression and perpetuation of OA.

While IL-1-induced iNOS mRNA upregulation, the most provocative finding of the experiment was the undetectable iNOS protein in chondrocytes treated with single or multiple

cytokines except for a combination of rcTNF- α + LPS (weak induction) and rcIL-1 β + rcTNF- α + LPS (strong induction). Similar to the finding of this study, normal HACs are reported to be recalcitrant to induction of iNOS even with multiple cytokines compared to all murine cell types in culture that exhibit a readily inducible iNOS (de Andrés *et al.*, 2013; Korhonen *et al.*, 2005). This finding suggests that normal CACs are recalcitrant to iNOS protein induction probably as a protective response from the deleterious effects of pathologically enhanced NO due to iNOS especially in the early phase of OA. Furthermore, this outcome suggests that other than proinflammatory cytokines, other factors such as mechanical stress could play a pivotal role in iNOS protein induction in OA joints. Moreover, mere excessive mechanical stress on articular cartilage has been reported to decrease synthesis of matrix components in chondrocytes through a NO-regulated pathway (Iimoto *et al.*, 2005) and promote *in vivo* cartilage damage (Ko *et al.*, 2013). Part of the signalling pathway cascade that leads to IL-1-induced NF- κ B activation and iNOS expression include the protein tyrosine kinases (PTK) and p38. However, NF- κ B activation which is essential for iNOS induction and p38 lie on two distinct pathways that seem to be independently required for IL-1-induced iNOS expression (Mendes *et al.*, 2002a; Mendes *et al.*, 2002b). While the inhibitory effects of PPS on the two pathways has been previously demonstrated (Sunaga *et al.*, 2012), by ICC, the present study confirms that indeed PPS impairs IL-1-induced NF- κ B p65 nuclear translocation but most interestingly the results demonstrate that PPS colocalizes with NF- κ B p65. Therefore, the observed inhibition of IL-1-induced iNOS mRNA upregulation and rcIL-1 β + rcTNF- α + LPS-induced iNOS protein expression by PPS is consistent with the inhibition of the p38 and NF- κ B p65 pathways. Although more experiments such as immunoprecipitation assays will be required to prove the binding of PPS to transcription factors and other intracellular proteins, the

observed colocalization with NF- κ B p65 may support the proposal that it may exert its action through direct interaction with transcription factors consequently blocking their downstream promoter activity (Ghosh, 1999; Ghosh *et al.*, 2010; Sathukhan *et al.*, 2002; Sunaga *et al.*, 2012). The detection of iNOS at mRNA level in monolayer cultured CACs in the absence IL-1 β observed in this study was unusual and in contrast with previous studies in mouse (Campo *et al.*, 2009), bovine (Mendes *et al.*, 2002a; Mendes *et al.*, 2002b), equine (Tung *et al.*, 2002) including some isolated HACs (Otero *et al.*, 2005; Palmer *et al.*, 1993). In order to confirm whether iNOS mRNA is normally detectable in normal cultured CACs, its expression in chondrocytes derived from two other dogs cultured in medium without IL-1 and passaged up to P3 was assessed. iNOS mRNA was verified to be detectable in all passages (P0-P3) of CACs evaluated with low FBS concentration appearing to enhance its expression. Whether the detection of iNOS at mRNA level in normal CACs could be related to the dedifferentiation of chondrocytes in monolayer culture remains to be determined since epigenetic “unsilencing” of iNOS gene due to loss of DNA methylation at specific cytosine-guanine dinucleotides (CpG) sites in human OA chondrocytes has been associated with iNOS expression (Cheung *et al.*, 2009; de Andrés *et al.*, 2013; Imagawa *et al.*, 2011; Roach *et al.*, 2005). However, these findings taken together suggest that iNOS in CACs is regulated by translational blockade which unlike in human cells has been reported to be strongly regulated at transcriptional level (Förstermann and Kleinert, 1995; Korhonen *et al.*, 2005; Mendes *et al.*, 2002b).

As part of cellular mediators of signal transduction, c-Jun together with c-Fos and ATF-2 (activating transcription factor) subunits form the complex dimeric transcription factors of AP-1 that bind to a common DNA site (Karin *et al.*, 1997; Whitmarsh and Davis, 1996). AP-1 just like NF- κ B seem to play a major role in mediating IL-1-induced early cellular responses

(Mendes *et al.*, 2002a; Mendes *et al.*, 2002b; Sunaga *et al.*, 2012). While AP-1 is not required for IL-1-induced iNOS expression in articular chondrocytes, it is strongly activated by IL-1 (Mendes *et al.*, 2002a; Mendes *et al.*, 2002b) and its activation is required for the expression of MMPs, such as MMP-13 and MMP-3, which promote cartilage degradation (Mendes *et al.*, 2002b; Zafarullah *et al.*, 1992). Particularly, JNK pathway has been shown to mediate the activation and transcription of c-Jun which is required for IL-1-induced MMP-13 upregulation (Mengshol *et al.*, 2000), making c-Jun a potential therapeutic target for OA treatment. The results of this study demonstrate for the first time that IL-1 β -induced c-Jun mRNA upregulation is inhibited by PPS. PPS was previously demonstrated to have no inhibitory effect on JNK activation but on ERK and p38 through inhibition of phosphorylation of these MAPKs (Sunaga *et al.*, 2012). Therefore, it is very reasonable to suggest that the observed inhibition of c-Jun mRNA by PPS could be an indication of PPS ability to directly interact with JNK thereby impairing the activation of c-Jun. The ICC results of this study also clearly demonstrate that PPS could impair IL-1-induced c-Jun nuclear translocation and activity in chondrocytes possibly through interaction subsequently repressing its MMP-13 promoter activity.

HIF-2 α (also designated endothelial PAS domain protein-1 or EPAS1), is a homolog of HIF-1 α and member of the basic helix-loop-helix/PAS transcription factor family. Normally, under normoxic culture condition HIF- α isoforms proteins are reported to be rapidly degraded in the cell as there is sufficient oxygen for the HIF-targeting prolyl hydroxylases (PHDs) to target them for von Hippel-Lindau-mediated proteosomal (pVHL) degradation, therefore neither HIF- α isoform is normally detectable (Jaakkola *et al.*, 2001; Lafont *et al.*, 2007; Thoms *et al.*, 2013; Thoms and Murphy, 2010). Consistent with results in HACs, the results of this

study confirmed that both HIF- α isoforms in monolayer CACs are constitutively expressed at the mRNA level but are not readily detectable at protein level under normoxic culture condition as they are rapidly degraded in the presence of sufficient oxygen (Coimbra *et al.*, 2004; Jaakkola *et al.*, 2001; Lafont *et al.*, 2007; Thoms *et al.*, 2013; Thoms and Murphy, 2010). While both HIF-1 α and HIF-2 α mRNA were upregulated by IL-1 β -stimulation, the preincubation of chondrocytes with PPS inhibited IL-1 β -induced upregulation of HIF-1 α in a dose-dependent manner whereas minimal inhibitory effect on HIF-2 α was observed except at the highest dose (40 μ g/mL). Both HIF-1 α and HIF-2 α have been shown to be significantly overexpressed in cytoplasmic and nuclear of synovial lining and stromal cells derived from RA and OA human patients relative to cells from normal nonarthritic cases (Giatromanolaki *et al.*, 2003), and the regression analysis results demonstrated a significant positive correlation of HIF- α isoforms mRNA expression in response to IL-1. HIF-1 α levels in chondrocytes have been shown in part to be regulated by the NF- κ B and p38 mediated pathways such that inhibitors of NF- κ B and p38 significantly abolish IL-1 or TNF- α -induced HIF-1 α upregulation (Coimbra *et al.*, 2004; Thoms *et al.*, 2013; Yudoh *et al.*, 2005). Therefore the observed inhibitory effect of PPS on IL-1 β -induced HIF-1 α mRNA upregulation observed in this study is consistent with NF- κ B and p38 inhibition. HIF-1 α mRNA has been shown to be higher in degenerated regions than in the intact regions of human OA articular cartilages, a finding which was associated with cellular response to catabolic stress aimed at production of anti-apoptotic factors or act as a chondroprotective factor to maintain chondrocytes viability (Yudoh *et al.*, 2005). In confirming the anti-catabolic effects of IL-1-upregulated HIF-1 α , another study using HACs cultured under hypoxic conditions, established these to be decreased cartilage degradation and MMP-13 expression (Thoms *et al.*, 2013). On the other hand, HIF-2 α has been

proposed as the most potent transactivator that enhances the promoter activities MMP-13 and iNOS including other catabolic genes involved in OA process (Saito *et al.*, 2010; Saito and Kawaguchi, 2010; Yang *et al.*, 2010). HIF-2 α mRNA and protein expression were found to be enhanced in P0 cultures of mouse articular chondrocytes stimulated with proinflammatory cytokines including IL-1 β , IL-17, IL-21 and TNF- α , and in human and mouse OA cartilage, with its ectopic expression triggering articular cartilage destruction in mice and rabbits (Yang *et al.*, 2010). While a significant positive correlation was observed between HIFs and iNOS mRNA expression, the present study does not prove any direct regulation of iNOS by HIFs, and in particular HIF-2 α since the active protein was also undetectable under normoxic culture conditions to correlate with iNOS mRNA transcription and translation. Moreover, in the present study iNOS protein was detected without detectable HIF-2 α protein levels. Contrary to the observed catabolic effects of HIF-2 α , under hypoxic culture condition, HACs have been confirmed to increase tissue production via HIF-2 α and inhibit cartilage destruction largely through HIF-1 α (Thoms *et al.*, 2013). These findings taken together suggest that HIF-2 α mRNA may be upregulated in response to IL-1 but this response may also be corresponding to IL-1-upregulated HIF-1 α since it has also been shown to be a potent regulator of autophagy in maturing mouse and HACs by acting as a brake to the autophagy accelerator function of HIF-1 α (Bohensky *et al.*, 2009). Whether the overexpressed HIF-2 α associated with induction of catabolic genes is due to its direct regulation of these genes leading to cartilage degradation or related to its functional dysregulation in OA process remains to be verified.

In conclusion, the study presented in this chapter demonstrated for the first time that PPS is a novel inhibitor of IL-1-induced iNOS, c-Jun and HIF-1 α mRNA upregulation, and IL-1 β + TNF- α + LPS-induced iNOS protein expression in CACs. In particular, the inhibitory

effects of PPS on iNOS and c-Jun in articular chondrocytes could translate to its beneficial effects in the prevention and treatment of OA joints. Furthermore, the results suggest that normal CACs may be recalcitrant to single or multiple cytokine induction of iNOS protein possibly as a protective mechanism to the deleterious effects of pathologically enhanced NO produced by iNOS.

3 Study 2 PPS restores the phenotype of dedifferentiated monolayer CACs cultured in alginate beads

3.1 Summary

Autologous chondrocyte transplantation (ACT) is a promising treatment option for the repair of isolated OA cartilage lesions. The procedure requires that chondrocytes are isolated from a small cartilage biopsy and expanded in monolayer cultures *in vitro* prior to implantation. However, when monolayer cultured, chondrocytes lose their stable phenotype and dedifferentiate to fibroblastic-like cells. The present study investigated the chondroinductive potential of PPS to completely restore the phenotype of dedifferentiated monolayer articular chondrocytes cultured in alginate beads. CACs were isolated from three cartilage samples and expanded in culture to establish P0 culture. Subsequent P1 chondrocytes were encapsulated in alginate beads and cultured for 18 days under normoxia condition in 20% DMEM supplemented with PPS concentrations of 0, 1, 5, 15 and 40 µg/mL with medium changes done every 72 hr. The concentration effects of PPS on type I, II and X collagen, aggrecan and Runx2 gene expression in alginate beads cultured chondrocytes were evaluated by RT-PCR and qPCR. Runx2, HIF-1 α and HIF-2 α protein expression were evaluated by Western blot and proteoglycan (PG) deposition was determined by Alcian blue stain. In the presence of PPS, dedifferentiated monolayer CACs cultured in alginate beads fully retained their phenotype as verified by enhanced synthesis of cartilage-specific marker genes, type II collagen and aggrecan mRNA with complete suppression of type I and X collagen at PPS concentrations of 15 and 40 µg/mL. Compared to the control, type II collagen and aggrecan

mRNA were significantly upregulated ($P < 0.05$) at 5, 15 and 40 $\mu\text{g/mL}$, and 5 and 15 $\mu\text{g/mL}$ PPS, respectively. PG deposition was significantly enhanced by PPS compared to the control with peak deposition notably at 5 $\mu\text{g/mL}$. Interestingly, HIF-1 α and HIF-2 α proteins were detectable at protein level for the first time under normoxia condition, an indication of stabilization of the HIF- α isoforms in alginate beads culture. The study presented in this chapter demonstrated for the first time that the phenotype of dedifferentiated monolayer CACs is restored by combining alginate encapsulation with culture in PPS without the addition of known chondrocytic growth factors. The findings of this study confirms PPS as novel chondroinductive factor with the potential to offer a solution to the major challenges that exist at the front-end of cartilage tissue engineering.

3.2 Introduction

For isolated focal OA cartilage lesions, several surgical treatment options that reduce pain and extend the useful life of the joint, prior to total joint replacement, are available (Athanasίου *et al.*, 2013). ACT has long been considered as a promising alternative strategy for the repair of small isolated OA cartilage lesions. The procedure requires that articular chondrocytes are isolated from a small biopsy, expanded in monolayer *in vitro* cultures, and then either directly injected into the defect or used to engineer implantable grafts (Barbero *et al.*, 2004). However, when expanded as monolayer cultures *in vitro*, articular chondrocytes lose their phenotype and dedifferentiate to fibroblastic-like cells synthesizing fibrocartilage proteins instead of hyaline cartilage proteins (Darling and Athanasίου, 2005; Freshney, 2010; Hamada *et al.*, 2013; Singh *et al.*, 2011). Fibrocartilage is undesirable for ACT as it does not possess the biochemical composition or structural organization to provide proper mechanical function within the joint environment and degrades with time due to insufficient load-bearing capacity (Buckwalter and Mankin, 1998; Darling and Athanasίου, 2005; Athanasίου *et al.*, 2013).

Therefore, in an attempt to restore the phenotype of dedifferentiated monolayer chondrocytes, three-dimensional (3D) hydrogel cultures have been employed. In general, 3D hydrogels provide a suitable condition for redifferentiation of chondrocytes because of their ability to mimic *in vivo* environment and support a spherical cell morphology which plays a major role in gene expression (Darling and Athanasίου, 2005; Glowacki *et al.*, 1983). Some previous studies have reported phenotype restoration in long-term 3D encapsulation cultures marked by complete suppression of type I collagen and re-expression of type II collagen and aggrecan to levels similar to P0 (Benya and Shaffer, 1982; Bonaventure *et al.*, 1994; Frondoza

et al., 1996; Lemare *et al.*, 1998). In contrast, other studies have demonstrated drastically higher type I collagen expression with reduced level of expression of type II collagen and aggrecan when expanded chondrocytes are encapsulated in alginate beads (Caron *et al.*, 2012; Darling and Athanasiou, 2005). To optimize the redifferentiation of chondrocytes, other studies have combined alginate encapsulation with culture in established chondrocytic growth factors (Ab-Rahim *et al.*, 2013; Caron *et al.*, 2012; Hsieh-Bonassera *et al.*, 2009; Xu *et al.*, 2014), and reduced oxygen tension to physiological level (Murphy and Polak, 2004; Murphy and Sambanis, 2001). While these studies demonstrated restoration of key chondrocyte phenotype markers, the undesirable type I collagen was still detectable and even reported higher in alginate than monolayer chondrocytes cultured in medium supplemented with chondrogenic growth factors (1% insulin-transferrin-sodium selenite medium supplement (ITS), 1% L-ascorbic acid-2-phosphate and 10 ng/ml transforming growth factor-beta (TGF- β 3)) (Caron *et al.*, 2012) whereas hypoxia showed no effect on type I collagen (Murphy and Polak, 2004; Murphy and Sambanis, 2001). Taken together, these findings provoke the desire to improve the current culture strategies or develop novel culture conditions if implantable phenotypically stable chondrocytes are to be established for successful cartilage tissue regeneration and repair of OA defects.

One strategy that requires further research involves combining alginate encapsulation with culture in drugs capable of modulating chondrocytes gene expression. PPS was recently shown to promote proliferation and chondrogenesis of human bone marrow-derived mesenchymal precursor cells (MPCs) while suppressing osteogenic expression and bone formation in micromass cultures (MMC) (Ghosh *et al.*, 2010). However, follow-up studies are currently lacking to clarify whether these chondroinductive effects may aid in restoring the

phenotype of dedifferentiated monolayer expanded articular chondrocytes in 3D hydrogel cultures for the purpose of cartilage tissue regeneration and repair of OA cartilage lesions. Therefore, the purpose of the this study was to investigate the chondroinductive potential of PPS to completely restore the phenotype of dedifferentiated monolayer articular chondrocytes *in vitro* since successful ACT and cartilage tissue regeneration therapy mandatorily depend on a cell population with a stable chondrocyte phenotype. The hypothesis was that PPS can completely restore the phenotype of dedifferentiated monolayer expanded chondrocytes marked by enhanced cartilage-specific genes, type II collagen and aggrecan, with indiscernible type I and X collagen.

3.3 Materials and Methods

3.3.1 Canine articular chondrocytes source and expansion culture

Chondrocytes were isolated from three cartilage samples of two dogs; a client-owned 10 months-old dog undergoing femoral head and neck ostectomy due to LCPD and a 3 year-old experimental dog as previously described. Chondrocytes from the 3 year-dog were isolated from both humeral head cartilages representing two independent samples. The use of samples from experimental dog was in accordance with Hokkaido University Institutional Animal Care and Use Committee guidelines (approval number: 12-0059). P0 cultures were established as previously described in study 1 with subsequent P1 chondrocytes used in all the experiments of this study. Cells were counted and viability determined as previously described.

3.3.2 Alginate encapsulation and culture of chondrocytes

Briefly, P1 chondrocytes were suspended in 1 mL of 1.25 % (w/v) sodium alginate solution (Wako Pure Chemical Industries Ltd, Product code: 192-09951) which was prepared as described elsewhere (Freshney, 2010). The suspension was diluted progressively until a homogenous cellular density of 1.1×10^7 cells/mL was reached of 5.0 mL final cell suspension volume. The cell suspension was expressed in drops through a 22-gauge needle into the gelation solution of 102 mM calcium chloride in 12-well plates (Corning) and allowed to polymerize for 15 min to form beads. The beads were washed three times with 0.15 M NaCl solution, twice with 10% DMEM, and then assigned specific treatment containing PPS (Biopharm) concentrations of 0 (CTL), 1, 5, 15 and 40 $\mu\text{g/mL}$ supplemented in 20% DMEM. In this study, 20% FBS was used based on two previous studies that showed that 20% FBS (Yu

et al., 2006) and human serum (Hsieh-Bonassera *et al.*, 2009) support redifferentiation of HACs. The beads were incubated and cultured under normoxia condition at 37°C in a humidified atmosphere of 5% CO₂ for 18 days with medium changes done every 72 hr. Each culture well contained ~75 beads equivalent to 1.1×10^7 cells.

3.3.3 RT-PCR gel electrophoresis and qPCR

Following recovery of cells from alginate beads, total RNA was extracted using TRIzol[®] Kit (Invitrogen), according to the manufacturer's instructions. 500 ng RNA was reverse transcribed into cDNA using M-MLV RT kit (Invitrogen) and amplified using PCR (TaKaRa Bio) according to manufacturer's recommended procedures to probe for Sox-9, type I, II and X collagen, aggrecan, runt-related transcription factor (Runx2), HIF-1 α and HIF-2 α , and parathyroid hormone related protein (PTHrP) mRNA expression levels. The PCR conditions for gel electrophoresis and qPCR were as described previously. The sequences, product size and accession codes for each of primers used in the experiments are provided in Table 3. Primer sequences were designed as previously described.

Table 3 Sequence of primers used for PCR to evaluate gene expression by chondrocytes in alginate culture

Name of gene	Domain	^a Primer	^b Fragment	Accession
GAPDH	664-683	5'-CTGAACGGGAAGCTCACTGG-3'	129 bp	NM_001003142.1
	773-792	5'-CGATGCCTGCTTCACTACCT-3'		
Sox-9	565-583	5'-GCCGAGGAGGCCACCGAACA-3'	179 bp	NM_001002978.1
	724-743	5'-CCCGGCTGCACGTCGGTTTT-3'		
Type I Collagen	150-169	5'-GTGGATACGCGGACTTTGTT-3'	164 bp	NM_001003187.1
	294-313	5'-GGGATACCATCGTCACCATC-3'		
Type II Collagen	4127-4146	5'-CACTGCCAACGTCCAGATGA-3'	215 bp	NM_001006951.1
	4322-4341	5'-GTTTCGTGCAGCCATCCTTC-3'		
Type X Collagen	1782-1801	5'-TTTCTCCTACCACGTGCATG-3'	117 bp	XM_003639401.1
	1879-1898	5'-GAAGCCTGATCCAGGTAGCC-3'		
Aggrecan	6569-6588	5'-ACTTCCGCTGGTCAGATGGA-3'	111 bp	NM_001113455.1
	6660-6679	5'-TCTCGTGCCAGATCATCACC-3'		
HIF-1 α	861-880	5'-GTACTTCACTGCACAGGCCA-3'	102 bp	NM_001287163.1
	943-962	5'-ACAAATCAGCACCAAGCACG-3'		
HIF-2 α	1248-1267	5'-TGCAAAGCACGGGGGCTACG-3'	72 bp	XM_531807.3
	1300-1319	5'-GGCTGCAGGTTGCGAGGGTT-3'		
Runx2	1168-1187	5'-TTACTTACCCCCGCCAGTC-3'	139 bp	XM_014118252.1
	1287-1306	5'-TATGGAGTGCTGCTGGTCTG-3'		
PTHrP	82-103	5'-GACTGCTGAGAAGATCCCCTTC-3'	73 bp	NM_001003303.1
	135-154	5'-TCGCGGGACCTTTTCCTTGA-3'		

^aPrimers for forward & reverse sense are presented in a 5' to 3' orientation, ^bThe expected fragment size, GAPDH = glyceraldehyde-3-phosphate dehydrogenase, HIF- = Hypoxia inducible factor -, Runx2 = Runt-related transcription factor, PTHrP = Parathyroid hormone related protein

3.3.4 Alcian blue stain for PG deposition analysis

Alginate beads were fixed in 4% formalin for 30 minutes. After fixation, the beads were sectioned and rinsed three times with 1 x PBS then stained for 30 minutes with 1% Alcian blue solution prepared in 0.1 N HCl. Thereafter, the beads were thoroughly rinsed with 0.1 N HCl before adding distilled water to neutralize the acidity. The stained sections were visualized under light microscope and images captured for PG deposition analysis.

3.3.5 Western blot analysis

Following recovery of cells from alginate beads, total protein was extracted using TRIzol[®] Kit (Invitrogen) and quantified as previously described. Briefly, 20 µg of protein was separated on 12% (w/v) SDS-PAGE and electroblotted to nitrocellulose membranes as previously described. The membranes were blocked as previously described then probed for target primary protein. The primary antibodies were antihuman Runx2 (C-19) goat polyclonal antibody (Santa Cruz Biotechnology, Cat. #: sc-8566), mouse monoclonal Anti-HIF-1α Clone H1α67 (Sigma-Aldrich) and EPAS-1 (HIF-2α) (C-16) goat polyclonal antibody (Santa Cruz Biotechnology) (1/200 dilution) and F-Actin rabbit polyclonal antibody (Bioss Antibodies) (1/1,000 dilution). Secondary antibodies (1/5,000 dilution) for actin and HIF-1α primary antibodies were Pierce[®] Goat anti-Rabbit Poly-HRP (Pierce Biotechnology) and Zymed[®] Rabbit anti-mouse IgG-HRP conjugate (Invitrogen), respectively. Anti-IgG goat Rabbit-Poly-HRP (R & D Systems) was used for Runx2 and HIF-2α. The blots were developed as previously described with Western Blot Ultra-Sensitive HRP substrate (Takara) and the protein-antibody reaction was visualized for chemiluminescent signal using FUJIFILM Luminescent Image Analyzer LAS-3000 (Fujifilm Life Science). All immunoreactive Western

blots were quantified by densitometric analysis using Image J analysis software (NIH) as per the request of the Image J developers following software calibration. Densitometric results are presented as intensities relative to actin (normalizer).

3.3.6 Immunocytochemistry: colocalization of Runx2 with PPS

Immunocytochemistry (ICC) was performed to investigate colocalization of PPS with Runx2, and to evaluate whether PPS could interfere with nuclear translocation and cellular distribution of Runx2. P1 chondrocytes (1×10^4 cells) were plated on 8-well Permanox[®] slides (Thermo scientific nunc) and incubated in 10% FBS DMEM for 24 hr. Cells were gently washed with 1 x PBS then incubated overnight in serum free DMEM with or without 20 $\mu\text{g/mL}$ TRITC-PPS. Cells were gently washed with cold 1 x PBS (2-8°C), fixed with cold methanol (-20°C) for 5 min at RT then washed thrice at 5 min interval with cold 1 x PBS containing 0.1% tween 20 (PBST). Non-specific antibody binding was blocked by incubating the methanol fixed cells for 1 hr at RT in 5% BSA (Sigma-Aldrich) prepared in 1 x PBST. Cells were incubated with anti-human Runx2 (C-19) (1/100 dilution) overnight at 4°C then washed as previously. Rabbit anti-goat IgG-FITC-conjugated (Santa Cruz Biotechnology; SC-2777) (1:1000 dilution) in 1% BSA was used to visualize antigen signal (1 hr, RT). Prolong[®] Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific) was used to stain the nucleus and mount the slides according to manufacturer's instructions. Slides were viewed using Zeiss LSM 700 confocal laser microscope (Zeiss) and images captured for analysis.

3.4 Data analysis

Quantitative data analysis was performed using SPSS version 16.0. ANOVA was used to compare the effects of PPS on gene expression between the treatments with equal variances assumed. Where significant difference was observed, *Post Hoc* multiple comparisons were performed using Fisher's Least Significant Difference (LSD). All quantitative data, unless specified is summarized as mean values (\pm 95% confidence interval (CI)) for three independent experiments. Significant difference was defined as $P < 0.05$.

3.5 Results

3.5.1 Alginate encapsulation of CACs leads to a spherical morphology

Following encapsulation of monolayer expanded CACs in alginate beads, the morphology of cells was monitored during specific days of the 18-days culture period. As expected, cells assumed a spherical morphology and maintained the shape throughout the culture period (Figure 9A-9D).

3.5.2 Alginate encapsulation of CACs suppresses Sox-9 mRNA expression; PPS selectively promotes a chondrocyte phenotype while suppressing chondrocyte hypertrophy and dedifferentiation

Type II collagen and aggrecan bands were distinctly expressed in all treatments. Surprisingly, the key positive cartilage regulator, Sox-9 mRNA was undetectable (Figure 10A-10C). Type I and X collagen mRNA bands were inhibited by PPS in a dose-dependent pattern with both bands completely suppressed at 15 and 40 $\mu\text{g/mL}$ (Figure 10B-10C). Although Runx2 mRNA was expressed, its band intensity was evidently reduced. Interestingly, PTHrP mRNA was undetectable in all treatments (data not shown). Quantitative analysis of gene expression by qPCR, demonstrated significantly upregulated type II collagen and aggrecan by PPS relative to the CTL at 5 ($P = 0.002$), 15 ($P = 0.03$) and 40 $\mu\text{g/mL}$ ($P = 0.036$), and 5 ($P = 0.012$) and 15 $\mu\text{g/mL}$ ($P = 0.019$), respectively (Figure 10D). Type I and X collagen were both significantly downregulated ($P < 0.05$) by PPS at all concentrations in a dose-dependent pattern relative to the CTL whereas Runx2 mRNA was only significantly downregulated at 1 and 5 $\mu\text{g/mL}$ of PPS relative to the CTL (Figure 10D).

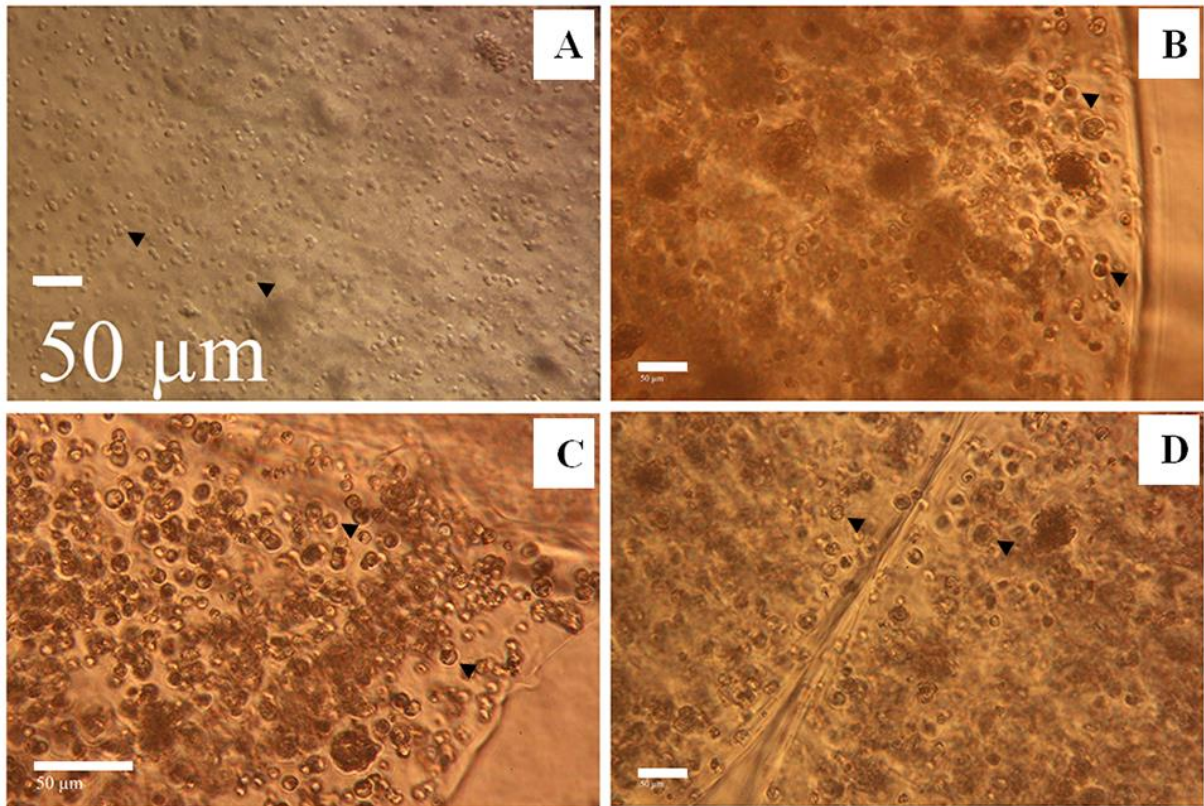


Figure 9 Microphotographs of first passage (P1) CACs cultured in alginate beads.

Morphology of cells on (A) day 1, (B) 7, (C) 11 and (D) 18. Alginate encapsulation of chondrocytes leads to a spherical morphology which corresponds to a chondrocyte-like phenotype *in vivo*. **Scale Bars: 50 µm.**

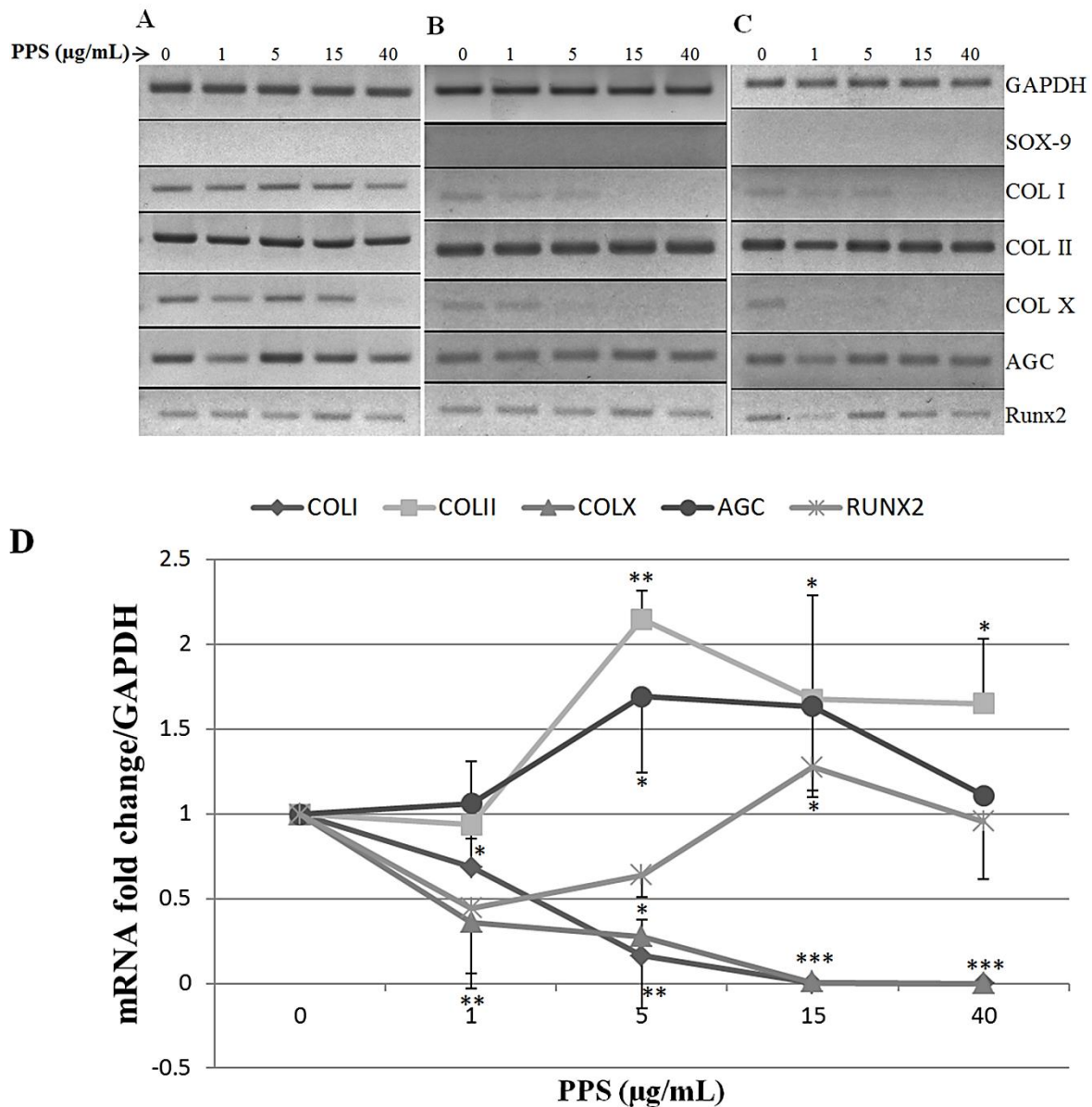


Figure 10 Effects of PPS on gene expression in alginate beads cultured CACs.

First passage canine articular chondrocytes isolated from; (A) femoral head cartilages of 10-month-old, (B) left and (C) right humeral head cartilages of 3-year-old dog were encapsulated in alginate beads and cultured for 18-days in 0 (control), 1, 5, 15 and 40 µg/mL of PPS. (D) Shows concentration effects of PPS on the target genes as quantified qPCR. PPS enhanced the expression of type II collagen (COL II) and aggrecan (AGC). Type I (COL I) and X collagen (COL X) were inhibited in a concentration-dependent manner whereas SRY-box 9 (Sox-9) was suppressed in all treatments. Significant difference was defined as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Results represent the mean (\pm 95% confidence interval) of three independent experiments. GAPDH; (internal control gene), L; 100bp DNA ladder.

To verify whether the suppression of Sox-9 mRNA was associated with alginate quality or specific to alginate encapsulation of CACs, we investigated the expression of Sox-9 in rat- and equine-isolated chondrocytes. Alginate was changed to a low viscosity grade (Sigma-Aldrich; Cat. #: A2158). Cryopreserved chondrocytes isolated from two other dogs were also analysed. As expected, in monolayer culture, Sox-9 mRNA was induced and restored but when the expanded cells were encapsulated in alginate beads for 10-days, Sox-9 mRNA was completely suppressed (Figure 11A). Similar to CACs, equine chondrocytes demonstrated induced Sox-9 mRNA in monolayer culture (Figure 11B) but it was suppressed in alginate beads (Figure 11C). Sox-9 mRNA was induced in chondrocytes isolated from three rats (Sprague Dawley) in both culture systems although the band intensity was evidently reduced in alginate beads compared to monolayer culture (Figure 11B and 11C). These findings taken together demonstrate that the culture of chondrocytes in alginate beads inhibits the expression of Sox-9 mRNA and this could be associated with the reduced cell-to-cell interactions in alginate beads culture compared to cells in MMC or pellet culture.

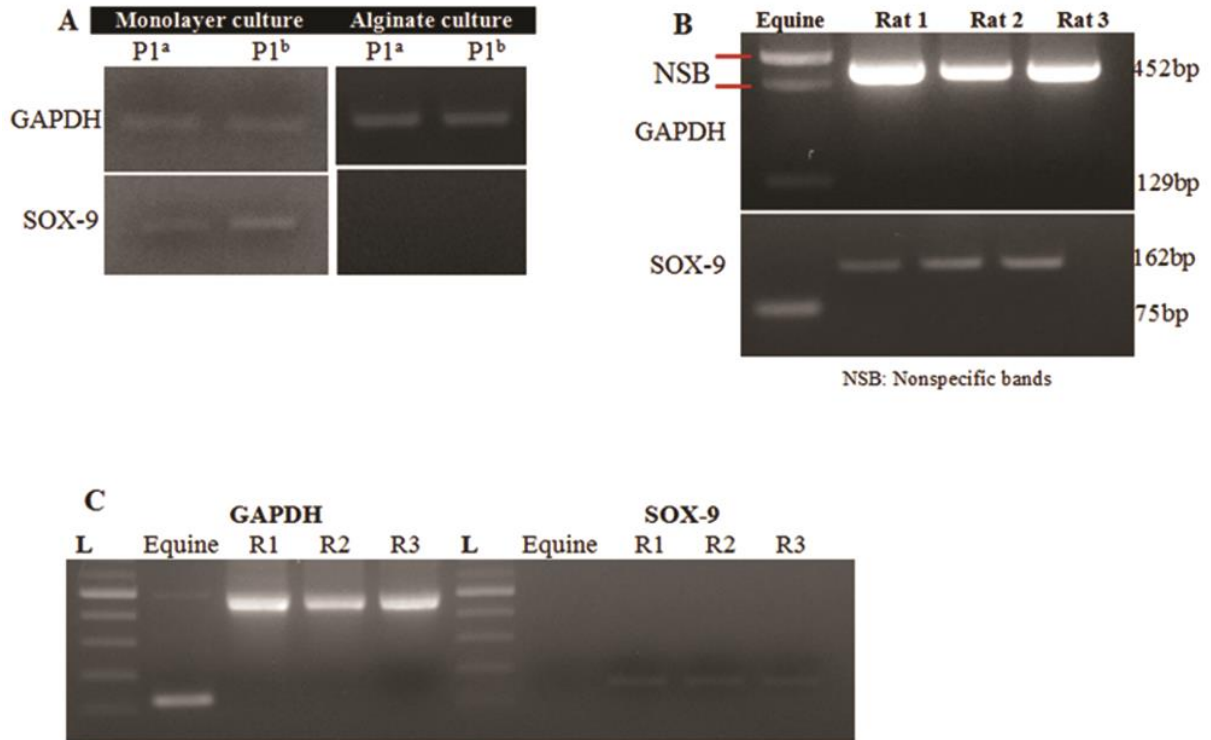


Figure 11 Effect of alginate encapsulation of chondrocytes on Sox-9 mRNA expression.

(A) First passage (P1) monolayer and alginate encapsulated canine articular chondrocytes (n = 2), (B) P1 monolayer equine (n = 1) and primary (P0) rat chondrocytes (n = 3), and (C) P2 alginate encapsulated equine chondrocytes (n = 1) and P1 rat chondrocytes (n = 3). P1^a and P1^b represent articular chondrocytes isolated from 11-months and 1-year-old dog, respectively. L; 100bp DNA ladder.

3.5.3 Sox-9 protein expression is reduced to low ‘physiological’ levels in alginate beads cultured CACs

To verify whether the suppression of Sox-9 at mRNA level by alginate encapsulation of CACs correlated with the protein level, Western Blot analysis using 20 µg of cellular protein lysates loaded per lane was performed as previously described with Rabbit polyclonal Anti-Sox-9 antibody (Abcam, Tokyo, Japan, Cat. #: ab26414) at 1/500 dilution. CACs cultured in MMC for 18 days in 20% FBS DMEM supplemented with PPS concentrations of 0, 5 and 40 µg/mL were used as positive control for Sox-9 protein expression. Surprisingly, while Sox-9 protein was detected in all the MMC, it could not be detected in all alginate beads cultured CACs with or without PPS (Figure 12A). To verify if the non-detection of Sox-9 protein was due to actual downregulation of the transcription factor in alginate encapsulated CACs, 80 µg of cellular protein lysates from each alginate cultured CACs sample was precipitated by acetone method and Western blot was repeated as previously described. Interestingly, Sox-9 protein expression was detected at 80 µg protein lysates load per lane in all alginate beads culture although the expression levels were very low compared to MMC loaded at 20 µg, highlighting that Sox-9 protein although downregulated is still involved at very low ‘physiological’ levels in maintaining the chondrocyte phenotype (Figure 12B).

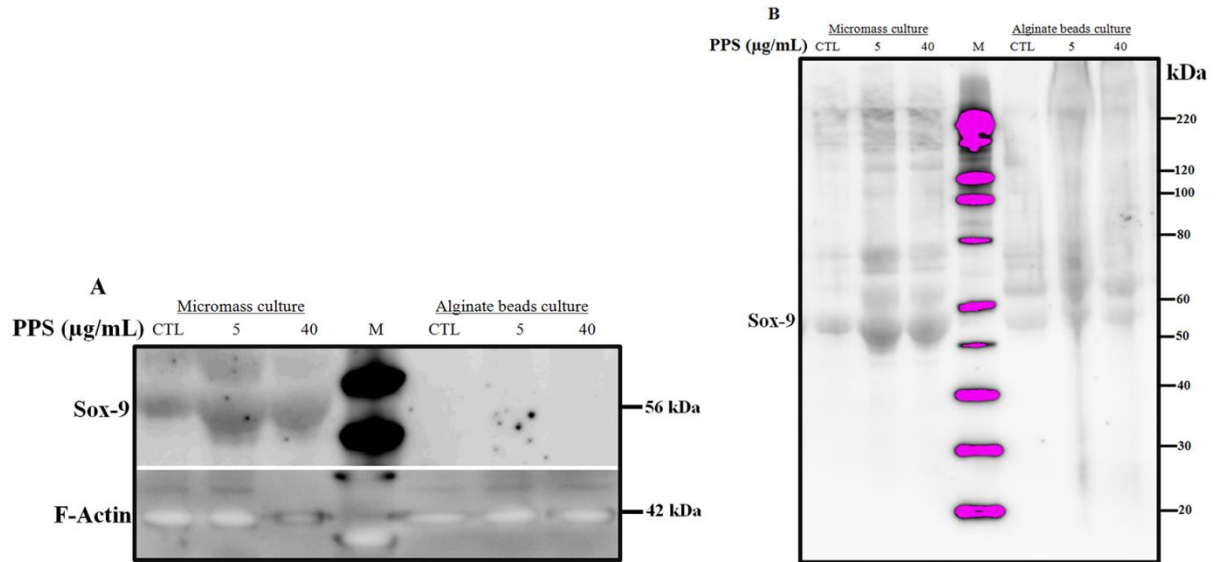


Figure 12 Sox-9 protein expression in CACs in MMC and alginate beads culture.

(A) Effect of MMC and alginate beads culture on Sox-9 protein expression in CACs. Cellular protein lysates were loaded at 20 µg per lane from each sample culture and Sox-9 protein was undetected in alginate cultured CACs. (B) However, when Western blot was repeated with 80 µg cellular protein lysates load per lane for alginate cultured CACs and 20 µg for MMC, Sox-9 protein was detected in alginates beads cellular lysates although at very low levels. M; Protein standards marker for blots. Predicted molecular weight for Sox-9: 56 kDa, and F-Actin: 42 kDa.

3.5.4 PPS promotes PG deposition in alginate cultured CACs

Alginate encapsulated CACs cultured in PPS were characterized by an enhanced Alcian blue staining indicating enhanced deposition of PG compared to CTL without PPS (Figure 13). The peak PG deposition was notably at 5 $\mu\text{g/mL}$ of PPS which was characterized by enhanced and homogenously stained matrix with almost all cells showing PG synthesis activity (Figure 13C). While PPS appeared to enhance PG synthesis, there was a decrease in PG deposition from 15 to 40 $\mu\text{g/mL}$ compared to beads treated with 5 $\mu\text{g/mL}$ as observed by reduced Alcian blue staining (Figure 13D and 13E).

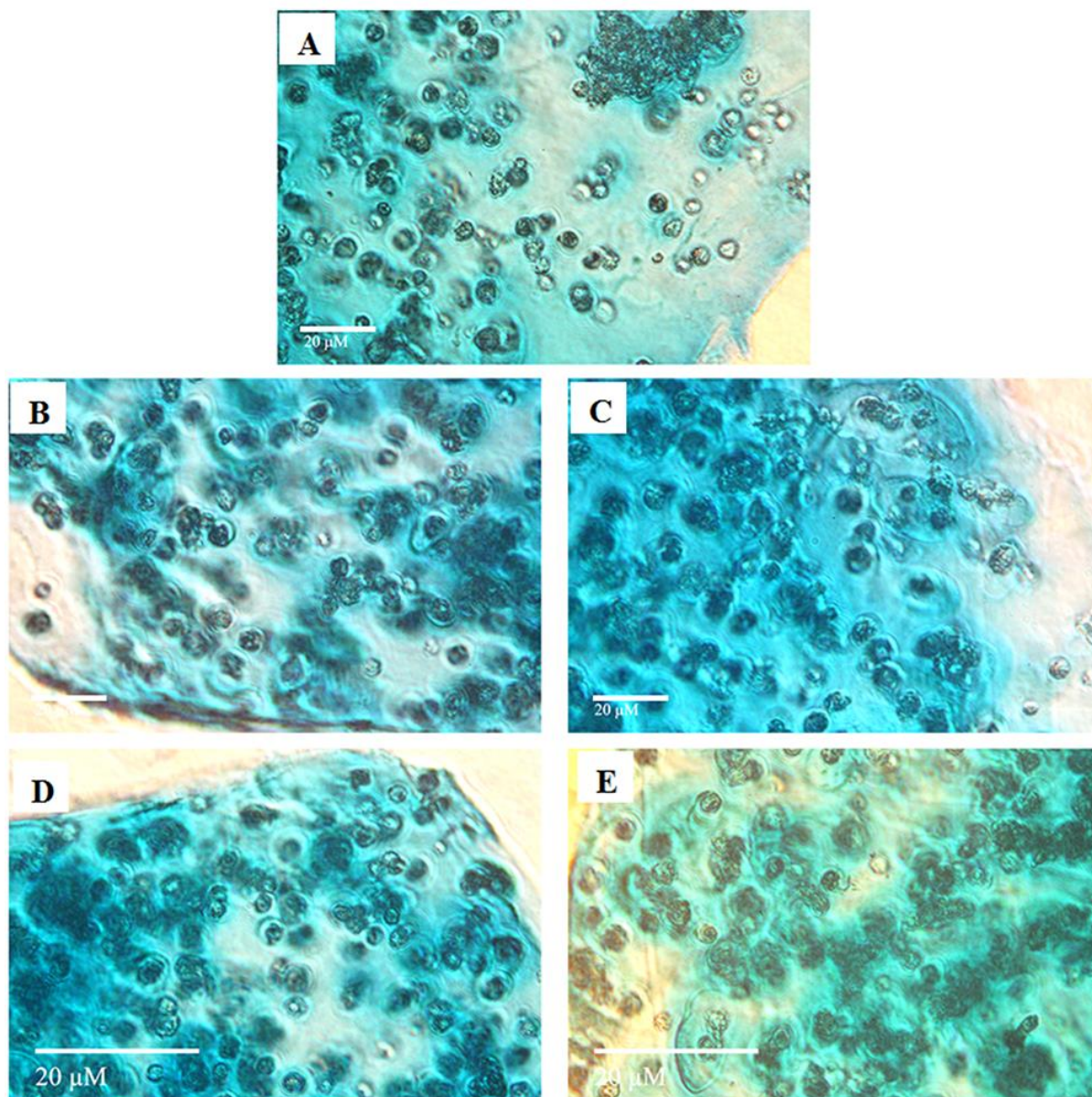


Figure 13 Effects of PPS on CACs PG deposition in alginate beads culture.

Photomicrographs of first passage (P1) articular chondrocytes cultured in (A) 0 (control), (B) 1, (C) 5, (D) 15 and (E) 40 $\mu\text{g/mL}$ of PPS for 18-days. Alginate beads were fixed with 4% formalin, sectioned and stained with 1% Alcian blue solution for PG deposition. PPS enhanced PG deposition with peak effect observed at 5 $\mu\text{g/mL}$ while concentrations >15 $\mu\text{g/mL}$ showed a slight decrease in PG deposition. Results representative of three independent experiments. **Scale Bars: 20 μm .**

3.5.5 Culture in alginate leads to HIFs protein stabilization; PPS has no effect on Runx2 and HIF-2 α protein expression but significantly stabilizes HIF-1 α expression in CACs

Normally, under normoxic culture condition, HIF- α isoforms proteins are reported to be rapidly degraded in the cell as there is sufficient oxygen for the HIF-targeting PHDs to target them for pVHL degradation, therefore neither HIF- α isoform is normally detectable (Jaakkola *et al.*, 2001; Lafont *et al.*, 2007; Thoms *et al.*, 2013; Thoms and Murphy, 2010). Nonetheless, HIF-1 α has been shown to be expressed in suspension cultures of human normal and OA articular chondrocytes under normoxic conditions (Coimbra *et al.*, 2004). However, little is known about the expression at protein level of HIF- α isoforms in dedifferentiated monolayer articular chondrocytes encapsulated in alginate beads under normoxic culture condition and their response to PPS. As expected, by RT-PCR, HIF-1 α and HIF-2 α bands were expressed and readily detectable at mRNA level (Figure 14A). Interestingly, both HIF-1 α and HIF-2 α protein were also detectable under normoxic culture condition in alginate beads culture, an indication of stabilization of the HIF- α isoforms (Figure 14A). HIF-2 α protein levels were not significantly different (ANOVA, $P = 0.74$) between the treatments (Figure 14B). In contrast, HIF-1 α protein was prominently expressed and significantly stabilized (ANOVA, $P < 0.0001$) at all PPS concentrations compared to the CTL (Figure 14B). The identity of the extra band at ~94 kDa level is unknown although a previous study which also observed two spaced bands under normoxia condition that became consolidated into a single band under hypoxia condition proposed this to be due to an increase in HIF-1 α protein degradation (Coimbra *et al.*, 2004). There was no significant difference (ANOVA, $P = 0.059$) in Runx2 protein expression between the treatments (Figure 14A and 14B).

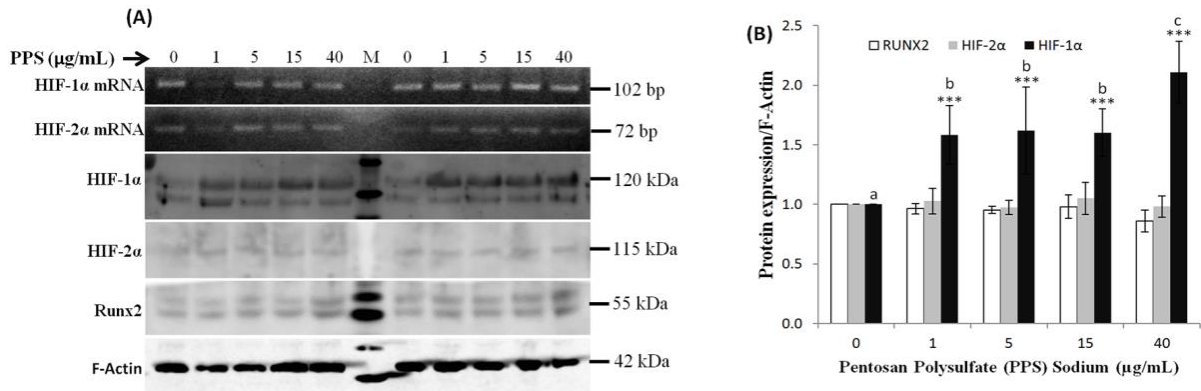


Figure 14 Effects of PPS on Runx2, HIF-1α and HIF-2α protein expression in alginate cultured CACs.

First passage (P1) articular chondrocytes were encapsulated in alginate beads and cultured in 0 (control), 1, 5, 15 and 40 μg/mL of PPS for 18-days. **(A)** Expression of HIF-α isoforms mRNA as detected by RT-PCR, and detection of HIF-1α, HIF-2α and Runx2 proteins by Western blot. **(B)** Bar graphs showing the concentration effects of PPS on HIF-1α, HIF-2α and Runx2 proteins as quantified by densitometry. Data represents mean ± 95% confidence interval of three independent experiments. Significant difference was defined as *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. Different letters represent significantly different treatments. M; Protein standards marker for blots and 100 bp DNA ladder for gels. Predicted band size; F-Actin (internal control): 42 kDa, Runx2: 55 kDa, HIF-1α: 120 kDa and HIF-2α: 115 kDa.

3.5.6 Runx2 colocalizes with PPS in CACs

As expected, chondrocytes without TRITC-PPS (CTL) predominantly showed nuclear localization of Runx2 protein (Figure 15A). Notably, chondrocytes incubated with TRITC-PPS predominantly demonstrated cytoplasmic and perinuclear colocalization of Runx2 (green) with TRITC-PPS (red) with evidently reduced Runx2 nuclear localization in some cells (Figure 15B). The merged image with white arrows (yellow/orange areas) clearly demonstrates that Runx2 protein colocalizes with TRITC-PPS (Figure 15B). To quantitatively verify the colocalization, colocalization analysis was performed on a pixel by pixel basis of Runx2 and TRITC-PPS intensity. The colocalization coefficient for Runx2 was 0.88 (88%) and that of TRITC-PPS was 0.44 (44%) with an overlap coefficient of 0.91 (91%), indicating that 88% of Runx2 protein colocalized with 44% of TRITC-PPS (Figure 15C).

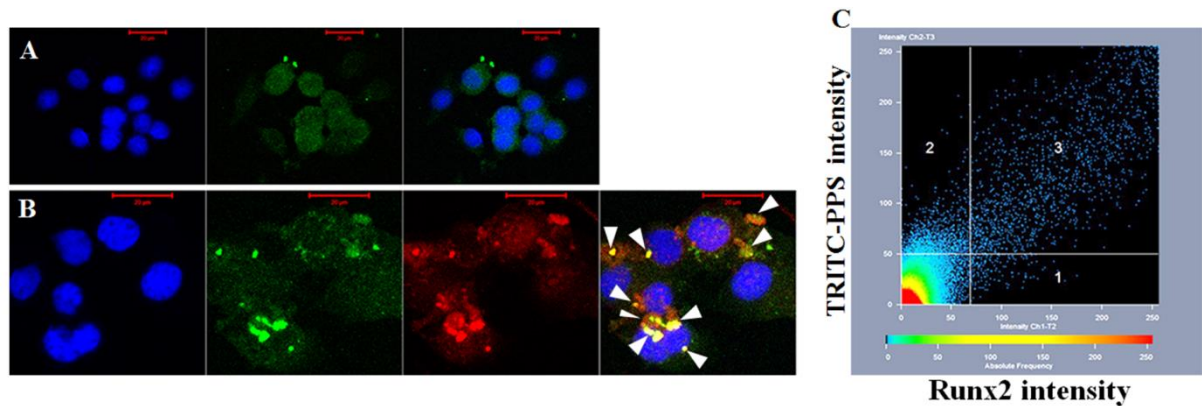


Figure 15 Colocalization analysis of Runx2 and PPS in immunofluorescence stained methanol fixed CACs.

First passage (P1) CACs were incubated overnight 0 (control) and 20 $\mu\text{g/mL}$ tetramethyl rhodamine (TRITC) labelled pentosan polysulfate (TRITC-PPS) in serum free DMEM. **(A)** Control cells showing predominantly nuclear localization of Runx2 protein (Green), while **(B)** chondrocytes treated with 20 $\mu\text{g/mL}$ of TRITC-PPS showing perinuclear and nuclear colocalization of TRITC-PPS (Red) and Runx2 protein. The merged section indicated by white arrows (Yellow areas) demonstrates Runx2 colocalization with TRITC-PPS. **(C)** Scatterplot representing Runx2 (green intensity) on the x-axis and TRITC-PPS (red intensity) on the y-axis. The three quadrants designated by crosshairs represent; quadrant 1 - pixels that have high Runx2 (green) intensities and low TRITC-PPS (red) intensities; quadrant 2 - pixels that have high TRITC-PPS (red) intensities and low Runx2 intensities (green), and quadrant 3 - pixels with high intensity levels in both Runx2 (green) and TRITC-PPS (red) considered to be colocalized (colocalization coefficient for Runx2; 0.88 and for TRITC-PPS; 0.44 with an overlap coefficient of 0.91). **Scale Bars: 20 μM .**

3.6 Discussion

For successful cartilage tissue regeneration and repair of OA defects, redifferentiation of chondrocytes following *in vitro* monolayer expansion has long been proposed as the best hope for returning dedifferentiated articular chondrocytes to their native articular cartilage mode of expression (Darling and Athanasiou, 2005; Athanasiou *et al.*, 2013). The study presented in this chapter is the first to demonstrate that the phenotype of dedifferentiated monolayer CACs is completely restored by combining alginate encapsulation with culture in standard medium supplemented with only PPS without the addition of known chondrocytic growth factors or maintaining the cells in culture for ≥ 4 weeks. The results demonstrated selective upregulation of type II collagen, aggrecan and PG deposition with complete suppression of type I and X collagen at mRNA level in the presence of PPS within 18-days of culture. These observed positive chondroinductive effects of PPS are in agreement with previous studies (Collier and Ghosh, 1989; Costesque *et al.*, 1986; Ghosh *et al.*, 2010; Weischer and Frey, 1978) and the inhibitory effects on type I and X collagen mRNA expression have also been previously reported in chondrogenic differentiated human MPCs in MMC (Ghosh *et al.*, 2010). Surprisingly, alginate encapsulation of CACs resulted in complete suppression of Sox-9 mRNA expression and significantly downregulated levels of Sox-9 protein expression which was only detectable at high cellular protein lysates load of 80 μ g. While Runx2 mRNA was significantly downregulated at PPS concentrations of 1 and 5 μ g/mL, by immunoblotting, there was no significant difference in Runx2 protein expression between the treatments. Nonetheless, the inhibitory effects of PPS on Runx2 mRNA expression observed in this study have also been previously reported in human MPCs (Ghosh *et al.*, 2010).

Intriguingly, in spite of using normoxic culture conditions, both HIF- α isoforms were detectable by immunoblotting, an indication of HIFs stabilization in 3D alginate beads culture. The stabilization of HIF-1 α protein expression under normoxia culture condition was previously reported in human normal and OA articular chondrocytes in suspension pellet culture (Coimbra *et al.*, 2004). However, the detection of both HIF- α isoforms at protein level in alginate beads culture is being reported for the first time. In agreement with previous studies, the study results also supports partial phenotype restoration of dedifferentiated monolayer chondrocytes cultured at high density in alginate beads. However, the addition of PPS was essential for the complete suppression of type I and X collagen gene expression. The outcome of this study confirms the working hypothesis and further verifies that PPS is indeed a suitable alternative novel chondroinductive factor to established growth factors like TGF- β that can induce not only chondrogenic differentiation of human MPCs as previously demonstrated by Ghosh and colleagues (2010), but can also promote the redifferentiation of dedifferentiated monolayer articular chondrocytes as verified by the results of this study.

The transcription factor Sox-9, is a key positive regulator of articular cartilage differentiation, chondrocyte proliferation, and transition to a non-mitotic hypertrophic state (Ikeda *et al.*, 2005; Leung *et al.*, 2011) that stimulates the transcription of type II collagen and aggrecan (Shen *et al.*, 2002). Alginate encapsulated P3 HACs after 4 weeks in 5% oxygen have been shown to fully regain type II collagen expression with aggrecan and Sox-9 levels exceeding encapsulated P0 chondrocytes levels cultured in 20% oxygen (Murphy and Polak, 2004). The induction of Sox-9 has also been demonstrated in dedifferentiated P5 HACs encapsulated in alginate beads in 7 days cultures (Caron *et al.*, 2012) and in adult P4 porcine chondrocytes cultured in alginate beads up to 4 weeks (Bernstein *et al.*, 2009). However,

earlier studies that investigated the use of alginate encapsulation to redifferentiate rabbit articular chondrocytes (Bonaventure *et al.*, 1994; Lemare *et al.*, 1998) including recent studies (Ab-Rahim *et al.*, 2013; Xu *et al.*, 2014) did not evaluate Sox-9 mRNA expression and therefore it is not clear whether the suppressive effects of alginate encapsulation of CACs on Sox-9 mRNA expression observed in this study also occurs in these chondrocytes. The observed induction of type II collagen in the absence of detectable Sox-9 mRNA strongly suggests that it may not be the key regulator of type II collagen promoter activity in CACs although in agreement with observations made by others (Aigner *et al.*, 2003; Takahashi *et al.*, 1998), it could still be involved at very low physiological levels in maintaining the chondrocyte phenotype. In support of the observed suppressive effects of alginate encapsulation of CACs on Sox-9 mRNA expression, Western blotting with 20 µg per lane of cellular protein lysates from alginate beads cultured CACs also showed no detectable Sox-9 protein compared to MMC chondrocytes that showed induced Sox-9 protein expression. Interestingly, Sox-9 protein was detected although at very low expression levels when the cellular protein lysates from alginate beads cultured CACs were loaded at 80 µg per lane, thus indicating that the transcriptional factor is still involved at very low ‘physiological’ levels in maintaining the chondrocyte phenotype. Moreover, these suppressive effects of alginate beads on Sox-9 mRNA expression were also observed in equine articular chondrocytes. While the exact molecular mechanism leading to the suppression of Sox-9 in alginate beads cannot be fully elucidated, it could be speculated that this could be due to the reported inhibitory effects of alginate on the chondroinductive factor, TGF-β and its ability to inhibit cell-to-cell interactions, a well-known chondroinductive factor that induces Sox-9 protein expression in MMC and pellet cultures as confirmed in this study. A previous review reported that TGF-β1 binds to alginate when

incubated in low pH environment by replacing Ca^{2+} , resulting in inactivation of the protein but this TGF- β 1-alginate bond is reversible when the beads are exposed to neutral conditions (pH 7.4), leading to the release of the active form of TGF- β 1 (Wee and Gombotz, 1998). This results in an altered cellular biochemical microenvironment in which cells are constantly exposed to varying levels of activated TGF- β 1 (Coleman *et al.*, 2007; Wee and Gombotz, 1998). Therefore, additional studies will be necessary to fully clarify the impact of unmodified alginate as a bioscaffold material for cartilage tissue engineering.

The transcription factor Runx2 drives the expression of type X collagen and stimulation of hypertrophy (Mueller and Tuan, 2008; Studer *et al.*, 2012). Therefore, the regulation of Runx2 expression is a potential target for preventing chondrocyte hypertrophy and promoting cartilage tissue formation. Although the mechanism by which PPS completely suppressed type X collagen mRNA was not fully elucidated in this study, ICC and colocalization analysis demonstrated that PPS colocalizes with Runx2 and may interfere with its nuclear translocation activity, suggesting that PPS may repress the promoter activity of Runx2 on type X collagen expression. In fact previous studies have suggested that PPS (Ghosh, 1999; Ghosh *et al.*, 2010; Sadhukhan *et al.*, 2002; Sunaga *et al.*, 2012) and other active GaGs (Campo *et al.*, 2009; Loeser *et al.*, 2009) exert their action through interaction with transcription factors subsequently repressing their promoter activity. PTHrP mRNA expression was also evaluated to verify whether the observed inhibition of type X collagen was due to PPS effects and not due to overexpression of PTHrP. PTHrP has been shown as one of the strong negative regulator that participates in a negative feedback loop with Indian Hedgehog (Ihh) to regulate chondrocyte hypertrophy by suppressing Ihh, a stimulatory factor of hypertrophy and chondrocyte proliferation that is regulated by Runx2 (Fischer *et al.*, 2010; Studer *et al.*, 2012).

However, PTHrP mRNA was undetectable in all alginate beads cultured CACs indicating that the observed inhibition of type X collagen was due to the effects of PPS. The non-detection of PTHrP could be due to limited cell-to-cell interactions in alginate culture since PTHrP mRNA has been shown to be continuously expressed in HACs pellet cultures (Fischer *et al.*, 2010) but low and undetectable in low cell density monolayer cultures (Pelosi *et al.*, 2013). PTHrP mRNA has been observed to be highly expressed in monolayer cultured CACs at high density but its expression is decreased at low density cultures and with passaging (unpublished observation). The results of this study taken together with findings of a previous study (Pelosi *et al.*, 2013) suggest that endogenous PTHrP is not involved in the regulation of chondrocyte hypertrophy.

In agreement with previous findings (Bohensky *et al.*, 2009; Thoms and Murphy, 2010), the results of this study demonstrate that HIF- α isoforms are normally expressed at mRNA level in cultured CACs. However, the most provocative finding of this study was the detection of HIF- α isoforms at the protein level under normoxia culture conditions, an indication of the HIFs stabilization. The detection of HIFs in this study could be strongly associated with reduced oxygen tension due to enhanced matrix proteins synthesis especially in the PPS treated beads. Chondrocytes in suspension cultures compared to monolayer cultures are assumed to experience differently low oxygen tension levels depending on the quantity of matrix deposited around them (Caron *et al.*, 2012; Coimbra *et al.*, 2004; Murphy and Sambanis, 2001). While there was no significant difference in HIF-2 α protein levels between the treatments, HIF-1 α protein was significantly stabilized and expressed at all PPS concentrations compared to the control. Previous studies have shown HIF-2 α as an anabolic factor that promotes a chondrocyte phenotype (Bohensky *et al.*, 2009; Lafont *et al.*, 2008,

2007; Murphy, 2010). HIF-2 α has been shown to be essential for hypoxic induction of the HACs phenotype at both the gene and protein level (Lafont *et al.*, 2007) by acting as a promoter of both Sox-9-dependent and independent factors important for key cartilage matrix synthesis (Lafont *et al.*, 2008, 2007). In fact, hypoxia has been shown to promote cartilage function by two complementary mechanisms involving the HIF- α isoforms in which HIF-2 α increases matrix tissue production and HIF-1 α inhibits cartilage destruction (Thoms *et al.*, 2013). The results of this study demonstrate for the first time that both HIF- α isoforms could be stabilized to detectable levels in CACs cultured in alginate beads under normoxic culture conditions and may therefore consequently accumulate in these cells translocating to the nucleus to activate the transcription of their target genes required for the survival and phenotype maintenance of CACs in what appears to be Sox-9-independent.

While the result of this study have a limited sample size of only three cartilages and the protocol was only evaluated in P1 chondrocytes, it is important to point out that this strategy was initially evaluated using cryopreserved P2 CACs that were alginate encapsulated from a solution of 2.0×10^6 cells/mL and cultured in 10% FBS DMEM supplemented with similar PPS concentrations. Similar suppressive effects of PPS on type I and X collagen gene expression were observed with the cells maintaining a chondrocyte phenotype. This suggests that the protocol is reproducible at least up to P2, hence more studies are required to evaluate it in higher passages (P3-P5) since ACT requires isolating chondrocytes from a small cartilage biopsy and passaging them several times to obtain a high cell population prior to implantation. This protocol could be further optimized by combining the observed chondroinductive effects of PPS with low physiological oxygen tension culture conditions to produce a phenotypically stable chondrocytes population. Furthermore, *in vivo* long-term animal model experiments will

be necessary to evaluate the ability of these constructs to maintain a chondrocyte phenotype and repair experimentally-induced articular cartilage defects with hyaline cartilage matrix.

The study presented in this chapter demonstrated for the first time that the phenotype of dedifferentiated monolayer CACs is completely restored by combining alginate beads with culture in standard medium supplemented with only PPS without the addition of established chondroinductive growth factors. While further studies will be required to fully elucidate the mechanism by which PPS restores the phenotype of chondrocytes, the findings of this study confirmed PPS as a novel suitable alternative chondroinductive factor to known chondrocytic growth factors that may offer a solution to the inherent challenges faced in cartilage tissue regeneration and repair of small OA cartilage lesions.

4 Study 3 Independent chondrogenic potential of cBMSCs in monolayer expansion culture decreases in a passage-dependent pattern

4.1 Summary

Although chondroinductive growth factors are considered necessary for chondrogenic differentiation of bone marrow-derived mesenchymal stem cells (BMSCs), independent and spontaneous chondrogenic differentiation has been previously demonstrated in adult horses, bovine calves and adult human BMSCs. Surprisingly, adult cBMSCs under similar culture conditions without dexamethasone and TGF- β previously failed to demonstrate a chondrogenic phenotype. However, it is not yet clear whether the failure of adult cBMSCs to exhibit independent chondrogenic potential is an indication of reduced chondrogenic potential related to extensive monolayer expansion cultures or specific to adult cBMSCs. Therefore, the present study was undertaken to clarify the findings of the previous study and to evaluate the independent chondrogenic potential of BMSCs sourced from young dogs in monolayer expansion cultures in the absence of known chondroinductive factors. BMSCs from three young dogs were expanded in monolayer culture up to P3 in 10% FBS-supplemented DMEM. At each passage (P0-P3), cBMSCs were evaluated by RT-PCR gel electrophoresis and qPCR for the expression of cartilage-specific gene markers, Sox-9, type II collagen and aggrecan, with type I and X collagen as chondrocyte dedifferentiation and hypertrophy gene markers, respectively, and HIF-2 α . cBMSCs exhibited a chondrogenic phenotype in the absence of

dexamethasone and TGF- β 1 previously shown to be necessary as verified by the expression of Sox-9, type II collagen and aggrecan. Sox-9 was significantly downregulated ($P < 0.05$) from P1-P3 compared to P0 while type II and X collagen, and aggrecan were significantly downregulated at P3 compared to P0. There was a significant ($P < 0.01$) negative correlation between passage number and Sox-9, type II collagen and aggrecan gene expression. Type I collagen was highly expressed after P0 culture but there was no significant difference ($P > 0.05$) between passages. Similarly, HIF-2 α showed no significant difference between passages. The results of this study demonstrated that the independent chondrogenic potential and phenotype retention of BMSCs decreases in a passage-dependent pattern and therefore caution should be exercised for future experiments evaluating the chondrogenic potential of BMSCs after extensive expansion cultures in standard growth medium.

4.2 Introduction

Currently, the clinical use of mesenchymal stem cells (MSCs) in veterinary medicine is in its early stages for the treatment of tendon, ligament, or OA cartilage lesions in horses or dogs (Fortier and Travis, 2011). The use of cBMSCs offers a significant promise as a multipotent source for cell-based therapies and could form the basis for the differentiation and cultivation of cartilage tissue grafts to regenerate and repair OA joint in dogs (Csaki *et al.*, 2007). In general, BMSCs have potential for extensive monolayer expansion *in vitro* and can undergo chondrogenesis when cultured with growth medium supplemented with different growth factors such as TGF- β , fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and bone morphogenetic proteins (BMP) (Bosnakovski *et al.*, 2004; Csaki *et al.*, 2007; Athanasiou *et al.*, 2013; Liao *et al.*, 2012; Majumdar *et al.*, 2001; Mwale *et al.*, 2006; O'Driscoll *et al.*, 1994; Solchaga *et al.*, 2010; Worster *et al.*, 2000). Although these growth factors are considered necessary, spontaneous independent chondrogenic differentiation of BMSCs derived from adult horses (Fortier *et al.*, 1998), bovine calves (Bosnakovski *et al.*, 2004; Bosnakovski *et al.*, 2006) and human (Ghosh *et al.*, 2010) has been demonstrated in the absence of these factors. BMSCs derived from adult horses were previously shown to exhibit a chondrogenic phenotype in monolayer culture system (Fortier *et al.*, 1998) whereas those derived from bovine calves (Bosnakovski *et al.*, 2004; Bosnakovski *et al.*, 2006) and human (Ghosh *et al.*, 2010) used the micromass/pellet culture system which is well-known to promote differentiation of MSCs towards the chondrocytic lineage by promoting strong cell-to-cell interaction because of contact mediated signalling, formation of junctional complexes and increased potential for exchange of homocrine factors. Surprisingly, BMSCs from adult dogs

failed to demonstrate chondrogenic phenotype in chondrogenic induction medium without dexamethasone and TGF- β or when cells were cultured in standard medium containing 10% fetal calf serum (FCS) in micromass/pellet culture or high density culture (Csaki *et al.*, 2007). However, it is not yet clear whether the failure of adult cBMSCs to undergo independent chondrogenic differentiation despite using MMC is an indication of reduced chondrogenic potential related to extensive monolayer expansion cultures or is specific to adult cBMSCs.

Therefore, the present study was undertaken to clarify the findings of the previous study and to evaluate the independent chondrogenic potential of BMSCs sourced from young dogs in monolayer expansion cultures in the absence of known chondroinductive factors. In the present study, it was hypothesized that cBMSCs undergo independent chondrogenic differentiation in monolayer cultures without exogenous supplementation with known chondroinductive factors previously considered necessary but the chondrogenic potential and phenotype retention significantly decreases in a passage-dependent pattern with extensive expansion.

4.3 Materials and Methods

4.3.1 Collection site and dog preparation

The study was performed using cBMSCs aseptically collected by the proximal femur approach from femoral bone marrow of three experimental Beagle dogs (mean age: 11.5 months; range: 11-12 months). The use of experimental dogs was in accordance with Hokkaido University Institutional Animal Care and Use Committee guidelines (approval number: 12-0059). Briefly, dogs were put under general anaesthesia (GA) using propofol at 6 mg/kg intravenously for induction and maintained on isoflurane and oxygen. The limb from where the bone marrow aspirate was collected was aseptically prepared by clipping the hair around the proposed site of collection, scrubbed with povidone iodine and then 70% ethanol applied to further disinfect the site.

4.3.2 cBMSCs isolation and culture

A modification of the protocol previously described by Soleimani and Nadri (2009) for isolation and culture of MSCs was used with slight modification which has been confirmed to obtain a purified population of MSCs with mesenchymal lineages differentiation ability. Briefly, mononuclear cells (MC) were collected by gradient centrifugation using a modification of LymphoprepTM protocol (1.077 ± 0.001 g/mL) (Axis Shield POC, Oslo, Norway). After centrifugation, MC were collected from the sample/medium interface and plated in polystyrene culture plates containing DMEM (GIBCO) supplemented with 10% FBS (Nichirei), 100 IU/mL of penicillin and 0.1 mg/mL of streptomycin and incubated at 37°C in 5% CO₂ in a humidified chamber without disturbing the plates for 4 days. Thereafter, non-adherent cells

were removed and discarded whereas the adherent cells were gently washed with 1 x PBS and fresh medium was added to the culture plates. Medium was changed every 48 hr until the cells reached about 80-90% confluence. At confluence, P0 monolayer colony forming unit cells (CFU-C) were gently washed twice with 1 x PBS and detached using pre-warmed TrypLE™ Select CTS™ (GIBCO). Cells were passaged up to P3 in 100 mm culture plates at seeding density of 1×10^4 cells/cm² per subculture. All subcultures were allowed to reach confluence of 80-90% before passaging. At each passage (P0-P3), 1×10^6 cells were collected and total RNA was extracted using TRIZol® Kit (Invitrogen), according to the manufacturer's instructions to evaluate the expression of chondrogenic phenotype genes. Cell viability and total cell density were determined using 0.5% Trypan blue stain and manual haemocytometer cell counting method. The cells morphology and proliferation characterization was determined using a light microscope. The isolated cells were confirmed to exhibit stemness character based on their ability to adhere to plastic culture plate surface, morphology, non-expression of CD34 and CD45, and ability to differentiate upon treatment with specific-lineage induction medium into chondrogenic, adipogenic and osteogenic lineage as verified by Alcian blue, Oil red O and Alizarin red S staining, respectively. Chondrogenic, adipogenic and osteogenic differentiation of cBMSCs was performed based on the validated protocol previously described for isolation and culture of MSCs (Zhu *et al.*, 2010) and a study that previously characterized multilineage differentiation potential of adult cBMSCs (Csaki *et al.*, 2007).

4.3.3 Multilineage differentiation potential of cBMSCs

Briefly, multilineage differentiation assessment was performed in monolayer culture by plating 2×10^4 P1 cells/well in 6-well culture plates in duplicate and culturing them to 100%

confluence in 10% FBS DMEM before induction. For chondrogenic differentiation, cells were cultured in commercial complete chondrogenic differentiation medium (CDM) (StemPro® Chondrogenesis Differentiation Kit, GIBCO BRL, Cat. #: A10071-01). The medium was changed every 72 hr and cells were cultured for 2 weeks (14 days). For adipogenic differentiation, confluent cells were also cultured for 14 days. The cells were treated for 72 hr with adipogenic induction medium then with adipogenic maintenance medium for 24 hr. The induction/maintenance cycle was repeated up to 14 days of culture. Adipogenic induction medium constituted of 10% FBS DMEM supplemented with 10^{-6} M dexamethasone (Sigma-Aldrich, Cat. #: D4902), 1 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich, Cat. #: 15879), 200 μ M Indomethacin (Wako Pure Chemical Industries Ltd, Cat. #: 093-02473) and 10 μ g/mL insulin (TaKaRa, Kit code: MK429) while adipogenic maintenance medium contained 10% FBS DMEM supplemented with 10 μ g/mL insulin (TaKaRa, Kit code: MK429). For osteogenic differentiation, confluent cells were treated in 10% FBS DMEM supplemented with 10^{-7} M dexamethasone (Sigma-Aldrich, Cat. #: D4902), 10 mM β -glycerophosphate disodium (Sigma-Aldrich, Cat. #: G5422) and 50 μ M ascorbic acid (Sigma-Aldrich, Cat. #: A4403) with medium changes done every 72 hr up to 14 days. Cells cultured in 10% FBS DMEM were used as a negative control. After 14 days of differentiation, medium was removed and cells assessed with lineage-specific stain. Chondrogenic-induced cells were stained with Alcian blue for PG deposition, adipogenic-induced cells by Oil red O for accumulation of lipid-rich vacuoles in the cells, and osteogenic-induced cells were stained by Alizarin Red S for calcium deposition into the ECM.

4.3.4 Chondrogenic phenotype analysis of monolayer passaged cBMSCs

Total RNA was quantified by spectrophotometry at 260 nm as previously described. Total of 500 ng RNA was reverse transcribed into cDNA using M-MLV RT kit (Invitrogen) and amplified by PCR using TaKaRa Ex taq (TaKaRa) according to manufacturer's recommended protocol. This technique was employed to detect mRNAs specific for Sox-9, type II collagen and aggrecan as cartilage-specific gene markers, type I and X collagen as chondrocyte dedifferentiation and hypertrophy gene markers, respectively, and HIF-2 α . The PCR conditions for RT-PCR and qPCR were as described previously. All target genes expression were normalised against the reference gene, GAPDH. The sequences, product size and accession codes for each primer used for the experiment are same as those indicated in Table 3.

4.4 Data analysis

Quantitative data was analyzed using SPSS Version 16.0. ANOVA was used to compare relative gene expression between passages. Where significant difference was observed, *Post Hoc* multiple comparisons were performed with Bonferroni. Bivariate correlation analysis was performed to model the relationship between passaging and relative gene expression. Significant difference was defined as $P < 0.05$.

4.5 Results

4.5.1 Multilineage, morphology and proliferation characterization of cBMSCs

On average, P0 cultures reached 80-90% confluence within 11 ± 2 day (mean \pm SD). The isolated cells exhibited mesenchymal stemness as verified by their lack of expression of CD34 and CD45 by immunofluorescence, an indication of no contamination from haematopoietic stem cells, and also they demonstrated multilineage differentiation ability into chondrogenic, adipogenic and osteogenic lineage as verified by Alcian blue, Oil red O, and Alizarin red S positive stain, respectively (Figure 16).

Subsequent passages (P1-P3) from all three dogs were able to reach confluence within 8 ± 2 days of passaging, indicating a high proliferation rate. Cell viability as determined using Trypan blue exclusion stain indicated viability of $>95\%$ at all passages. The morphological events of cBMSCs as observed using a light microscope demonstrated the appearance of adherent elongated large, spindlyoid fibroblastic-like cells on the surface of the culture plates with many non-adherent cells on day 4 of P0 culture (Figure 17B). Following removal of non-adherent cells and medium change, there was a rapid morphological change of cells to small rounder to bipolar spindle-shaped cells that established colony forming unit-fibroblasts (CFU-F) and proliferated in circular patches (Figure 17C). Subsequent passages (P1-P3) were characterized by bipolar to polygonal spindle-shaped cells with notably reduced CFU-F ability especially from P2-P3 when compared to P0 (Figure 17D-17F).

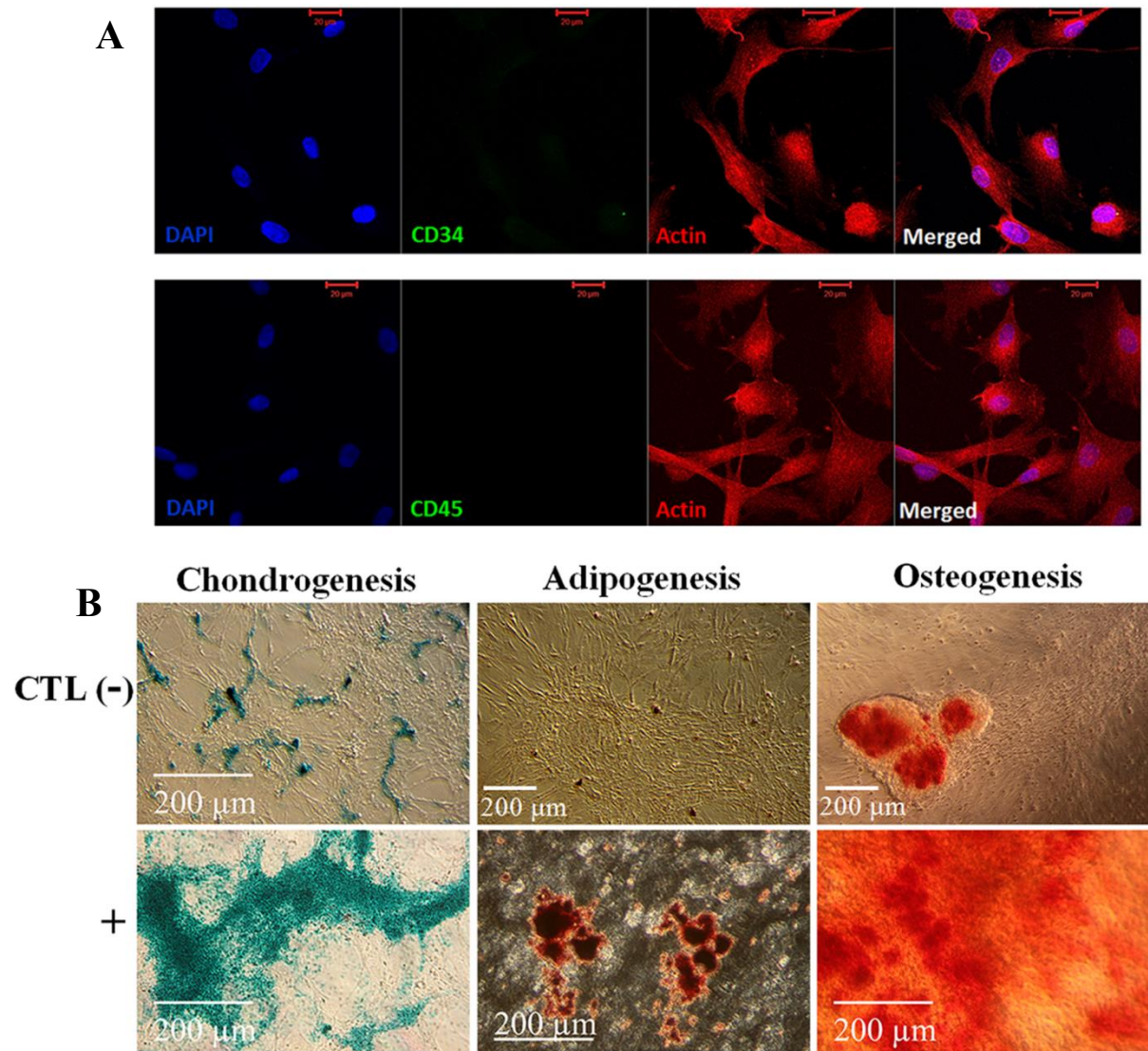


Figure 16 Surface markers evaluation and multilineage differentiation potential of cBMSCs cultured in lineage-specific induction medium.

(A) Immunofluorescence staining of P1 cBMSCs showed that the cells lacked hematopoietic stem cell markers CD34 and CD45. (B) The cells differentiated into chondrogenic lineage cells (lower left) stained with Alcian blue, adipogenic lineage cells (lower middle) stained with Oil red O, and osteogenic (calcium depositing) lineage cells (lower right) stained with Alizarin red S. Control chondrogenic (upper left) and osteogenic cells (upper right) showed some limited lineage differentiation ability compared to adipogenic control cells that all stained negative. Cytoskeleton was stained with rhodamine phalloidin (Thermo Fisher Scientific). **Scale Bars indicated in images.**

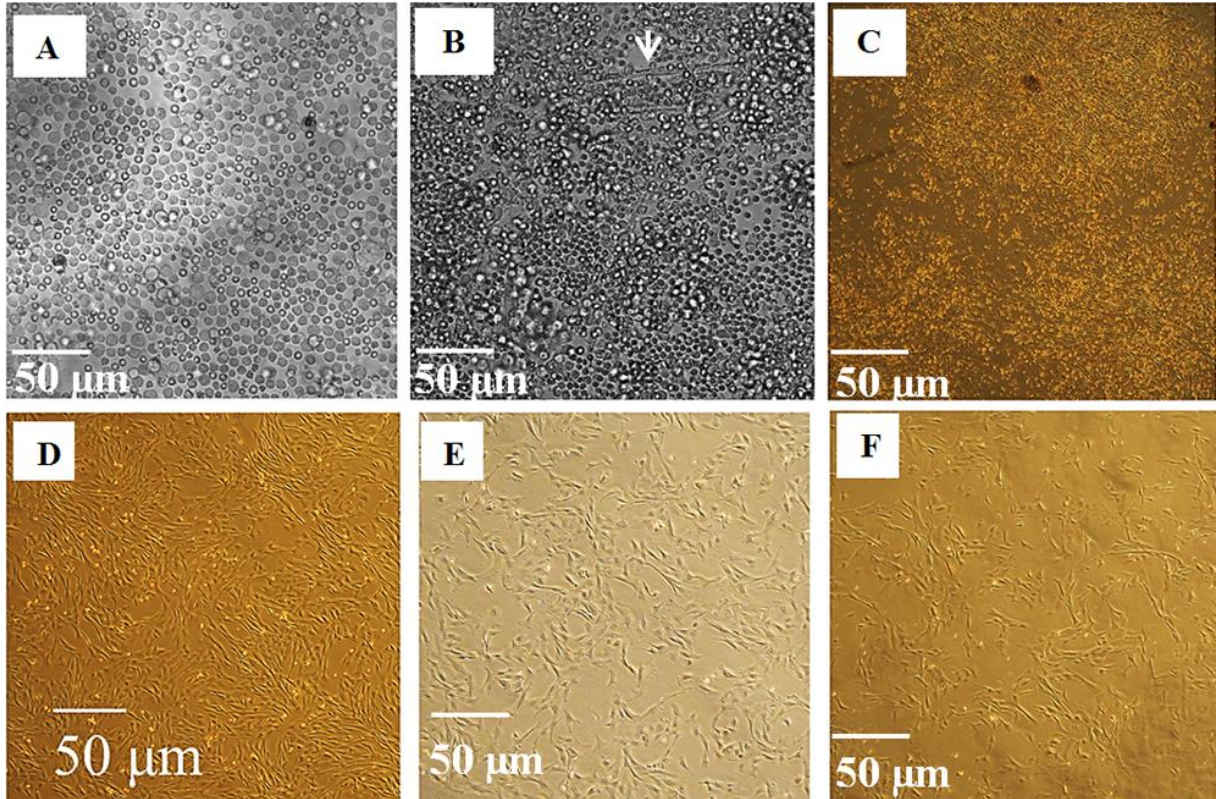


Figure 17 Photomicrographs of monolayer cultured cBMSCs maintained in 10% FBS DMEM.

(A) Day 1, primary culture (P0) showing non-adherent mononuclear cells immediately following seeding, (B) Day 4, P0 culture showing appearance of adherent elongated spindle-shaped fibroblastic-like cells just before removing non-adherent cells, (C) Day 10, P0 culture showing circular patches of small bipolar colony forming unit-fibroblasts (CFU-F), (D) Day 4, first passage (P1) culture showing bipolar to polygonal shaped cells, (E) Day 5, second passage (P2) and (F) Day 6, third passage (P3) showing larger polygonal shaped cells with reduced CFU-F formation. **Scale Bars: 50 µm.**

4.5.2 Chondrogenic potential of cBMSCs in monolayer culture in the absence of chondroinductive factors

cBMSCs demonstrated a chondrogenic phenotype at all passages (P0-P3) as verified by the expression of cartilage-specific gene markers, Sox-9, type II collagen and aggrecan using RT-PCR gel electrophoresis (Figure 18). Notably, there was a passage-dependent decrease in cells retention of a chondrogenic phenotype as demonstrated by decreased Sox-9, type II collagen and aggrecan mRNA bands expression especially for Sox-9 which was barely detectable in some passages albeit cells continuously expressing distinct type II collagen and aggrecan genes (Figure 18). The observed passage-dependent decrease in chondrogenic potential of cBMSCs appeared to be related to the reduced CFU-F formation from P1-P3. Although cBMSCs demonstrated a chondrogenic phenotype, they concomitantly expressed type I and X collagen genes, with the former being predominantly expressed at all passages compared to other genes while the latter demonstrated a passage-dependent downregulation. HIF-2 α gene was expressed at all passages and the band intensity appeared to be enhanced with subsequent passaging (Figure 18).

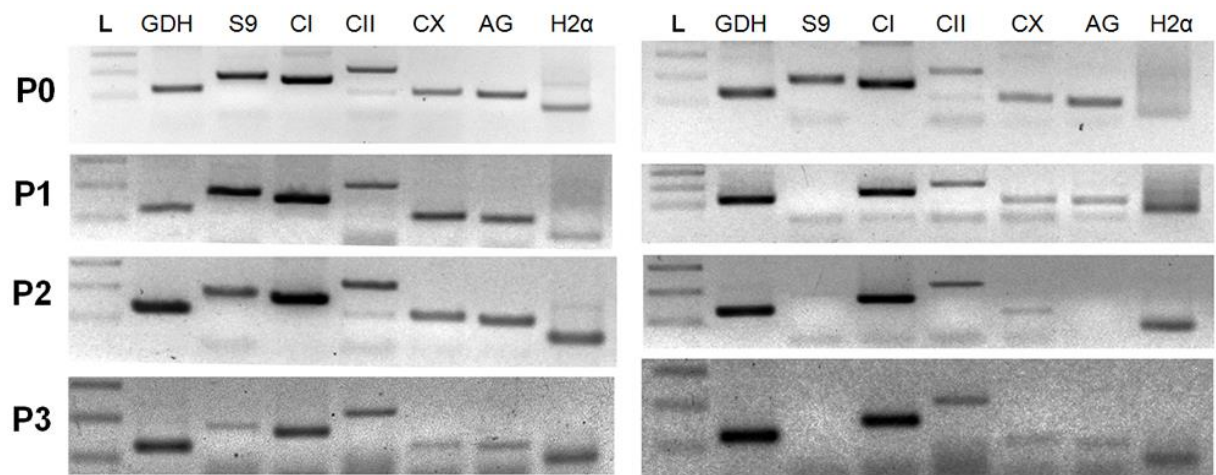


Figure 18 Expression of chondrogenic specific-gene markers in monolayer cultured cBMSCs.

Sox-9 (S9), type II collagen (CII) and aggrecan (AG), dedifferentiation gene marker, type I collagen (CI), chondrocyte hypertrophy gene marker, type X collagen (CX) and hypoxia inducible factor-2α (H2α) were evaluated by RT-PCR gel electrophoresis in monolayer cultured canine bone marrow-derived mesenchymal stem cells (cBMSC). RNA was collected from monolayer expansion cultures of cBMSC at every passage from P0 to P3. Results represent two experiments of three independent experiments for each passage evaluated. L; 100bp DNA ladder.

To verify and quantify the findings of RT-PCR gel electrophoresis, qPCR was performed. Sox-9, type II collagen and aggrecan were all downregulated in a passage-dependent pattern (Figure 19). However, compared to P0, only Sox-9 was significantly downregulated ($P < 0.05$) at all passages (P1-P3) while type II collagen was only significantly downregulated at P3 compared to P0 whereas aggrecan was significantly downregulated at P2-P3 compared to P0. Type I collagen expression was markedly upregulated from P1-P3 compared to P0 but there was no significant difference ($P > 0.05$) between the passages (Figure 19). Interestingly, type X collagen expression followed a similar pattern to cartilage-specific genes and was significantly downregulated ($P < 0.05$) at P3 compared to P0. There was no significant difference in HIF-2 α gene expression between the passages although it showed a passage-dependent increase from P1-P3 (Figure 19).

Bivariate correlation analysis demonstrated a significant negative correlation between passage number and Sox-9 (Pearson Correlation = -0.802, $P = 0.002$), passage number and type II collagen (Pearson Correlation = -0.789, $P = 0.002$), passage number and type X collagen (Pearson Correlation = -0.599, $P = 0.04$) and, passage number and aggrecan (Pearson Correlation = -0.754, $P = 0.005$). Type I collagen (Pearson Correlation = 0.566, $P = 0.055$) and HIF-2 α (Pearson Correlation = 0.157, $P = 0.626$) showed a non-significant positive correlation with passage number (Table 4).

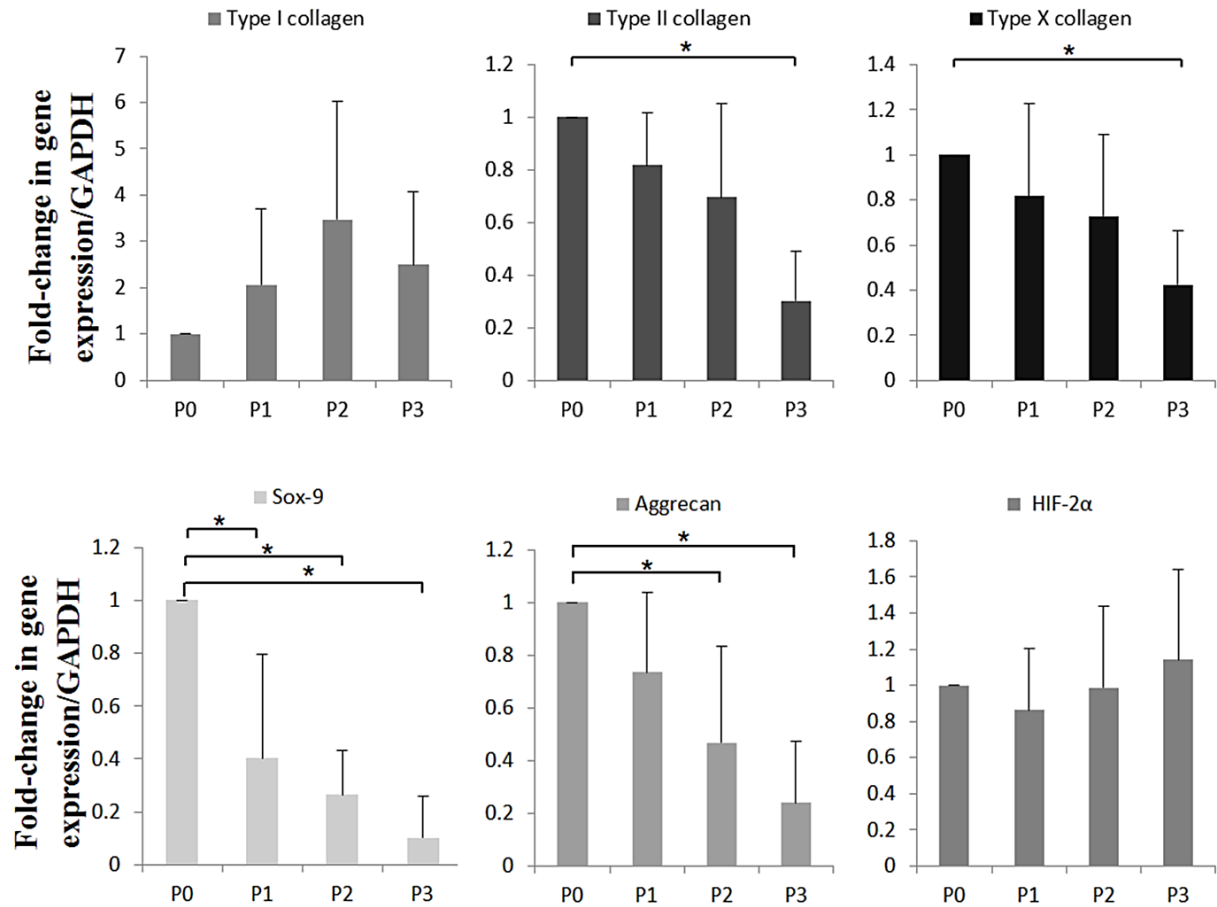


Figure 19 Bar graphs showing the fold-changes in gene expression between passages of cBMSCs monolayer expansion cultures.

Gene expression for Sox-9, type I, II and X collagen, aggrecan, and hypoxia inducible factor-2alpha (HIF-2α) were compared between passages with the primary culture (P0) as the calibrator to which fold-changes between the passages were determined. Gene expressions were normalized with the reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All fold-changes in gene expression are expressed as mean ± 95% CI (confidence interval) of three independent experiments. Significant difference was defined as $*P < 0.05$.

Table 4 Bivariate correlation analysis between passaging and gene expression of cBMSCs in monolayer expansion cultures

		Passage	Sox-9	Type I collagen	Type II collagen	Type X collagen	Aggrecan	HIF-2 α
Passage	Pearson							
	Correlation	1	-0.802**	0.566	-0.789**	-0.599*	-0.754**	0.157
	Sig. (2-tailed)		0.002	0.055	0.002	0.04	0.005	0.626
	N	12	12	12	12	12	12	12
Sox-9	Pearson	-						
	Correlation	0.802**	1	-0.769**	0.659*	0.32	0.399	-0.341
	Sig. (2-tailed)	0.002		0.003	0.02	0.31	0.199	0.278
	N	12	12	12	12	12	12	12
Type I collagen	Pearson							
	Correlation	0.566	-0.769**	1	-0.213	-0.324	-0.401	-0.179
	Sig. (2-tailed)	0.055	0.003		0.507	0.304	0.197	0.577
	N	12	12	12	12	12	12	12
Type II collagen	Pearson	-						
	Correlation	0.789**	0.659*	-0.213	1	0.192	0.433	-0.524
	Sig. (2-tailed)	0.002	0.02	0.507		0.55	0.16	0.08
	N	12	12	12	12	12	12	12
Type X collagen	Pearson							
	Correlation	-0.599*	0.32	-0.324	0.192	1	0.684*	0.166
	Sig. (2-tailed)	0.04	0.31	0.304	0.55		0.014	0.607
	N	12	12	12	12	12	12	12
Aggrecan	Pearson	-						
	Correlation	0.754**	0.399	-0.401	0.433	0.684*	1	0.361
	Sig. (2-tailed)	0.005	0.199	0.197	0.16	0.014		0.249
	N	12	12	12	12	12	12	12
HIF-2 α	Pearson							
	Correlation	0.157	-0.341	-0.179	-0.524	0.166	0.361	1
	Sig. (2-tailed)	0.626	0.278	0.577	0.08	0.607	0.249	
	N	12	12	12	12	12	12	12

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

4.6 Discussion

The results of the study presented in this chapter demonstrated that cBMSCs have the potential to undergo independent chondrogenesis in monolayer cultures in the absence of dexamethasone and TGF- β 1 previously shown to be necessary for chondrogenic differentiation of adult cBMSCs (Csaki *et al.*, 2007). The cells did not express CD34 and CD45 hematopoietic stem cell surface markers and were confirmed to have MSCs character based on their ability to adhere to plastic culture plate under standard culture conditions and differentiate into chondrogenic, adipogenic, and osteogenic lineages *in vitro* as per previously defined criterion (Dominici *et al.*, 2006). Morphologically, from P0-P3, cBMSCs progressed from being elongated spindle-shaped, to small bipolar cells then to large polygonal fibroblastic-like cells, a characteristic morphology similarly described in other studies (Bosnakovski *et al.*, 2004; Csaki *et al.*, 2007; Fortier *et al.*, 1998; Kadiyala *et al.*, 1997). In this study, cBMSCs demonstrated independent chondrogenic differentiation within 8 (\pm 2) days of monolayer culture as verified by the expression of cartilage-specific gene markers, Sox-9, type II collagen and aggrecan. The independent chondrogenic potential and retention of chondrogenic phenotype of cBMSCs significantly decreased in a passage-dependent pattern marked by decreased expression of Sox-9, type II collagen and aggrecan mRNA with highly expressed type I collagen. The observed concomitant expression of type I and X collagen is an indication of loss of spatiotemporal chondrogenic differentiation signals in cBMSCs during *in vitro* cultures which results in formation of a fibrocartilage-like phenotype instead of a hyaline cartilage phenotype.

Chondrogenesis *in vivo* is characterized by condensation of mesenchyme cells that enlarges through appositional growth by recruiting the cells from the outer layer, which

differentiate into chondroblasts and begins secreting the molecules that form the ECM (Cancedda *et al.*, 1995; Athanasiou *et al.*, 2013; Studer *et al.*, 2012). However, in the present study, condensing of cells using pellet culture or MMC was not used instead the potential of cBMSCs to undergo independent chondrogenesis was evaluated in monolayer expansion cultures. There was a notable passage-dependent decrease in cells ability to form distinct CFU-F as they proliferated mostly as large polygonal-shaped fibroblastic-like cells especially from P2-P3 and this appeared to correlate with the reduced chondrogenic potential. Independent chondrogenic differentiation of BMSCs from adult horses has been previously reported in monolayer culture with medium supplemented with only 10% FBS and was verified by a chondrocytic phenotype shift in expression from type I to type II collagen, and an increase in quantity and molecular size of PGs synthesized over time (Fortier *et al.*, 1998). Surprisingly, a study using cBMSCs from adult dogs showed no expression of type II collagen and cartilage-specific PG in high density monolayer culture in chondrogenic induction medium without TGF- β 1 or in cell culture medium containing 10% FCS (Csaki *et al.*, 2007). To the contrary, this study demonstrates that cBMSCs have the ability to undergo independent chondrogenic differentiation but the chondrogenic potential and phenotype retention decreases significantly in a passage-dependent pattern as demonstrated by decreased expression of Sox-9, type II collagen and aggrecan with increased expression of type I collagen. Taken together, this significant negative correlation between passaging and chondrogenic potential observed in this study may account for the reported failure of cBMSCs from adult dogs to exhibit chondrogenesis in the absence of dexamethasone and TGF- β 1, in which cells were passaged 3 to 4 times prior to differentiation (Csaki *et al.*, 2007). Previously, BMSCs from bovine calves culture expanded up to P2 were shown to undergo independent chondrogenesis in pellet culture

and during the process chondrogenic growth factors like TGF- β 1 and β 2, BMP-6, and FGF-2 showed remarkable influence in an autocrine and/or paracrine manner (Bosnakovski *et al.*, 2006, 2004). To the contrary, another study using BMSCs from bovine calves expanded up to P4 prior to differentiation failed to exhibit a chondrogenic phenotype (Mauck *et al.*, 2006), an indication that independent chondrogenic potential of BMSCs reduces with extensive passaging. In providing further support to the findings of this study, the chondrogenic potential of human MSCs has also been shown to be vulnerable to monolayer expansion cultures. Human adult BMSCs expanded in standard growth medium (DMEM-LG + 10% FBS) supplemented with FGF-2 prior to differentiation in aggregate cultures in chondrogenic medium supplemented with dexamethasone and TGF- β 1 exhibited chondrogenic differentiation at all passages tested compared to cells that were expanded in only standard growth medium prior to differentiation in aggregate cultures that differentiated along the chondrogenic lineage after P1 but exhibited only marginal differentiation after P4 and failed to form cartilage after P7 (Solchaga *et al.*, 2010). Taken together, these findings suggest that caution be taken when evaluating the *in vitro* chondrogenic potential of MSCs intended for cartilage tissue regeneration and repair of OA cartilage because their chondrogenic potential reduces significantly with extensive expansion cultures in standard growth medium.

Sox-9 is a known positive regulator of articular cartilage differentiation, chondrocyte proliferation, and transition to a non-mitotic hypertrophic state and is necessary for chondrogenic differentiation both before and after mesenchymal condensations (Ikeda *et al.*, 2005; Leung *et al.*, 2011). It stimulates transcription of a number of genes characteristic for the articular cartilage matrix, including type II collagen, and it is expressed predominantly in mesenchymal condensations throughout the embryo before and during the deposition of

cartilage (Shen *et al.*, 2002). In this study, Sox-9 gene was highly expressed in P0 cBMSCs and this was paralleled with increased expressional levels of type II collagen, aggrecan and type X collagen. Although Sox-9 is essential for the induction and maintenance of chondrocyte phenotype differentiation, it seems other factors may be involved in regulating the promoter activity of type II collagen and aggrecan and this is in agreement with observations made elsewhere (Takahashi *et al.*, 1998). Distinct type II collagen and aggrecan gene expression were observed in some cultures where Sox-9 was barely detectable. Nonetheless, the results overall demonstrated a significant positive correlation between Sox-9 and type II collagen gene expression but not between Sox-9 and aggrecan gene expression.

The expression of type I collagen is normally considered as a premature cartilage marker during cartilage differentiation *in vivo* which at a later stage of chondrification is then replaced by type II collagen as the differentiation of MSCs proceeds (Karlsson *et al.*, 2007). However, type I collagen is also associated with *in vitro* dedifferentiation of monolayer cultured articular chondrocytes (Freshney, 2010; Hamada *et al.*, 2013; Murphy and Sambanis, 2001). A non-significant positive correlation was observed between passage number and type I collagen expression with a significant negative correlation observed between passage number and type II collagen expression, an indication of a major shift to a fibroblastic-like phenotype. This phenotype shift is similar to findings by other studies that have evaluated chondrogenic differentiation of MSCs (Bosnakovski *et al.*, 2004; Ghosh *et al.*, 2010; Karlsson *et al.*, 2007; Mwale *et al.*, 2006) which makes monolayer culture system for chondrogenic differentiation undesirable for cartilage tissue engineering.

Similar to the results of this study, the expression of type X collagen during *in vitro* chondrogenic differentiation of MSCs has been previously reported by others (Adesida *et al.*,

2012; Ghosh *et al.*, 2010; Mwale *et al.*, 2006; Studer *et al.*, 2012; Weiss *et al.*, 2010). Interestingly, type X collagen showed a significant negative correlation with passage number and a positive significant correlation with aggrecan gene expression. This aberrant expression of type X collagen during early stages of BMSCs chondrogenic differentiation has previously raised questions on its use as a marker for chondrogenesis and chondrocyte hypertrophy of BMSCs differentiation (Mwale *et al.*, 2006). Hypertrophic chondrocytes secrete type X collagen which establishes the framework for subsequent calcification and endochondral ossification (Narcisi *et al.*, 2012; Studer *et al.*, 2012). In articular cartilage chondrocytes, the expression of type X collagen indicates the terminal stage of chondrocyte differentiation and is characterized by increase in cell volume, ECM remodelling and expression of hypertrophy related factors including Runx2, alkaline phosphatase (ALP) and Ihh (Mueller and Tuan, 2008; Studer *et al.*, 2012). While Sox-9 is a suggested negative regulator of type X collagen with its over-expression reported to significantly downregulate expression of type X collagen (Leung *et al.*, 2011), a non-significant positive correlation between Sox-9 and type X collagen mRNA expression was observed in the present study. In immature chondrocytes, there is restricted and reciprocal expression of the collagen X gene in hypertrophic chondrocytes and Sox-9 which epitomise the precise spatiotemporal control of gene expression *in vivo* as chondrocytes progress through phases of differentiation (Leung *et al.*, 2011). However, during *in vitro* chondrogenesis as observed in this study and other studies, the critical spatiotemporal cues observed *in vivo* are not present and the majority of the MSCs population continues to express both type II and X collagen concomitantly (Studer *et al.*, 2012). This *in vitro* aberration in MSCs has been partly attributed to altered DNA methylation status at 2 CpG sites of type X collagen. Methylation-based type X collagen gene silencing is established in cartilage tissue

and HACs but altered in MSCs at 2 CpG sites and their demethylation during *in vitro* chondrogenesis may facilitate induction of type X collagen (Zimmermann *et al.*, 2008).

Lastly, the expression of HIF-2 α mRNA in monolayer cultured cBMSCs under normoxia conditions was evaluated. HIF-2 α mRNA was expressed in all culture passages with no significant difference between the passages or significant correlation with passaging. Increased expression of HIF-2 α mRNA has been reported to improve chondrogenic differentiation of human BMSCs and stem cells derived from the infrapatellar fat pad under hypoxic conditions (Adesida *et al.*, 2012; Khan *et al.*, 2007). Therefore, the results of this study may reflect its important role during and after chondrogenic differentiation.

The present study is limited by lack of protein expression analysis to verify whether the observed phenotype based on mRNA expression correlates with the active protein synthesis by these BMSCs. Nonetheless, whether the observed mRNA transcription of the selected genes evaluated does not result in translation of the active proteins, the results clearly demonstrate the passage-dependent epigenetic changes that occur in cBMSCs in monolayer expansion cultures.

In summary, the study presented in this chapter demonstrated that cBMSCs exhibit independent chondrogenic differentiation potential in monolayer expansion cultures in the absence of dexamethasone and TGF- β 1 previously shown to be necessary. The findings of the study confirm that independent chondrogenic potential and chondrogenic phenotype retention of cBMSCs in monolayer expansion cultures in standard growth medium of 10% FBS DMEM significantly decreases in a passage-dependent pattern. Therefore, caution should be exercised when evaluating the chondrogenic potential of BMSCs intended for cartilage tissue regeneration and repair of OA defects especially after extensive expansion cultures in 10% FBS DMEM.

5 Study 4 Effects of PPS and PSGAG on chondrogenesis of cBMSCs in alginate and micromass culture

5.1 Summary

Mesenchymal stem cells (MSCs) are a potential alternative source of differentiated chondrocytes for cartilage tissue regeneration and repair of isolated OA cartilage lesions. In this study, the effects of PPS and PSGAG on chondrogenesis of cBMSCs in alginate culture and MMC were investigated with a view of improving *in vitro* chondrogenic differentiation culture conditions. CDM was supplemented with PPS or PSGAG at concentrations of 0 (positive control; PC), 1, 3 and 5 µg/mL. cBMSCs cultured in 10% DMEM were used as negative control (NC). Chondrogenic lineage phenotype was analyzed by qPCR for alginate cultures and Alcian blue staining for PG synthesis was used for MMC. Surprisingly, in alginate culture, both PPS and PSGAG showed no significant effect on type II collagen, aggrecan and HIF-2α mRNA expression and thus both did not promote chondrogenesis in alginate culture. PPS had no significant effect on type I collagen whereas PSGAG significantly upregulated ($P < 0.05$) type I collagen at all concentrations relative to other treatments. Interestingly, PPS demonstrated a dose-dependent inhibitory effect on type X collagen mRNA with significant inhibition observed at 5 µg/mL compared to the NC. On the other hand, PSGAG showed an inverse effect on type X collagen with 1 µg/mL significantly inhibiting its expression while increase in the concentration correspondingly increased type X collagen expression. Contrary to the observations in alginate cultures, in MMC, PPS synergistically enhanced chondrogenesis and PG deposition whereas PSGAG inhibited chondrogenesis and promoted a fibrocartilage-

like phenotype with reduced PG deposition. The findings of this study demonstrated that PPS synergistically enhances chondrogenesis of cBMSCs in MMC but showed no significant effect in alginate cultures, suggesting that the response of cBMSCs to chondroinductive factors is culture system-dependent and varies significantly between alginate beads culture and MMC. This finding has serious implications as it cautions that the response of MSCs to different culture systems be considered prior to *in vitro* cartilage tissue engineering efforts intended for regeneration and repair of OA cartilage lesions.

5.2 Introduction

Pluripotent MSCs have potential for extensive monolayer expansion *in vitro* cultures and can be differentiated into a variant of cells under specific-lineage inductive medium and culture conditions (Ghosh *et al.*, 2010; Caskie *et al.*, 2007; Bosnakovski *et al.*, 2004). These undifferentiated cells can differentiate into different generative cells such as bone, cartilage, adipose, tendon and other cells of the mesenchymal lineage under appropriate stimuli. Therefore, MSCs are a potential alternative source of differentiated chondrocytes from different sources including bone marrow-derived MPCs for cartilage tissue regeneration and repair of isolated OA cartilage lesions (Ghosh *et al.*, 2010; Caskie *et al.*, 2007). Regrettably, previous studies have revealed that *in vitro* chondrogenic differentiated MSCs just like monolayer cultured articular chondrocytes concomitantly express the undesirable dedifferentiation and hypertrophic markers, thus raising questions on their suitability for cartilage tissue regenerative therapy.

Therefore, these challenges at the front-end of cartilage tissue regeneration demands that novel *in vitro* culture conditions are developed to engineer a cell population with a stable chondrocyte phenotype. In the present study the effects of PPS and PSGAG on chondrogenic differentiation of MSCs were evaluated with a view of improving the culture conditions for cartilage tissue engineering. A recent study showed that PPS promotes human MPCs proliferation and chondrogenesis in pellet or MMC (Ghosh *et al.*, 2010). Similar to PPS, PSGAG is a semi-synthetic polysulfated chondroitin sulfate that has been shown to diminish articular cartilage matrix molecule degradation, improve lameness score (Fujiki *et al.* 2007), and enhance matrix molecule synthesis (Glade 1990). However, there are no studies that have

evaluated its effects on chondrogenesis of MSCs.

Generally, the use of bioscaffold 3D culture systems is increasingly being employed to better mimic the *in vivo* chondrocyte environment. The use of alginate culture provides a natural material for 3D encapsulation of cells which has been shown to support chondrogenesis and redifferentiation of chondrocytes (Singh *et al.*, 2011; Freshney, 2010; Lemare *et al.*, 1998; Bonaventure *et al.*, 1994). Therefore, the objective of the study was to investigate the effects of PPS and PSGAG on chondrogenesis of cBMSCs in alginate culture and MMC. It was hypothesized that the combined culture of cBMSCs in alginate beads and MMC in CDM supplemented with either PPS or PSGAG would promote chondrogenic differentiation and improve culture conditions for cartilage tissue engineering.

5.3 Materials and Methods

5.3.1 Treatment of cBMSCs in alginate beads and micromass culture

In this study, monolayer expanded P0 cBMSCs described in study 3 were expanded twice with subsequent P1 and P2 cells being differentiated into chondrogenic lineage using StemPro[®] CDM (GIBCO BRL) exogenously supplemented with either PPS (Biopharm Australia) or PSGAG (Adequan[®] PSGAG - 100 mg/mL; Novartis Animal Health Inc, Tokyo, Japan) at concentrations of 1, 3 and 5 µg/mL in alginate beads and MMC. The NC cells were cultured in 10% DMEM while the PC group was cultured in CDM. cBMSCs at P1 and P2 derived from each of the three dogs were encapsulated and cultured independently in the presence or absence of PPS and PSGAG, giving a total of six experiments, each considered as an independent chondrogenesis assay.

5.3.2 Alginate beads encapsulation and culture of cBMSCs

The encapsulation of cBMSCs in alginate beads was as previously described in study 2. Alginate beads culture has been reported to have the potential to differentiate MSCs into chondrocytic lineage (Coleman *et al.*, 2007). The cBMSCs-alginate beads were established as previously described for CACs with the only difference being that in this experiment, a suspension of 2×10^6 cells/mL was used. The beads were washed three times with 0.15 M NaCl then twice with 10% DMEM before being assigned specific treatment and incubated at 37°C in a humidified atmosphere of 5% CO₂. Alginate beads per culture-well contained an equivalent of 1×10^6 cells. The cultures were maintained for 20-days with medium changes done every 72 hr. On day-20, cells were recovered from alginate beads according to the

protocol described elsewhere (Freshney, 2010) and total RNA was extracted using TRIzol[®] Kit (Invitrogen), according to the manufacturer's instructions.

5.3.3 Micromass culture of cBMSCs

MMC were established according to instructions of STemPro[®] Chondrogenesis Differentiation Kit (GIBCO BRL). Briefly, cells were suspended in serum-free DMEM to generate a cell solution of 1.6×10^7 cells/ mL. MMC were established in duplicate by seeding 20- μ L droplets of cell solution in the centre of 12-well plate. The plates were incubated for 3 hr to allow cell adherence then 10% DMEM was gently added to each well and plates incubated overnight. After 24 hr, the medium was gently removed and each well was assigned specific treatment as described above and incubated at 37°C in 5% CO₂. Medium was replenished every 72 hr. Chondrocyte phenotype was assessed by Alcian blue stain analysis for PG deposition at 12- and 20-days culture period.

5.3.4 Chondrogenic phenotype analysis of cBMSCs by qPCR

Total RNA was quantified by spectrophotometry at 260 nm. Total of 500 ng RNA was reverse transcribed into cDNA using M-MLV RT kit (Invitrogen) according to manufacturer's recommended protocol. qPCR was performed as described previously with KAPA SYBR[®] FAST qPCR kit (KAPA biosystems) to determine the relative mRNA expression of the selected target genes; Sox-9, type II collagen, and aggrecan as chondrocyte-specific genes, type I and X collagen as chondrocyte dedifferentiation and hypertrophy genes, respectively, and HIF-2 α . All PCR reactions were validated, normalized and quantified as previously described with the NC group as the calibrator. The primers used in the study are same as those provided in Table 3 for the previous studies.

5.3.5 Chondrogenic phenotype analysis of cBMSCs by Alcian blue stain

After 12- and 20-days of culture MMC pellets were evaluated for PG deposition by Alcian blue stain analysis. Briefly, at 12- and 20-day culture, the medium was removed from culture plates, pellets were gently washed once with 1 x PBS and then fixed in 4% formaldehyde solution for 30 min. After fixation, the pellets were rinsed with 1 x PBS and stained with 1% Alcian blue solution prepared in 0.1 N HCl for 30 min. The pellets were rinsed three times with 0.1 N HCl, before adding distilled water to neutralize the acidity. The pellets were visualized under light microscope and images captured for PG analysis. Blue staining of the pellets indicate synthesis of cartilage sulfated aggrecan, a marker of chondrocyte phenotype.

5.4 Data analysis

Quantitative data was entered into a Microsoft Excel[®] spreadsheet. The data was then transferred to SPSS version 16.0 for descriptive and analytical statistics. ANOVA was used to determine significant difference in the mRNA expression between the treatments. Where significant difference was observed, *Post Hoc* multiple comparisons was performed using Bonferroni test to determine significantly different treatments. Unless specified, data is summarized as mean \pm 95% CI. Significant difference was defined as $P < 0.05$.

5.5 Results

5.5.1 Chondrogenic phenotype profile of cBMSCs cultured in alginate beads

Overall, there was no significant difference ($P > 0.05$) between PPS treated beads when compared to NC and PC groups (Figure 20). In contrast, PSGAG significantly upregulated ($P < 0.05$) type I collagen mRNA at all concentrations compared to NC, PC and PPS treated beads (Figure 20). Both PPS and PSGAG had no significant effect on type II collagen, aggrecan and HIF-2 α mRNA expression although at 3 $\mu\text{g/mL}$ both drugs tended to increase the expression of the three genes. PPS showed a dose-dependent inhibitory effect on type X collagen with the hypertrophy gene significantly inhibited at 5 $\mu\text{g/mL}$ compared to the NC. Interestingly, PSGAG demonstrated an inverse inhibitory effect on type X collagen with a lower concentration of 1 $\mu\text{g/mL}$ significantly inhibiting the hypertrophy marker compared to the NC whereas increase in concentration from 1 to 5 $\mu\text{g/mL}$ correspondingly increased the gene expression (Figure 20). The most provocative finding of the results was the undetectable Sox-9 mRNA. Nonetheless, cBMSCs still exhibited a chondrocytic phenotype expressing cartilage-specific genes; type II collagen and aggrecan.

The undetected Sox-9 mRNA expression by qPCR analysis was further verified by RT-PCR ethidium bromide gel electrophoresis which showed no Sox-9 mRNA bands in all the alginate cultured cBMSCs albeit cells expressing type II collagen and aggrecan (Figure 21A). To clarify whether the non-detection of Sox-9 mRNA was related to alginate culture, P1 cBMSCs were cultured under monolayer condition for 20-days in CDM. Sox-9 mRNA was highly induced in monolayer culture suggesting that alginate encapsulation of cBMSCs may have suppressive effect on Sox-9 mRNA expression (Figure 21B).

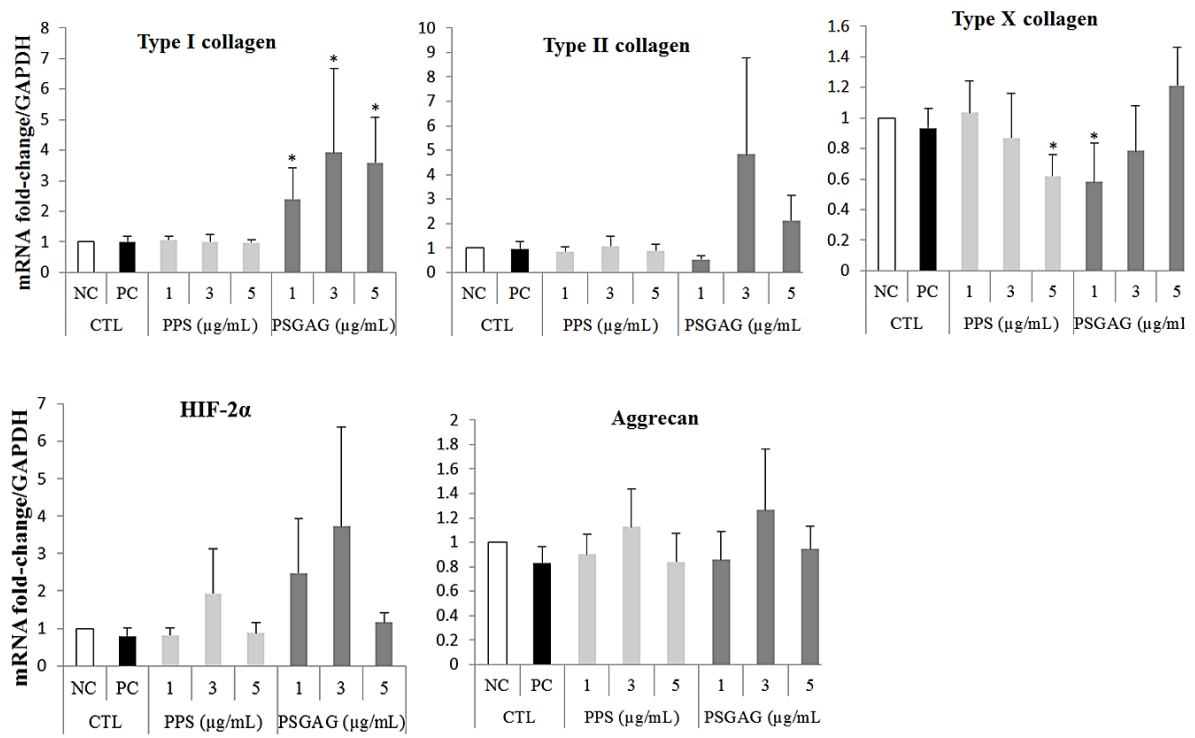


Figure 20 Effects of PPS and PSGAG on chondrogenesis of cBMSCs in alginate beads.

Chondrogenic differentiation medium was supplemented with either PPS or PSGAG at 0 (PC), 1, 3 and 5 $\mu\text{g/mL}$. Chondrogenic phenotype gene expression was quantified by real-time PCR targeting chondrocyte-specific genes; type II collagen and aggrecan, dedifferentiation and hypertrophy genes; type I collagen and X collagen, respectively, and hypoxia inducible factor-2 α subunit (HIF-2 α). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as reference gene. Control (CTL) groups were; negative control (NC); 10% DMEM, and positive control (PC); CDM. Data represent the mean \pm 95% confidence interval (95% CI) of 6 independent experiments (n = 6). Significant difference was defined as $*P < 0.05$ (Bwalya *et al.*, 2017b).

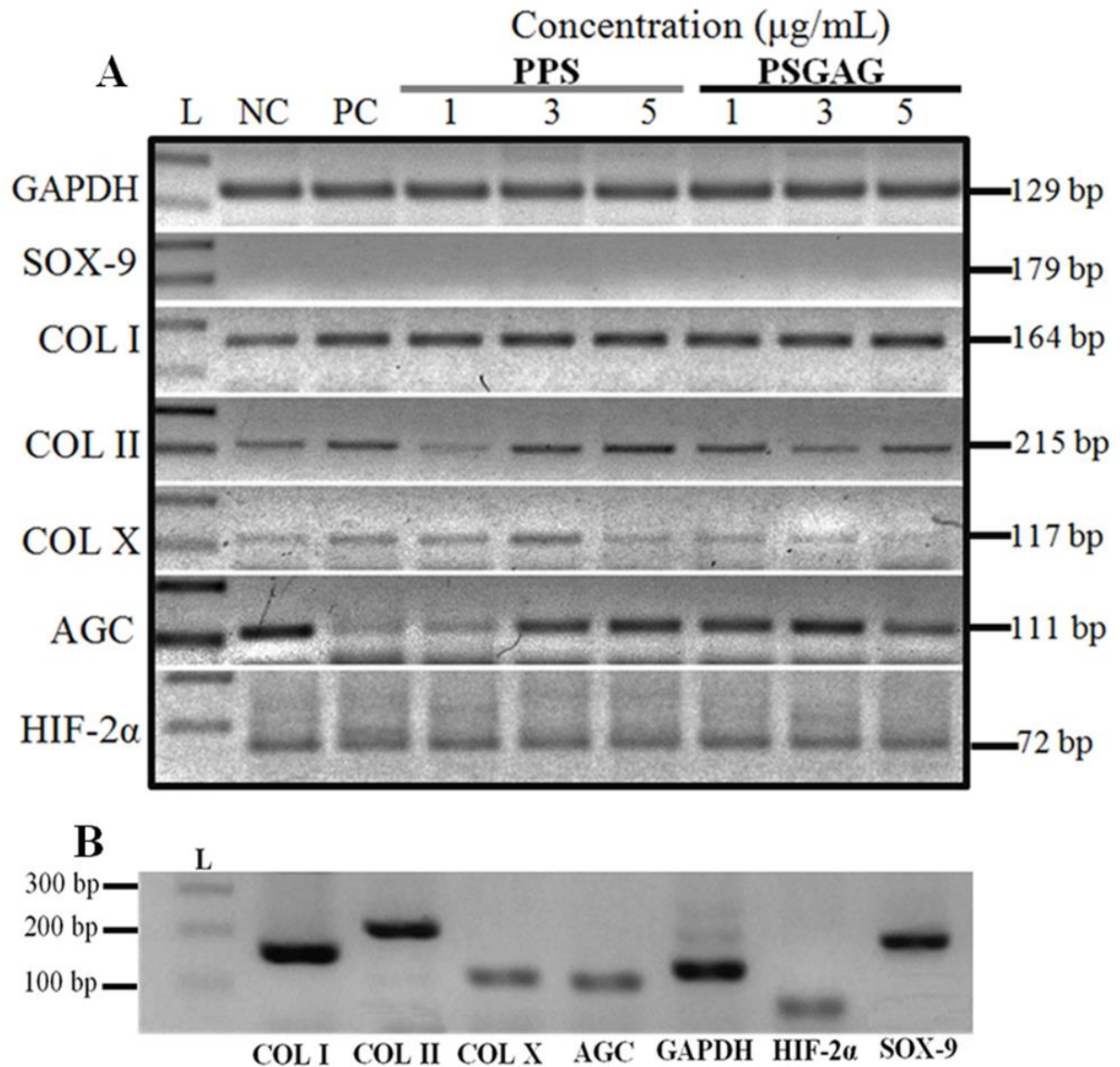


Figure 21 Alginate encapsulation of cBMSCs suppresses Sox-9 mRNA expression.

(A) Alginate encapsulated cBMSC presented in Figure 20 were further evaluated for Sox-9 mRNA expression reverse transcriptase-PCR (RT-PCR) ethidium bromide gel electrophoresis since initial assaying by qPCR showed no detectable Sox-9 mRNA signal albeit cells expressing type II collagen and aggrecan. RT-PCR verified that Sox-9 mRNA was suppressed in cBMSC cultured in alginate beads whereas type I, II and X collagen, aggrecan and Hypoxia Inducible Factor-2 α subunit (HIF-2 α) mRNA bands were all detectable. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as reference gene. L; 100 bp DNA ladder. Gels shown are from one representative experiment of six independent experiments (n = 6). (B) Shows that contrary to alginate beads culture, cBMSCs cultured as monolayer in CDM express Sox-9 mRNA and exhibit a chondrogenic phenotype (Bwalya *et al.*, 2017b).

5.5.2 PPS and PSGAG effects on PG deposition in cBMSCs

The effect of PPS and PSGAG on PG deposition in 12- and 20-days MMC was assessed by Alcian blue stain analysis. In 12-days cultures in the NC group most cells predominantly proliferated as monolayer fibroblastic-like cells with limited chondrogenic condensation. Chondrogenic condensation of cBMSC was more pronounced in PC compared to the NC although PG deposition was not different (Figure 22A-22B). When compared to the NC and PC, both PPS and PSGAG tended to enhance chondrogenic pelleting and PG deposition in a dose-dependent pattern (Figure 22C-22H).

In 20-days culture, cBMSC cultured in 10% FBS DMEM predominantly proliferated as fibroblastic-like monolayer cells with limited chondrogenic pellets that stained poorly with Alcian blue indicating reduced PG deposition and chondrogenic differentiation (Figure 23A). While cells cultured in CDM continued to form more compact chondrogenic pellets, some pellets stained poorly with Alcian blue stain. Interestingly, PPS demonstrated significantly enhanced chondrogenic pelleting and PG deposition at all concentrations when compared to the NC, PC and PSGAG, an indication of its potential to promote chondrogenic differentiation of cBMSC (Figure 23C-23E). In contrast, in 20 days culture, PSGAG inhibited chondrogenic pelleting and PG deposition in a dose-dependent pattern with most pellets being characterized by less condensed and sparsely arranged fibroblastic-like cellular aggregates which stained poorly with Alcian blue, an indication of its potential to inhibit chondrogenic differentiation of cBMSC (Figure 23F-23H).

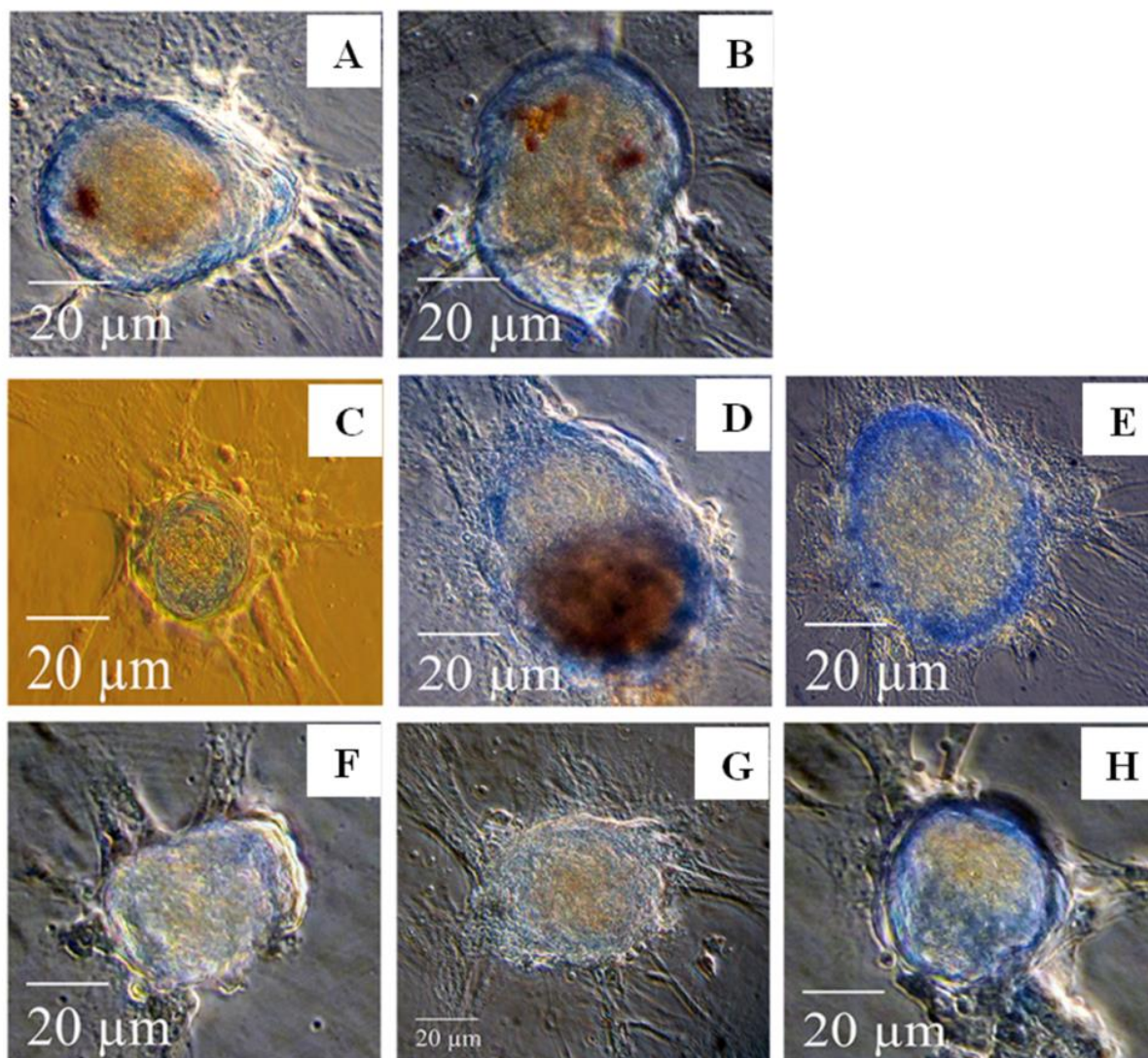


Figure 22 Effects of PPS and PSGAG on chondrogenesis of cBMSCs in micromass culture maintained for 12-days.

Photomicrographs represent the treatment conditions as; **(A)** 10% DMEM – negative control (NC), **(B)** chondrogenic differentiation medium (CDM) – positive control (PC), **(C)** 1.0 $\mu\text{g/mL}$ PPS, **(D)** 3.0 $\mu\text{g/mL}$ PPS, **(E)** 5.0 $\mu\text{g/mL}$ PPS, **(F)** 1.0 $\mu\text{g/mL}$ PSGAG, **(G)** 3.0 $\mu\text{g/mL}$ PSGAG and **(H)** 5.0 $\mu\text{g/mL}$ PSGAG. The supplementation of PPS and PSGAG to CDM tended to enhance cell aggregation and PG deposition at 12-days compared to NC and PC although the difference between PPS and PSGAG was not remarkable. PG deposition was evaluated by Alcian blue stain. Results are from one representative of six independent experiments ($n = 6$). **Scale bars: 20 μm** (Bwalya *et al.*, 2017b).

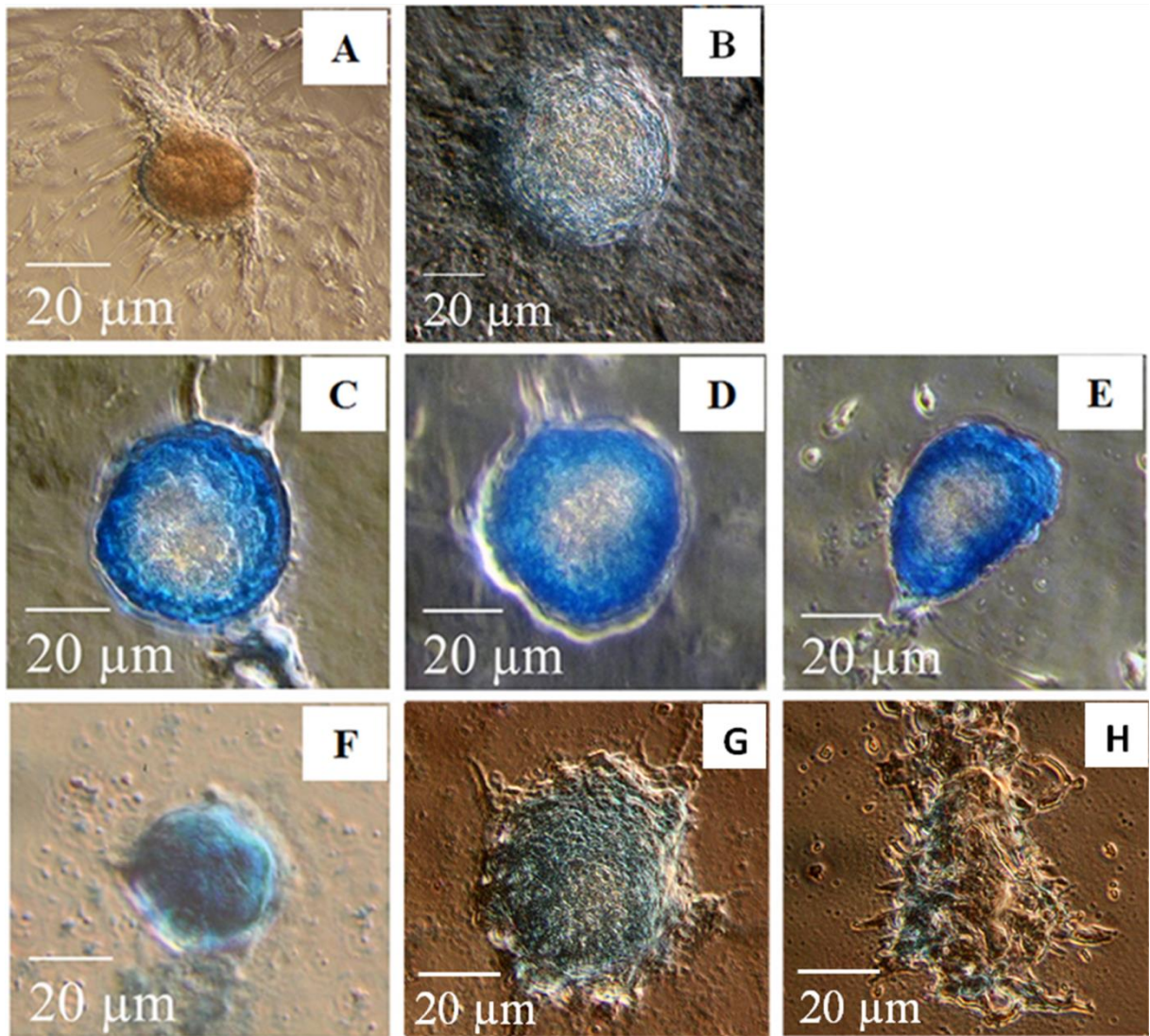


Figure 23 Effects of PPS and PSGAG on chondrogenesis of cBMSCs in micromass culture maintained for 20-days.

Photomicrographs represent the treatment as; (A) 10% DMEM – negative control (NC), (B) chondrogenic differentiation medium (CDM) – positive control (PC), (C) 1.0 µg/mL PPS, (D) 3.0 µg/mL PPS, (E) 5.0 µg/mL PPS, (F) 1.0 µg/mL PSGAG, (G) 3.0 µg/mL PSGAG and (H) 5.0 µg/mL PSGAG. In 20-days culture, addition of PPS to CDM significantly enhanced chondrogenic pelleting and PG deposition at all concentrations compared to NC, PC and PSGAG. In contrast, PSGAG inhibited chondrogenic pellet formation in a dose-dependent pattern leading to formation of fibroblastic-like pellets that stained poorly with Alcian blue. Results represent six independent experiments (n = 6). **Scale bars: 20 µm** (Bwalya *et al.*, 2017b).

5.6 Discussion

The objective of this study was to investigate the effects of PPS and PSGAG on chondrogenic differentiation of cBMSCs cultured in alginate beads and MMC with a view of improving *in vitro* culture conditions for cartilage tissue engineering. It was hypothesised that PPS and PSGAG would enhance chondrogenic differentiation of cBMSCs while concomitantly suppressing hypertrophy and dedifferentiation. The present study demonstrated that the response of cBMSCs to chondroinductive factors is culture system-dependent and varies significantly between alginate culture and MMC. The most provocative finding of the study was the observed suppressive effect of alginate encapsulation of cBMSCs on Sox-9 mRNA, a known positive regulator of articular cartilage differentiation. Nonetheless, cBMSCs cultured in alginate beads still demonstrated a chondrocyte phenotype expressing type II collagen and aggrecan with no significant difference in the genes expression between PPS and PSGAG treated beads, and cells cultured in 10% DMEM (NC) and CDM (PC). PPS had no significant effect on type I collagen mRNA expression in alginate cultures whereas PSGAG significantly upregulated the gene at all concentrations. PPS inhibited type X collagen in a dose-dependent pattern with the highest concentration significantly inhibiting the hypertrophy gene compared to the NC. The observed inhibitory effects of PPS on type X collagen are in agreement with findings of a previous study in human MPCs (Ghosh *et al.*, 2010). In contrast, PSGAG had an inverse effect on the hypertrophy gene with the lowest concentration significantly inhibiting the gene while increase in concentration correspondingly increased type X collagen mRNA expression. The results also demonstrated extensive expression of HIF-2 α mRNA in alginate beads cultured cBMSCs with both PPS and PSGAG having no significant effect on its

expression when compared to the NC and PC. To the contrary, in MMC, PPS significantly enhanced chondrogenic condensation and PG deposition, a confirmation of its potential to promote chondrogenic differentiation in pellet or aggregate cultures. In contrast, this study demonstrates for the first time that PSGAG has inhibitory effects on chondrogenic condensation and PG deposition in cBMSCs, an indication of its potential to suppress chondrogenic differentiation of MSCs.

As explained in the preceding studies, Sox-9 is a known positive regulator of articular cartilage differentiation, chondrocyte proliferation, and transition to a non-mitotic hypertrophic state. It is highly activated and predominantly expressed in mesenchymal condensations before and during the deposition of cartilage (Shen *et al.*, 2002; Studer *et al.*, 2012) and is necessary for chondrogenesis both before and after mesenchymal condensations (Ikeda *et al.*, 2005; Leung *et al.*, 2011). It activates specific enhancer elements in cartilage matrix genes and stimulates transcription of cartilage matrix genes, type II collagen and aggrecan (Ikeda *et al.*, 2005; Athanasiou *et al.*, 2013; Studer *et al.*, 2012). While it has been reported that alginate supports chondrogenic differentiation of MSCs, the findings of this study strongly suggests that the encapsulation of cBMSCs in alginate beads may have inhibitory effect on the process. This unusual finding is contrary to a previous study which demonstrated an increase in Sox-9 with BMP treated human MSCs encapsulated in alginate beads cultures (Majumdar *et al.*, 2001). However, there are no studies that have evaluated Sox-9 expression in cBMSCs cultured in alginate beads and therefore this observation suggests a difference in response of MSCs sourced from different species to alginate since when cBMSCs are cultured as monolayer at a high seeding density, Sox-9 mRNA is induced and restored. In study 2, using two different grades of alginate also verified these Sox-9 mRNA suppressive effects of alginate

encapsulation of CACs which also demonstrated significantly low Sox-9 protein expression by Western blot. Therefore, albeit Sox-9 being an established regulator and enhancer of type II collagen during chondrogenesis, it may not be the only key regulator of type II collagen promoter activity but could still be involved at very low physiological levels in maintaining a chondrocyte phenotype and this finding is in agreement with observations made elsewhere (Aigner *et al.*, 2003; Takahashi *et al.*, 1998).

The expression of type II collagen and aggrecan in alginate encapsulated cBMSCs cultured in only 10% DMEM demonstrates independent chondrogenesis in the absence of chondroinductive factor previously reported to be necessary (Coleman *et al.*, 2007; Athanasiou *et al.*, 2013; Mwale *et al.*, 2006; Nöth *et al.*, 2007). However, similar to findings by others (Bosnakovski *et al.*, 2006, 2004; Ghosh *et al.*, 2010; Mauck *et al.*, 2006; Mwale *et al.*, 2006; Nöth *et al.*, 2007; Studer *et al.*, 2012), chondrogenic differentiated cBMSCs in alginate beads also concomitantly expressed type I and X collagen in the presence or absence of both PPS and PSGAG. These findings taken together indicate that the use of cBMSCs as a source of differentiated chondrocytes population for cartilage tissue regeneration still requires a lot of effort in establishing suitable *in vitro* culture conditions that can best promote chondrogenesis without the cells progressively undergoing hypertrophy or dedifferentiation. While PPS showed no significant effect on type I collagen mRNA expression in alginate cultured cBMSCs, in study 2, it was demonstrated to significantly inhibit type I collagen mRNA expression in alginate beads cultured CACs at higher concentrations (15 - 40 µg/mL) compared to the concentration range used for cBMSCs (1 - 5 µg/mL). Moreover, PPS has also been shown to inhibit type I collagen gene expression in human MPCs cultured in MMC system (Ghosh *et al.*, 2010). Similar to the findings in monolayer cultures, HIF-2 α mRNA was found

to be extensively expressed in cBMSCs cultured in alginate beads. While both PPS and PSGAG had no significant effect on HIF-2 α mRNA expression in alginate culture, it was upregulated by both drugs at a concentration of 3 μ g/mL which also showed a non-significant positive correlation with type II collagen and aggrecan. As previously stated, increased expression of HIF-2 α mRNA has been reported to improve chondrogenic differentiation of human BMSCs and stem cells derived from the infrapatellar fat pad under hypoxic conditions (Adesida *et al.*, 2012; Khan *et al.*, 2007).

In contrast to alginate beads culture, in MMC, PPS significantly enhanced cBMSCs chondrogenic condensation and PG deposition compared to NC, PC and PSGAG. This observation confirms the results of a previous study which demonstrated that PPS promotes proliferation and chondrogenic differentiation of human MPCs (Ghosh *et al.*, 2010). In contrast, PSGAG inhibited chondrogenic pelleting and PG deposition in a dose-dependent pattern and resulted in chondrogenic pellets that were sparsely arranged with a fibroblastic-like phenotype that poorly stained with Alcian blue indicating a decreased PG deposition. This effect of PSGAG on chondrogenic differentiation of cBMSCs in MMC system could be associated with its previously reported inhibitory effect on growth of cell cultures (Glade, 1990). Therefore, PSGAG may ultimately fail to stimulate MSCs replication, a prerequisite for cartilage tissue regeneration especially in long-term pellet culture systems. The observed fibroblastic-like pellet phenotype in MMC could also be associated with the observed significant upregulation of type I collagen mRNA at all PSGAG concentrations in alginate beads culture. These findings taken together clearly demonstrate that the response of MSCs is dependent on the culture system and varies significantly between alginate and MMC. Of note was that PG deposition was higher in 12-days MMC in NC than in 20-days culture. Notably, in

20-days culture, cells in NC proliferated mostly as monolayer fibroblastic-like cells with limited chondrogenic pelleting and PG deposition as indicated by poor Alcian blue stain. Overall, while cells in the PC continued to form compact chondrogenic pellets and exhibited a positive homogenous Alcian blue stain indicating PG deposition in 20-days culture, some pellets showed reduced PG deposition albeit chondrogenic pelleting when compared to 12-days MMC. The reduced chondrogenic potential of cBMSCs cultured for 20-days in medium without chondroinductive factors indicates that such cultures generally progress into a mixed fibroblastic-like phenotype synthesizing type I and X collagen whereas the reduced PG deposition in some chondrogenic pellets cultured in CDM could indicate formation of mixed phenotype pellets in long-term adherent MMC conditions.

In summary, the study in this chapter demonstrated that while PPS and PSGAG share a similar chemical structure, their effects on chondrogenesis of cBMSCs varies significantly. The study demonstrated that the response of cBMSCs to chondroinductive factors is culture system-dependent and varies significantly between alginate culture and MMC. While PPS had no significant effect on chondrogenesis of cBMSCs in alginate beads, in MMC it promoted chondrogenesis as indicated by enhanced chondrogenic condensation and PG deposition. In contrast, PSGAG upregulated type I collagen in alginate beads culture and inhibited chondrogenic condensation and PG deposition in MMC resulting in sparsely arranged fibroblastic-like pellets with decreased PG deposition. Therefore the use of PSGAG in cartilage tissue engineering may be limited. While the present study confirms PPS as suitable alternative chondroinductive factor that may be used in cartilage tissue regeneration and repair of OA joints, caution must be exercised when extrapolating results from one culture system to another as a major variation of MSCs response to different culture systems exists.

6 General Conclusions

Pentosan polysulfate (PPS) is a semi-synthetic sulfated polysaccharide derived from wood of beech plant, *Fagus sylvatica* that is available for the relief of various medical conditions including thrombi and interstitial cystitis in humans, and OA in dogs and horses. Although the mechanism of action of PPS remains to be fully elucidated, it has been demonstrated to have a positive anabolic effect on articular chondrocytes, reduce cartilage destruction by inhibiting the synthesis and activity of cytokine-driven catabolic mediators of OA, and to significantly increase some anti-inflammatory cytokines, and was recently shown to promote chondrogenic differentiation and proliferation of human MPCs. Based on these attributes, PPS has long been considered as a prospective disease modifying osteoarthritic drug (DMOAD) although until the findings of the studies presented in this dissertation, its effects on some novel therapeutic targets of OA such as iNOS, c-Jun and HIF- α isoforms remained unknown. On the other hand, bone marrow-derived mesenchymal stem cells (BMSCs) are considered a potential alternative source of differentiated chondrocytes for cartilage tissue regeneration and repair of small OA cartilage defects. Defining *in vitro* culture conditions to differentiate BMSCs as well as redifferentiating the phenotype of dedifferentiated articular chondrocytes into functional and phenotypically stable chondrogenic cells prior to implantation is the current focus for successful cartilage tissue engineering. Therefore, the present study was designed to investigate the effects of PPS on iNOS, c-Jun and HIF- α isoforms recently identified as novel potential targets for the treatment of OA, and to evaluate its chondroinductive potential to promote the redifferentiation of dedifferentiated monolayer expanded articular chondrocytes as well as chondrogenic differentiation of cBMSCs intended

for cartilage tissue regeneration *in vitro*. The major findings of the studies can be summarized as follows;

1. IL-1 β -induced iNOS, c-Jun, HIF-1 α and HIF-2 α mRNA expression in first passage (P1) canine articular chondrocytes (CACs). However, the preincubation of P1 CACs with PPS significantly inhibited IL-1 β -induced iNOS, c-Jun and HIF-1 α mRNA upregulation with minimal inhibitory effect observed on IL-1 β -induced HIF-2 α mRNA upregulation. Furthermore, normal CACs demonstrated recalcitrance to single IL-1 β , TNF- α or LPS-induction of iNOS protein expression including to a combination of IL-1 β + TNF- α , IL-1 β + LPS except for TNF- α + LPS and IL-1 β + TNF- α + LPS suggestive of a protective mechanism from iNOS detrimental effects on OA progression. Preincubation of CACs with PPS significantly abrogated IL-1 β + TNF- α + LPS-induced iNOS protein expression. The results also confirmed that PPS colocalized with the transcriptional factors, NF- κ B p65 and c-Jun reducing their nuclear translocation and localization activity in IL-1-induced CACs. These results demonstrated for the first time that PPS is a novel inhibitor of IL-1 β -induced iNOS, c-Jun, and HIF-1 α mRNA upregulation, and iNOS protein-induction and thus may translate to its beneficial effects in treatment OA and prevention of disease progression.
2. For successful cartilage tissue regeneration and repair of OA defects by autologous chondrocyte transplantation (ACT), redifferentiation of dedifferentiated articular chondrocytes following *in vitro* monolayer expansion

has long been proposed as the best hope for returning chondrocytes to their native articular cartilage mode of expression prior to implantation. Dedifferentiated monolayer P1 CACs cultured for 18 days by encapsulation in alginate beads under normoxia condition in 20% DMEM supplemented with only PPS demonstrated a full retain to their 'native' cartilage phenotype as verified by enhanced synthesis of cartilage-specific gene markers, type II collagen and aggrecan mRNA with complete suppression of type I and X collagen. PG deposition, a marker of articular cartilage phenotype was also significantly enhanced by PPS compared to the control. Alginate encapsulation of CACs resulted in Sox-9 suppression, which was associated to the reduced cell-to-cell interactions in alginate beads cultured cells. HIF-1 α and HIF-2 α proteins were detected at protein level for the first time under normoxia condition an indication that culture of chondrocytes in alginate beads may stabilize the HIF- α isoforms at the protein level leading to promotion of a chondrocyte phenotype. The results demonstrated that the phenotype of dedifferentiated articular chondrocytes may be restored by combining alginate encapsulation with culture in standard medium supplemented with only PPS without the addition of known chondrocytic growth factors. Therefore, this study verifies that PPS is a novel alternative chondroinductive factor with the potential to offer a solution to the major challenges that exist in cartilage tissue engineering efforts.

3. Although chondroinductive growth factors such as TGF- β are considered necessary for chondrogenic differentiation of BMSCs, independent and

spontaneous chondrogenic differentiation has been previously demonstrated in adult horses, bovine calves and adult human BMSCs but not in canine BMSCs (cBMSCs). This study was undertaken to evaluate the independent chondrogenic potential of cBMSCs sourced from young dogs in monolayer expansion cultures in the absence of known chondroinductive factors. The results showed for the first time that cBMSCs exhibit independent chondrogenic differentiation in the absence of dexamethasone and TGF- β 1 previously shown to be necessary as verified by the expression of Sox-9, type II collagen and aggrecan. Sox-9 was significantly downregulated from P1-P3 compared to P0 while type II and X collagen, and aggrecan were significantly downregulated at P3 compared to P0. Sox-9, type II collagen and aggrecan gene expression showed a significant negative correlation with passaging, which may explain why after extensive passaging cBMSCs fail to exhibit independent chondrogenesis. Type I collagen was highly expressed after P0 indicating a phenotype drift to a fibroblastic-like phenotype. HIF-2 α mRNA was expressed in all cBMSCs passages, which may reflect its important regulatory role in the chondrogenesis process. The results demonstrated that independent chondrogenic potential and phenotype retention of cBMSCs is passage-dependent and decreases with extensive passaging in standard medium, thus caution must be exercised when evaluating the chondrogenic potential of BMSCs intended for cartilage tissue engineering and repair of OA cartilage lesions.

4. MSCs are a potential alternative source of differentiated chondrocytes for

cartilage tissue regeneration and repair of small OA cartilage defects. Both PPS and PSGAG had no significant effect on type II collagen, aggrecan and HIF-2 α mRNA expression in alginate cultured cBMSCs indicating that both failed to promote chondrogenesis in this culture model. In addition, PPS had no effect on type I collagen whereas PSGAG upregulated type I collagen mRNA expression. PPS had a dose-dependent inhibitory effect on type X collagen mRNA while PSGAG showed an inverse effect with increased concentration correspondingly increasing type X collagen mRNA expression. In contrast to alginate beads culture, PPS enhanced chondrogenesis and PG deposition in cBMSCs in MMC whereas PSGAG inhibited chondrogenesis and promoted a fibrocartilage-like phenotype with reduced PG deposition. The result verified PPS as a chondroinductive factor that may enhance chondrogenesis of cBMSCs in MMC but appears to have no effect in alginate beads culture. This finding has serious implications in cartilage tissue engineering as it indicates that the response of MSCs to chondroinductive factors is culture system-dependent and varies significantly between alginate and MMC.

The findings in this dissertation will contribute in a positive way to the body of knowledge on the effects and possible mechanism of action of PPS on novel therapeutic targets for OA treatment and prevention of disease progression. Furthermore, by demonstrating and verifying the positive *in vitro* chondroinductive effects of PPS on articular chondrocytes and BMSCs, the findings of this study will surely have a positive impact in influencing the future focus for cartilage tissue regeneration and repair efforts of OA joint defects in both humans and companion animals.

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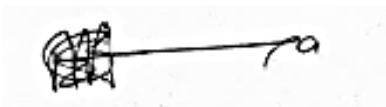
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Hokkaido University, July 2017

A handwritten signature in black ink, appearing to read 'Eugene Chisela Bwalya', with a stylized flourish at the end.

Dr. Eugene Chisela Bwalya