Univerza v Ljubljani
Veterinarska fakulteta

Metka Voga

VPLIV IZBRANIH DEJAVNIKOV NA PROLIFERACIJO IN DIFERENCIACIJO MEZENHIMSKIH MATIČNIH CELIC IZ MAŠČOBNEGA TKIVA PSOV IN MAČK

Doktorska disertacija

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EFFECT OF SELECTED FACTORS ON PROLIFERATION AND DIFFERENTIATION OF CANINE AND FELINE ADIPOSE DERIVED MESENCHYMAL STEM CELLS

Doctoral dissertation

Ljubljana, 2021

Metka Voga,

Vpliv izbranih dejavnikov na proliferacijo in diferenciacijo mezenhimskih matičnih celic iz maščobnega tkiva psov in mačk

Delo je bilo opravljeno v Laboratoriju za genomiko na Inštitutu za predklinične vede Veterinarske fakultete v Ljubljani. Raziskovalno delo je bilo delno izvedeno tudi na Odseku za nanostrukturne materiale Inštituta Jožef Štefan v Ljubljani in na Zavodu Republike Slovenije za transfuzijsko medicino v Ljubljani.

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Lenja Voga, 6 let

»I AM AMONG THOSE WHO THINK THAT SCIENCE HAS GREAT BEAUTY. A SCIENTIST IN HIS LABORATORY IS NOT ONLY A TECHNICIAN, HE IS ALSO A CHILD PLACED BEFORE NATURAL PHENOMENA WHICH IMPRESS HIM LIKE A FAIRY TALE«

- Marie Curie

IZVLEČEK

Ključne besede: mezenhimske matične celice – citologija; celična proliferacija – fiziologija; celična diferenciacija – fiziologija; celične kulture – metode; biomateriali – metabolizem; svilni fibroin – metabolizem; hondrogeneza – fiziologija; psi; mačke

Matične celice so v zadnjih letih zaradi svojih edinstvenih lastnosti postale vir razvoja regenerativne medicine in tkivnega inženirstva. Ker različne lastnosti mezenhimskih matičnih celic (MSC) v pogojih *in vitro* lahko vodijo v potencialno različen terapevtski učinek, je temeljnega pomena odkrivanje vplivov različnih dejavnikov na značilnosti MSC. Uveljavljena podlaga za gojenje MSC je plastična osnova, vendar se zaradi boljšega posnemanja naravnega celičnega okolja za gojenje celic vedno bolj poudarja pomen uporabe celičnih nosilcev. V doktorski disertaciji smo raziskavo sistematično razdelili na dva dela. V prvem delu smo celice gojili na običajni plastični podlagi in proučevali vpliv vrste, spola in poznih pasaž na proliferacijski in diferenciacijski potencial MSC iz maščobnega tkiva (AMSC) psov in mačk. V drugem delu smo celice gojili na celičnih nosilcih iz svilnega fibroina (SF) in proučevali, kako SF vpliva na proliferacijski in diferenciacijski potencial AMSC. Rezultati prvega dela naše raziskave so pokazali, da AMSC mačk izkazujejo slabši proliferacijski in diferenciacijski potencial kot AMSC psov in da tudi pozne pasaže bolj negativno vplivajo na AMSC mačk kot na AMSC psov. Dodatno smo pokazali, da AMSC mačk v manjši meri izražajo preiskovane celične označevalce in v manj optimalnih pogojih izkazujejo slabšo živost. Z rezultati prvega dela raziskave smo pokazali, da vrsta živali lahko bistveno vpliva na značilnosti AMSC v pogojih *in vitro* in da bi živalsko vrsto morali upoštevati kot spremenljivko pri pripravi celic za regenerativno zdravljenje. Rezultati drugega dela naše raziskave so pokazali, da SF omogoča adhezijo celic in vodi usodo AMSC v smeri hondrogene diferenciacije, ki smo jo potrdili z analizo celične morfologije, barvanjem zunajceličnega matriksa diferenciranih celic ter kvantifikacijo izražanja genov, značilnih za hrustančne celice. Z rezultati drugega dela raziskave smo pokazali, da je hondrogeno diferenciacijo celic moč doseči brez posebnih pogojev gojenja celic, ki so sicer potrebni za hondrogenezo MSC na običajni plastični podlagi. Rezultati doktorske disertacije omogočajo poglobitev pomena poznavanja razlik matičnih celic med živalskimi vrstami in hkrati predstavljajo pomembno osnovo za prihodnje študije in potencial za uvajanje novih zdravljenj z matičnimi celicami v veterinarski klinični medicini.

ABSTRACT

Key words: mesenchymal stem cells – cytology; cell proliferation – physiology; cell differentiation – physiology; cell culture techniques – methods; biomaterials – metabolism; silk fibroin – metabolism; chondrogenesis – physiology; dogs; cats

Due to their unique properties, stem cells have become an important source for the development of regenerative medicine and tissue engineering in recent years. Since different properties of mesenchymal stem cells (MSCs) *in vitro* can lead to potentially different therapeutic effects, investigating the influence of various factors on the properties of MSCs is essential. The established basis for the cultivation of MSCs is a plastic surface, but due to the better mimicking of the natural cellular environment, the use of cell carriers for cell cultivation is being increasingly developed. In this dissertation, our studies are systematically divided into two parts. In the first part, cells were grown on a conventional plastic surface and the influence of species, sex and late passages on the proliferation and differentiation potential of canine and feline adipose derived MSCs (AMSCs) were investigated. In the second part, cells were grown on silk fibroin (SF) cell carriers and influence of SF on the AMSCs was investigated. The results of the first part of our study showed that feline AMSCs had poorer proliferation and differentiation potential than canine AMSCs and that late passages had a more negative effect on feline AMSCs than on canine AMSCs. In addition, feline AMSCs expressed fewer examined cell surface markers and exhibited lower viability under less optimal conditions. The results of the first part of the study showed that animal species can significantly affect the properties of AMSCs *in vitro* and that animal species should be considered when preparing cells for regenerative treatment. The results of the second part of our study showed that SF enables cell adhesion and directs AMSC fate towards chondrogenic differentiation, which was confirmed by the analysis of cell morphology, staining of extracellular matrix of differentiated cells and quantification of genes, characteristic of chondrocytes. With the results of the second part of the study, we have shown that chondrogenic AMSC differentiation can be achieved without the special cell culture conditions that are otherwise required for chondrogenesis of AMSCs on a conventional plastic surface. The results of the dissertation allow us to deepen the knowledge about the importance of the stem cell differences between species and at the same time represent basis for further studies and the potential for the introduction of new stem cell therapies in clinical veterinary medicine.

KAZALO VSEBINE

SEZNAM OKRAJŠAV

AMSC	Mezenhimske matične celice iz maščobnega tkiva *(Adipose–derived mesenchymal stem cells)*
$CD8^+$	Označevalec pripadnosti 8^+ *(Cluster of differentiation 8^+)*
CD14	Označevalec pripadnosti 14 *(Cluster of differentiation 14)*
CD29	Označevalec pripadnosti 29 *(Cluster of differentiation 29)*
CD34	Označevalec pripadnosti 34 *(Cluster of differentiation 34)*
CD44	Označevalec pripadnosti 44 *(Cluster of differentiation 44)*
CD45	Označevalec pripadnosti 45 *(Cluster of differentiation 45)*
$CD56^+$	Označevalec pripradnosti 56^+ *(Cluster of differentiation 56^+)*
CD73	Označevalec pripadnosti 73 *(Cluster of differentiation 73)*
CD79α	Označevalec pripadnosti 79 α *(Cluster of differentiation 79α)*
CD90	Označevalec pripadnosti 90 *(Cluster of differentiation 90)*
CD105	Označevalec pripadnosti 105 *(Cluster of differentiation 105)*
CD	Število celičnih podvojitev *(Cell doubling)*
CDT	Čas, ki je potreben, da se število celic podvoji *(Cell doubling time)*
DNK	Deoksiribonukleinska kislina
ESC	Embrionalne matične celice *(Embrional stem cells)*
FBS	Fetalni goveji serum *(Foetal bovine serum)*
HLA–DR	Humani levkocitni antigen – izotip DR
HSC	Hematopoetske matične celice *(Hematopoietic stem cells)*
IDO	Indolamin–2,3–dioksigenaza
IL10	Interlevkin 10
IPSC	Inducirane pluripotentne matične celice *(Induced pluripotent stem cells)*
MAPK	Z mitogenom aktivirana protein kinaza
MSC	Mezenhimske matične celice *(Mesenchymal stem cells)* / Mezenhimske stromalne celice *(Mesenchymal stromal cells)* / Medicinske signalizirajoče celice *(Medicinal signaling cells)*
PGE2	Prostaglandin E2
p16	Protein 16

p53	Protein 53
PRP	Krvna plazma obogatena s trombociti *(Platelet rich plasma)*
RGD	Zaporedje aminokislin arginin–glicin–aspartat
RNK	Ribonukleinska kislina
ROS	Reaktivne kisikove spojine *(Reactive oxygen species)*
SF	Svilni fibroin
TGF – β	Transformirajoči rastni dejavnik – β *(Transforming growth factor – β)*
TNF – α	Dejavnik tumorske nekroze – α *(Tumor necrosis factor – α)*
2D	Dvodimenzionalen
3D	Trodimenzionalen

1 UVOD

1.1 PREGLED LITERATUTE IN PREDSTAVITEV PROBLEMATIKE

Matične celice so v zadnjih letih v splošni javnosti in na strokovnem področju vzbudile več zanimanja kot katerokoli drugo področje biologije. Eden izmed razlogov za to je obet, da bo poznavanje njihovega delovanja vodilo v globje razumevanje biologije celice in s tem možnost razvoja zdravljenja številnih degenerativnih bolezni [1]. Matične celice so nediferencirane celice, sposobne samoobnove in razvoja v specializirane celice posameznega tkiva [2]. Zaradi svojih edinstvenih lastnosti so postale vir razvoja regenerativne medicine in tkivnega inženirstva – interdisciplinarnega področja, ki kombinira znanje in tehnologijo celic, biomaterialov in biokemijskih dejavnikov, za namen regeneracije poškodovanega tkiva ali konstruiranja umetnega tkiva oziroma organa [3]. Presaditev matičnih celic je v rutinski klinični uporabi že od leta 1968 [4], ko je bila prvič opravljena presaditev kostnega mozga z namenom obnove hematopoetskega sistema, prizadetega zaradi radiacije ali kemoterapije pri zdravljenju malignih obolenj. Danes matične celice veljajo za bodoči temelj medicine v prihodnosti s potencialom nadomestitve katerekoli vrste celic v našem telesu [5].

1.1.1 Matične celice glede na stopnjo razvoja organizma

Glede na stopnjo razvoja matične celice lahko razdelimo na totipotentne ali omnipotentne, pluripotentne, multipotentne in unipotentne [6]. Totipotentne matične celice imajo pomembno vlogo v razvoju organizma, saj predstavljajo vir vseh znotraj– in zunajzarodnih tkiv [7]. Prisotne so takoj po oploditvi in še tri do štiri delitve za tem. Nadaljnje delitve vodijo zgodnji embrionalni razvoj v nastanek blastociste in nižjo potentnost celic. Embrioblast blastociste predstavlja vir pluripotentnih embrionalnih matičnih celic (angl., Embrional stem cells, ESC), ki so sposobne razvoja v celice vsakega od treh kličnih listov – ektoderma (iz katerega se razvijeta koža in živčni sistem), mezoderma (iz katerega se razvijejo kri, kosti mišice, hrustanec, maščoba) in endoderma (iz katerega se razvijeta dihalni in prebavni trakt). Nastanek kličnih listov je eden od najpomembnejših dogodkov embrionalnega razvoja. Iz pluripotentnih nastanejo multipotentne matične celice, sposobne diferenciacije v več vrst celic, ki pripadajo samo enemu od treh kličnih listov. Lastnost nekaterih odraslih matičnih celic, da se lahko razvijejo v le eno vrsto celic, posedujejo unipotentne matične celice, npr. epidermalne matične celice, ki se diferencirajo v keratinocite [6].

1.1.2 Matične celice glede na izvor

Glede na izvor matične celice ločimo na embrionalne, inducirane pluripotentne in odrasle matične celice. Prva izolacija ESC iz embrioblasta blastociste miši leta 1981 [8] je vodila v možnost raziskovanja izražanja in delovanja genov med embrionalnim razvojem ter procesa celične diferenciacije, s hkratno vizijo uporabe matičnih celic za zdravljenje bolezni in določanja genov, ki bi lahko bili tarča za potencialna zdravila na področju regenerativne medicine. Z izolacijo ESC človeka leta 1998 [9] se je teoretična možnost zdravljenja bolezni z matičnimi celicami približala realnosti. Vendar pa je uporaba ESC z vidika potrebe po uničenju zarodkov za namen njihove izolacije omejena zaradi etičnih, moralnih in kulturnih zadržkov. Za nadaljevanje raziskovanja možnosti zdravljenja z matičnimi celicami se je pojavila potreba po iskanju drugih virov matičnih celic. Možnost pridobitve pluripotentnih matičnih celic brez uporabe zarodka sta leta 2006 prva odkrila japonska znanstvenika Takahashi in Yamanaka [10]. Inducirane pluripotentne matične celice (angl. Induced pluripotent stem cells, IPSC) so nastale z reprogramiranjem odraslih mišjih fibroblastov v inducirano pluripotentno stanje. Z retrovirusno transdukcijo genov Oct3/4, Sox2, c – Myc in Klf4, sicer pomembnih pri vzdrževanju značilnih lastnosti ESC, so nastale celice, ki so bile v morfologiji, samoobnovitvenih sposobnostih in diferenciacijskem potencialu podobne ESC. Kljub pomembnemu preboju v znanosti na področju matičnih celic je uporaba IPSC za terapevtske namene vprašljiva zaradi retrovirusne transdukcije onkogenov, ki z možnostjo sprožanja sprememb na kromosomih lahko vodi v tumorogenezo [10]. Alternativa ESC in IPSC so multipotentne matične celice, ki se nahajajo v odraslem organizmu. V primerjavi z embrionalnimi, odrasle matične celice kažejo podobne samoobnovitvene zmožnosti, a imajo hkrati bolj omejen diferenciacijski potencial za razvoj v specializirane vrste celic [11]. Odrasle matične celice se tvorijo med ontogenezo in ostanejo znotraj niše tkiv in organov odraslega organizma, kjer pod fiziološkimi ali patološkimi pogoji obnavljajo populacijo tkivnih celic [6].

1.1.3 Mezenhimske matične celice

Mezenhimske matične celice (angl., Mesenchymal stem cells, MSC), imenovane tudi mezenhimske stromalne celice (angl., Mesenchymal stromal cells, MSC), so multipotentne odrasle matične celice. Z vidika pomena mezenhima, ki je embrionalno vezivo mezodermalnega izvora v prenatalnem obdobju, so MSC ostanek embrionalnega veziva, ki se

v nediferencirani obliki nahajajo v številnih organih in igrajo vloge celične diferenciacije, nadomeščanja celic oz. popravila poškodb in imunomodulacije [12]. Leta 1968 je bila prvič omenjena osteogena populacija celic, izolirana iz kostnega mozga s fibroblastom podobno morfologijo [13]. Nadaljne študije so pokazale tudi zmožnost pritrditve teh celic na plastično podlago za gojenje celic in edinstveno zmožnost diferenciacije v različne vrste specializiranih celic [14, 15]. Slabi dve desetletji kasneje so celice poimenovali MSC [12]. Rezultati začetnih študij so pokazali sposobnost MSC za diferenciacijo v osteoblaste, adipocite in hondrocite *in vitro* [16]. Kasneje je bilo ugotovljeno, da so MSC zmožne tudi diferenciacije v celice drugih tkiv mezodermalnega izvora, kot so celice tetiv in ligamentov [17], kardiomiociti [18] in miociti [19]. Nadaljne študije so pokazale, da so MSC sposobne diferenciacije tudi v celice endodermalnega in ektodermalnega izvora. Rezultati raziskav, ki so pokazali sposobnost diferenciacije MSC v celice kože [20], pigmentni epitelij mrežnice [21], celice pljuč [22], hepatocite [23], ledvične tubule [24], Langerhansove otočke [25] in nevrone [26], nakazujejo na to, da MSC niso zgolj multipotentne, saj posedujejo zmožnost diferenciacije v tkiva izvirajočih iz vseh treh kličnih listov in torej izkazujejo pluripotenten značaj.

MSC se nahajajo v vseh ožiljenih tkivih in so zaradi svojih edinstvenih lastnosti predmet številnih predkliničnih in kliničnih raziskav po vsem svetu (ClinicalTrials.gov). Kljub temu pa je njihov izvor še vedno neznanka. Fiziološka vloga MSC je najbolj proučevana v kostnem mozgu, ki predstavlja pomemben vir MSC in kjer imajo MSC pomembno vlogo v ohranjanju niše hematopoetskih matičnih celic (angl., Hematopoietic stem cells, HSC) – celic v kostnem mozgu, iz katerih v procesu hematopoeze nastanejo specializirane krvne celice [27]. Fenotip MSC so v večih raziskavah povezali s periciti – perivaskularnimi celicami kapilar in manjših žil [28-31], ki so v celični kulturi zaradi sposobnosti adhezije na plastiko, enake morfologije ter enakega proliferacijskega in diferenciacijskega potenciala neločljivi od MSC [32]. Povezanost MSC s periciti je bila dodatno potrjena tudi z izražanjem istih površinskih označevalcev [32]. Ali so periciti zgolj predhodniki MSC ali pa bi jim tudi z vidika funkcionalnosti lahko rekli MSC, zaenkrat ostaja nerešeno vprašanje [33]. Vendar periciti niso edine perivaskularne celice, pri katerih je bil pokazan potencial za razvoj v MSC. Fibroblastom podobne progenitorne celice, ki se nahajajo v tuniki adventiciji – zunanji ovojnici stene večjih arterij in žil – so bile prav tako prepoznane kot predhodnice MSC. Na podlagi raziskav tako obstajata dva perivaskularna vira MSC – periciti kapilar in majhnih žil ter adventicijske celice v steni večjih

žil [34]. Kljub nerešenemu problemu določitve natančne definicije so se MSC zaradi svojih edinstvenih lastnosti, odsotnosti večjih etičnih zadržkov pri njihovi uporabi, lahki dostopnosti in enostavni manipulaciji v pogojih *in vitro* izkazale kot najustreznejše za uporabo v terapevtske namene. Mednarodno združenje za celična zdravljenja je z namenom poenotene karakterizacije MSC ljudi in primerljivosti rezultatov raziskav z njimi leta 2006 izdalo minimalna merila za njihovo identifikacijo. Zahtevana merila vključujejo sposobnost adhezije MSC na plastiko v standardni celični kulturi, izražanje celičnih označevalcev CD105, CD73, CD90, odsotnost izražanja celičnih označevalcev CD45, CD34, CD14 (ali CD11b), CD79α (ali CD19) in HLA–DR ter sposobnost diferenciacije v osteoblaste, hondroblaste in adipocite [16].

1.1.4 Terapevtski potencial MSC

Z vidika sposobnosti diferenciacije MSC v specializirane celice različnih tkiv je moč sklepati, da je njihov terapevtski potencial posledica njihove diferenciacije v celice tkiv [35, 36]. Vendar so rezultati kasnejših raziskav pokazali, da je terapevtski učinek MSC predvsem posledica njihovega imunomodulatornega delovanja. Predpostavljeno je, da mesto nahajanja MSC v perivaskularni niši igra ključno vlogo v zaznavanju poškodb lokalnih ali oddaljenih tkiv, na katere se MSC odzovejo z migracijo proti mestu poškodbe in sodelovanjem v procesu celjenja [37]. Na podlagi tega je bil leta 2017 predstavljen predlog, da bi poimenovanje »mezenhimske matične celice« spremenili v »medicinske signalizirajoče celice« (angl., Medicinal signaling cells, MSC) [38]. Imunomodulatorno delovanje MSC vključuje parakrino signalizacijo, izločanje zunajceličnih veziklov, z apoptozo pogojeno imunomodulacijo ter prenos mitohondrijev v tarčne celice.

1.1.4.1 Parakrina signalizacija

Vse več je dokazov, da primarno delovanje MSC temelji na parakrinem izločanju (t.j. izločanju dejavnikov v svojo neposredno okolico) signalnih molekul, ki vplivajo na funkcionalne spremembe imunskih celic, kot so monociti in makrofagi [39], dendritične celice [40], celice T [41], celice B [42] in naravne celice ubijalke [43, 44]. Med najpomembnejše in najbolj proučevane dejavnike, ki omogočajo imunomodulatorni učinek MSC, se uvrščajo transformirajoči rastni dejavnik–β (angl., Transforming growth factor–beta, TGF–β), indolamin–2,3–dioksigenaza (angl., Indolamine–2,3–dioxygenase, IDO), prostaglandin E2

(angl., Prostaglandine E2, PGE2), interlevkin 10 (angl., Interleukin 10, IL10) in dejavnik tumorske nekroze–α (angl. Tumor necrosis factor–α, TNF– α). Učinek parakrine signalizacije MSC se kaže v zaviranju delovanja dendritičnih celic [45], polarizaciji makrofagov – pretvorbi vnetnega v protivnetni tip makrofagov [46-52], aktivaciji regulatornih celic T [53-55], zaviranju aktivacije in proliferacije naravnih celic ubijalk [43] ter celic T [45, 53] in B [56] in posledičnem zaviranju citotoksičnega in protitelesnega imunskega odziva [45]. MSC tako na podlagi parakrine signalizacije z vplivom na delovanje imunskega sistema omogočajo spreminjanje poteka in posledic nekaterih bolezni.

1.1.4.2 Izločanje zunajceličnih veziklov

Poleg parakrinega izločanja topnih molekul, MSC izločajo tudi številne molekule v zunajceličnih veziklih – heterogeni populaciji membranskih veziklov, ki se iz celice sproščajo z brstenjem membrane v notranjost ali zunanjost celice [57]. Zunajcelični vezikli so glede na mesto nastanka v celici in velikost razdeljeni v eksosome (vezikli endocitotskega izvora velikosti 30 – 150 μm), mikrovezikle (vezikli, ki nastanejo z brstenjem membrane, velikosti 100 – 1000 nm) in apoptotska telesca (vezikli, sproščeni med programirano celično smrtjo, velikosti 50 nm – 5 μm) [58]. Zunajcelični vezikli so prenašalci informacijske ribonukleinske kisline (RNK), male interferenčne RNK, beljakovin in mitohondrijev, ki zaradi membranske zaščite vezikla lahko prepotujejo daljše razdalje znotraj telesa [59, 60]. V večih raziskavah je bilo pokazano, da je učinek delovanja zunajceličnih veziklov MSC podoben učinku parakrine signalizacije [61-63], zaradi česar zunajcelični vezikli predstavljajo potencial za izkoriščanje terapevtskih učinkov MSC brez uporabe samih celic [64, 65].

1.1.4.3 Z apoptozo pogojena imunomodulacija

Eden izmed načinov terapevtskega delovanja MSC je tudi imunomodulacija, pogojena z apoptozo. Fagocitoza odmrlih celic igra pomembno vlogo v zmanjševanju vnetja in obnavljanju funkcije tkiva in tudi v adaptivnem imunskem odzivu v vnetem tkivu [66]. Tudi MSC so po intravenski aplikaciji celic v telo podvržene apoptozi. V eni od raziskav [67] so pokazali, da so MSC podvržene apoptozi po fizičnem stiku s citotoksičnimi $CD56^+$ naravnimi celicami ubijalkami in $CD8^+$ celicami T. Odmrle MSC fagocitirajo makrofagi, posledica česar je polarizacija makrofagov in modulacija adaptivnega imunskega sistema [67-69]. Z apoptozo

pogojena imunomodulacija je tudi ena izmed možnih razlag za dolgotrajno terapevtsko delovanje MSC, ki nasprotuje dokazom o njihovi kratkoživosti po intravenski aplikaciji [69].

1.1.4.4 Prenos mitohondrijev

Rezultati novejših raziskav kažejo, da je eden izmed možnih načinov delovanja MSC tudi prenos organelov med celicami s pomočjo nanocevk. Prenos mitohondrijev med somatskimi celicami in MSC je bil pokazan na mišjih modelih bolezni pljuč [70] in ledvic [71]. Prenos mitohondrijev med celicami je povezan z različnimi fiziološkimi in patološkimi stanji in bi lahko predstavljal potencial za zdravljenje različnih patoloških stanj v prihodnosti.

1.1.5 MSC v veterinarski regenerativni medicini

MSC so v veterinarski medicini v večji meri uporabljane eksperimentalno za zdravljenje številnih bolezni. Med te sodijo bolezni kit, tetiv in sklepov, bolezni ustne votline in zob, bolezni prebavnega trakta, jeter, ledvic in srca, bolezni dihal, bolezni kože in oči, bolezni živčnomišičnega sistema in bolezni spolnih organov. Izkoriščanje kompleksnih imunomodulacijskih sposobnosti MSC se je tako izkazalo za uspešno tudi pri zdravljenju različnih bolezni psov in mačk. Pri psih po uspešnosti zdravljenja najbolj stopajo v ospredje ortopedske bolezni [72-77], medtem ko se je pri mačkah kot najbolj obetavna indikacija za zdravljenje z MSC izkazal kronični gingivostomatitis [78-81]. Med bolezni z obetavnimi rezultati zdravljenja z MSC pri obeh vrstah živali se uvršča tudi kronična vnetna bolezen črevesa [82, 83].

Ker različen potencial MSC v pogojih *in vitro* lahko vodi v različen terapevtski učinek, so za namen presoje zdravljenja z MSC v veterinarski klinični medicini v teku številne raziskave, ki se osredotočajo na preiskovanje vplivov dejavnikov na lastnosti celic *in vitro.* Za namen uporabe MSC v terapevtske namene je potrebna izolacija, karakterizacija in namnožitev MSC. Pri psih in mačkah so bile MSC izolirane iz številnih tkiv, kot so maščobno tkivo [84-88], kostni mozeg [85, 86, 89-91], sklepna tekočina [84, 85, 92], popkovnica [93], Whartonova žolica [94], periferna kri [95], mišično tkivo in pokostnica [96]. Zaradi lahke dostopnosti, minimalnega posega, potrebnega za odvzem tkiva in velikega števila celic ob izolaciji, je maščobno tkivo najbolj uporabljano tkivo za izolacijo MSC [97]. Matičnost izoliranih celic je določena s sposobnostjo pritrditve celic na plastiko, trilinijskega diferenciacijskega potenciala in

izražanjem ustreznih površinskih označevalcev [16]. MSC živali so, tako kot MSC ljudi, sposobne adhezije na plastiko in difereneciacije v kostne, hrustančne in maščobne celice, vendar v primerjavi z MSC ljudi ne izražajo enakih površinskih označevalcev. Minimalna merila za identifikacijo MSC živali zaenkrat še niso določena. Ker je za namen zdravljenja živali z MSC potrebna obsežna namnožitev celic izven organizma, sta dve osnovni in pogosto proučevani lastnosti v pogojih *in vitro* njihov proliferacijski in diferenciacijski potencial. V večih raziskavah je bilo pokazano, da na proliferacijski in diferenciacijski potencial MSC *ex vivo* vplivajo različni dejavniki, kot so tkivo izvora MSC [84, 86, 89, 98], anatomsko mesto tkiva [88, 99, 100], starost živali [99, 101-103] in število pasaž [104-107]. Potencialni dejavnik vpliva na MSC bi lahko bila tudi vrsta živali. Posamezne študije, narejene na MSC posamezne vrste živali, so med seboj težko primerljive zaradi nestandardiziranih postopkov izolacije, karakterizacije in gojenja celic ter zaradi individualne sestave celičnih gojišč, uporabljanih med posameznimi laboratoriji. Posameznih raziskav, v katerih bi neposredno proučevali vpliv vrste, je zelo malo [108-110] in temeljijo predvsem na primerjavi MSC živali z MSC ljudi. Tudi vpliv spola je v veterinarski regenerativni medicini redko proučevan. Vpliv spola je bil pokazan v nekaterih raziskavah iz področja biologije matičnih celic in njihovega terapevtskega učinka [111-114].

Napredek v pridobivanju znanja s področja uporabe MSC in mehanizmov njihovega delovanja omogoča izrazito napredovanje razvoja ne le humane, ampak tudi veterinarske regenerativne medicine. Zato je proučevanje celic izjemnega pomena, saj nam omogoča vpogled v celično fiziologijo in procese tkiv izven organizma in posledično presojo uporabe MSC za zdravljenje.

1.1.6 Proučevanje MSC v dvo– in trodimenzionalnih modelih *in vitro*

1.1.6.1 Dvodimenzionalne kulture

Celične kulture predstavljajo model za proučevanje celic in procesov v tkivih izven organizma in so uveljavljene na področju raziskovanja celične in molekularne biologije, mutageneze in karcinogeneze ter učinkov zdravil in toksičnih komponent na celice [115]. Za proučevanje MSC je zaradi njihove sposobnosti adhezije na plastiko uveljavljena uporaba dvodimenzionalne (2D) podlage iz plastike. Prednosti gojenja MSC na plastiki so stroškovna nezahtevnost [116], preprostost gojenja ter možnost nadzora nad pogoji v kulturi [115]. Vse celice so izpostavljene

enakim količinam hranilnih snovi, rastnih in ostalih dejavnikov ter se posledično nahajajo v isti fazi celičnega cikla [116], kar omogoča enostavno interpretacijo ponovljivih rezultatov [117]. Priprava celic na nadaljne analize je nezahtevna [115]. Kljub prednostim uporabe 2D plastične podlage za proučevanje MSC se vedno bolj poudarja vprašanje ustreznosti uporabe 2D podlag. Ugotovljeno je bilo, da gojenje MSC na plastični podlagi vodi v heterogeno populacijo celic in lahko vpliva na njihove lastnosti [118, 119]. 2D podlaga iz plastike se namreč bistveno razlikuje od fiziološkega okolja v telesu, zato je podobnost celic, gojenih na plastiki, s celicami v naravnem okolju v telesu vprašljiva.

1.1.6.2 Vpliv naravnega okolja na delitev MSC

Pomembno vlogo pri ugotavljanju in primerjavi značilnosti celic v pogojih *in vitro* in *in vivo* igra poznavanje njihovega naravnega okolja in mehanizmov njihovega delovanja. Samoobnovitvena sposobnost in sposobnost diferenciacije v specializirane celice tkiv sta edinstveni lastnosti matičnih celic, ki sta posledici celičnih delitev znotraj njihovega naravnega okolja, kjer neprestano poteka ravnotežje med mirujočimi in delečimi se matičnimi celicami. Delitev matičnih celic je lahko simetrična, pri kateri nastaneta dve hčerinski matični celici, ali asimetrična, pri kateri nastaneta ena hčerinska identična matična celica in ena hčerinska progenitorna celica [120]. Celični cikel sestavljajo štiri faze – faza S (faza podvojitve DNK) in faza M (faza delitve) ter vmesni kontrolni fazi rasti G1 in G2. Napredovanje celičnega cikla je strogo nadzorovano s pomočjo kontrolnega sistema celičnega cikla, ki zagotavlja ohranjanje integritete celičnega genoma. Pomemben vpliv na celično delitev imajo mitogeni – zunajcelične signalne molekule, ki povzročajo napredovanje celičnega cikla skozi fazo G1 in s tem začetek diferenciacije. ESC imajo v primerjavi s somatskimi celicami značilno krajšo fazo G1 in posledično manj omejen diferenciacijski potencial [11]. Podobno velja tudi za IPSC [121]. Odrasle matične celice v izogib vplivom mitogenov in s tem diferenciaciji izstopijo iz celičnega cikla v fazo mirovanja G0 ter tako ohranjajo populacijo matičnih celic v tkivu [122]. Vstop v celični cikel in s tem začetek diferenciacije matičnih celic sovpada z zmanjšanjem proliferacije in povečanim izražanjem genov, značilnih za specifične celične linije [123]. Poleg mitogenov ima pomemben vpliv na celično delitev tudi vezava celic na površino – zunajcelični matriks. V naravnem okolju se matične celice povezujejo s proteini zunajceličnega matriksa preko transmembranskih adhezijskih proteinov – integrinov, ki so tesno povezani s citoskeletnima

elementoma aktinom in miozinom. Vezava integrinov na zunajcelični matriks omogoča ustvarjanje stika (fokalne adhezije) med celico in površino, ter mehanotransdukcijo – prenos informacij iz zunajceličnega matriksa v celico. Pomen integrinov je tudi v aktivaciji signalne poti pri nastanku ciklinov G1, ključnih regulatornih proteinov, pomembnih za vstop celice v celični cikel. Vezava celice na podlago ima torej pomembno vlogo za njeno delitev [124]. Zunajcelični matriks je ena izmed najpomembnejših komponent mikrookolja – niše, ki regulirajo delovanje MSC, t.j. adhezijo, migracijo, proliferacijo, diferenciacijo in preživetje MSC [120].

1.1.6.3 Niša MSC

Niša matičnih celic je specifično anatomsko mesto v visoko specializiranem mikrookolju, ki z vodenjem in nadzorovanjem njihove usode regulira delovanje matičnih celic pri nastanku, vzdrževanju in popravilu tkiva [125]. Ključna naloga niše je ohranjanje stalne zaloge matičnih celic in dinamično uravnavanje njihove samoobnove in diferenciacije z namenom zagotavljanja homeostaze in regeneracije tkiva. V niši so matične celice obdane s podpornimi celicami, zunajceličnim matriksom in intersticijsko tekočino. Strukturni proteini, kot so kolagen, fibronektin in laminin, dajejo zunajceličnemu matriksu mehanske lastnosti, ki celicam omogočajo adhezijo in mehanotransdukcijo. Tako so matične celice izpostavljene številnim zunanjim dejavnikom, kot so medcelične interakcije, interakcije celic z zunajceličnim matriksom, fizikalno–kemijski dražljaji (npr. temperatura, delni tlak kisika) in drugi topni dejavniki, npr. rastni dejavniki in citokini [5]. Kombinacija organizacije celičnega citoskeleta in delovanja zunajceličnih bioaktivnih molekul spodbuja ohranjanje matičnih celic znotraj niše in migracijo progenitornih celic iz niše ter njihovo diferenciacijo v specializirane celice tkiva [120]. Celično mikrookolje tako prispeva k prostorsko in časovno kompleksni signalizaciji, ki usmerja celični fenotip. Celica je skupaj z zunajceličnim matriksom, rastnimi dejavniki, hormoni in drugimi molekulami povezana v celoto, ki vodi delovanje posameznih organov in celotnega organizma [126]. Na spremembe v okolju, npr. na poškodbo ali bolezen, se celice odzivajo z izločanjem proteaz, rastnih dejavnikov in drugih signalnih molekul, ki usmerjajo znotrajcelično in medcelično komunikacijo in tako vodijo procese proliferacije, sinteze proteinov, migracije in apoptoze. Celični odzivi na spremembe v tkivu so posledica neposredne povezave z zunajceličnim matriksom ali vpliva signalnih molekul, ki izvirajo iz zunajceličnega

matriksa. Posledica izgube niše je izguba matičnih celic in s tem zmanjšanje regenerativnih zmožnosti [5]. Medsebojni vpliv matičnih celic in njihove niše ustvarja dinamični sistem, po katerem se zgledujejo modeli niš *in vitro*, ustvarjeni z namenom terapevtske rabe matičnih celic [125].

1.1.6.4 Trodimenzionalne kulture

Trodimenzionalni (3D) načini gojenja celic posnemajo ključne mehanske in biokemijske lastnosti naravnega celičnega okolja in posledično omogočajo boljši vpogled v fiziološko delovanje MSC [127], kar je še posebno pomembno z vidika uporabe MSC za terapevtske namene [118]. Študije vpliva 3D okolja na MSC so pokazale, da 3D okolje celicam nudi boljše možnosti za izkazovanje bioloških mehanizmov, vključujoč število celic, živost, morfologijo, proliferacijo, diferenciacijo, odzivanje na okoljske signale, medcelično komuniciranje, migracijo, stimulacijo angiogeneze, izogibanje imunskemu sistemu, izražanje genov in sintezo proteinov in se je tako z vidika posnemanja naravnega celičnega okolja izkazalo kot bolj ustrezno za gojenje celic [115]. Obstaja več različnih načinov gojenja celic v 3D modelih, ki jih lahko razdelimo na 2 skupini – 3D kulture z ali brez uporabe nosilcev. 3D kulture brez uporabe nosilcev vključujejo kulturo viseče kapljice, ki omogoča agregacijo celic na podlagi gravitacije, magnetno levitacijo oz. magnetno lebdenje, pri čemer je agregacija celic posledica izpostavljenosti magnetnim nanodelcem, ter gojenje celic na okroglih ploščah z nizko adhezivno prevleko, ki se običajno uporablja za proučevanje tumorskih celic [127]. V 3D kulturah z uporabo nosilcev oziroma biomaterialov se uporabljajo štiri osnovne skupine materialov – polimerni, keramični, kovinski in kompozitni materiali [128], med katerimi so najpogosteje uporabljani hidrogeli, polimerni materiali, hidrofilna steklena vlakna in organoidi [127]. Prednost uporabe 3D nosilcev je predvsem v možnosti posnemanja zunajceličnega matriksa *in vivo* [115]. Z napredovanjem razvoja 3D modelov za gojenje celic *in vitro* se večajo tudi možnosti za premostitev razlik med pogoji gojenja celic *in vitro* in živalskimi modeli. Ena izmed pomembnih prednosti uporabe 3D celičnih kultur je, da omogočajo simulacijo delovanja celice v naravnem okolju, a je hkrati proučevanje celice omogočeno v pogojih *in vitro*, zaradi česar je verjetna predpostavka, da se bo potreba po uporabi živalskih modelov v prihodnosti zmanjševala [127].

1.1.7 Biomateriali

1.1.7.1 Namen uporabe biomaterialov

Namen razvoja biomaterialov je posnemanje naravnega zunajceličnega matriksa za zagotavljanje potrebnih lastnosti celičnega okolja za vodenje usode matičnih celic znotraj njihove niše. Biomaterial mora zagotavljati mikrookolje, ki posnema fiziološko nišo in matičnim celicam omogoča pretvorbo vpliva strukturnih lastnosti biomateriala v biokemijske signale. Biomateriali lahko služijo kot bioadhezivne površine za namen adhezije in proliferacije celic v 2D celičnih kulturah, ali kot 3D celični nosilci za gojenje celic v okolju, ki bi omogočalo prostorsko in časovno zahtevne celične procese, potrebne za regeneracijo določenega tkiva [129].

1.1.7.2 Vpliv lastnosti biomaterialov na MSC

Za vodenje celične usode so ključne mehanske, površinske in kemijske lastnosti biomateriala [120]. Znano je, da so matične celice občutljive na mehanske lastnosti biomaterialov in da imajo zmožnost zaznavanja trdne podlage, četudi niso v neposrednem stiku z njo [130]. Njihov oprijem na podlago je odvisen od elastičnosti biomateriala, kar kaže na to, da že najmanjše spremembe mehanskih lastnosti biomateriala lahko vplivajo na diferenciacijo matičnih celic. Različna elastičnost biomateriala tako različno vpliva na celično adhezijo, proliferacijo in njihov diferenciacijski potencial. Večja trdnost biomateriala pogojuje večji potencial za osteogeno diferenciacijo, ki je posledica povečane aktivacije integrinov, mehkejši biomateriali pa večajo celični potencial za adipogeno ali hondrogeno diferenciacijo, ki sta posledici povečanega izražanja kolagena tipa II in lipaze lipoproteinov, označevalcev hrustančnih in maščobnih celic [131]. Matične celice se ne vežejo neposredno na površino biomateriala, ampak se v raztopini z vsebnostjo proteinov, npr. v mediju za gojenje celic, zaradi počasnejšega gibanja v primerjavi s proteini vežejo na površino biomateriala posredno preko vezave na predhodno vezane proteine. Vezava celic na proteine je odvisna od razporeditve in konformacije proteinov, slednji pa sta odvisni od omočljivosti in kemijske sestave biomateriala. Različne polarne skupine na površini biomateriala različno vplivajo na vezavo proteinov in lahko izzovejo denaturacijo ali spremembo orientacije proteinov. Ustrezna orientacija proteina omogoča prepoznavanje in vezavo površinskih celičnih receptorjev na vezavna mesta na proteinu. Za vodenje celične adhezije je torej posebnega pomena manipulacija proteinov,

vezanih na površino biomateriala [130]. Rezultati številnih študij kažejo tudi na vpliv kemijskih lastnosti površine biomateriala na smer celične diferenciacije [132-134]. Obdelava površine biomateriala z različnimi kemijskimi skupinami, npr. metilno (–CH3), amino (–NH2), tiolno (–SH), hidroksilno (–OH) ali karboksilno (–COOH) skupino lahko različno vpliva na celično usodo in vodi MSC v smeri adipogene, osteogene ali hondrogene diferenciacije [133, 134]. Smer celične diferenciacije pa je ob dodatku iste kemijske skupine lahko različna v 2D ali 3D okolju [130]. 2D ali 3D okolje lahko torej neodvisno od funkcionalne kemijske skupine različno vpliva na usodo MSC.

1.1.7.3 Ogrodje biomaterialov za enkapsulacijo celic

Poleg mehanskih, površinskih in kemijskih lastnosti je za biomaterial pomembno tudi samo ogrodje oz. konstrukcija materiala, ki omogoča enkapsulacijo celic. 3D biomateriali so lahko mikroporne, nanofibrozne ali hidrogelne sestave. Mikroporni nosilci sicer omogočajo enkapsulacijo celic, vendar zaradi velikosti por (100 µm), večjih od povprečnega premera celice (10 µm), predstavljajo ukrivljeno 2D mikrookolje. Nanofibrozni nosilci z vsebnostjo fibrilarnih proteinov zunajceličnega matriksa zagotavljajo boljši približek naravnega celičnega okolja, vendar so njihove mehanske lastnosti preslabe, da bi obvladovale stres, potreben za mehanotransdukcijo. Hidrogeli nimajo omenjenih omejitev, zato predstavljajo ustrezen biomaterial za razvoj zunajceličnemu matriksu podobnega okolja. Mrežasta struktura medsebojno povezanih polimernih verig omogoča visoko vsebnost vode, transport kisika, hranilnih in odpadnih snovi ter drugih topnih molekul. Hidrogeli so lahko sestavljeni iz številnih naravnih ali sintetičnih materialov, ki nudijo širok razpon različnih mehanskih in kemijskih lastnosti [126]. Naravni hidrogeli v primerjavi s sintetičnimi ne le omogočajo, ampak tudi spodbujajo njihovo delovanje. Naravne hidrogele navadno sestavljajo proteini zunajceličnega matriksa, kot so kolagen, fibrin, hialuronska kislina, ali pa sestavine iz drugih bioloških virov kot so hitozan [135], alginat [136], amnijska membrana [137] in svila [138].

Področje uporabe biomaterialov v tkivnem inženirstvu se usmerja v razvoj univerzalnih biomaterialov, s katerimi bi bilo moč voditi usodo celic v katerokoli želeno smer [139] in ki bi bili ustrezni za vnos v telo [129]. Zaenkrat ne poznamo biomateriala, ki bi zadostil potrebam vseh vrst tkiv, zato po vsem svetu potekajo raziskave v smeri iskanja univerzalnega

biomateriala za uporabo v regenerativni medicini. Eden izmed obetavnih biomaterialov za uporabo v tkivnem inženirstvu je svilni fibroin (SF).

1.1.8 Svilni fibroin

Zaradi svojih mehanskih lastnosti, elastičnosti, prilagodljivih strukturnih in morfoloških lastnosti ter biokompatibilnosti [138] v ospredje številnih raziskav s področja biomaterialov stopa SF. Svilni proteini so prisotni v žlezah sviloprejk, pajkov, škorpijonov, pršic in čebel. Naravni biopolimer, ki ga proizvaja sviloprejka *Bombyx mori*, je najpogosteje uporabljena, komercialno dostopna in najbolje proučena svila, ki je sicer že dolga leta v uporabi v tekstilni industriji in v medicini kot šivalni material [138]. Svila je sestavljena iz dveh proteinov – fibroina, ki predstavlja glavno komponento svile, in sericina, ki povezuje vlakna SF med seboj. Sericin je imunogen in se ga med pripravo nosilca SF odstrani. SF sestoji iz lahke (25 kDa) in težke polipeptidne verige (350 kDa), med seboj povezanih z disulfidno vezjo. Na obe verigi se nekovalentno pripenja še dodatni glikoprotein p25, ki utrjuje integriteto svile. Hidrofobne domene težke verige sestojijo iz ponavljajočih se polipeptidnih enot, med seboj povezanih z vodikovimi vezmi. Hidrofobne domene tvorijo kristale iz β–plošč, zavzemajo do 70 % celotne strukture proteina ter svili dajejo moč in stabilnost. Hidrofobne domene so obdane s hidrofilnimi neponavljajočimi se amorfnimi regijami iz lahkih verig, ki svili omogočajo prožnost. Neurejene hidrofilne domene prej pridejo v stik z vodo, kisikom in encimi in so posledično bolj dovzetne za razgradnjo v primerjavi s hidrofobnim delom. Razgradnja in mehanske lastnosti svile so tako močno odvisne od sekundarne strukture SF. Kemijski in fizikalni dejavniki v žlezi sviloprejk vodijo v spremembo lastnosti svile, ki je potrebna za njeno izločanje. S spreminjanjem pH, koncentracije ionov ali strižnih sil v žlezi se sekundarna struktura SF pretvarja v topno – neurejeno, ali netopno – urejeno konformacijo iz β–plošč ter se tako spremenja v sol ali gel stanje. Mehanizem spreminjanja topnosti svile znotraj žlez sviloprejke je slabo poznan, ugotovljeno pa je, da ima pri tem pomembno vlogo kalcij, katerega koncentracija se v različnih delih žleze spreminja. Z dodatkom $CaCl_2$ je možno doseči pretvorbo SF iz sol v gel stanje. Konformacijske in reološke spremembe SF v žlezah sviloprejk nastajajo z namenom potovanja tekočega SF skozi lumen in nastajanja vlakna v distalnem delu predilne žleze. Možnost spreminjanja in prilagajanja molekularne strukture in morfologije svilnega proteina omogoča uporabo SF tudi v tkivnem inženirstvu [140]. V zadnjih letih se SF

testira in uporablja kot biomaterial v tkivnem inženirstvu v različnih oblikah kot so filmi, vlakna, mreže, hidrogeli in porozni 3D nosilci [138].

1.1.9 Vpliv svilnega fibroina na proliferacijo in diferenciacijo MSC

SF v različnih oblikah, kot so filmi [141, 142], vlakna [143], mreža vlaken [144] ali 3D nosilec [145, 146], omogoča proliferacijo in diferenciacijo MSC. Proliferacija in diferenciacija MSC na SF sta odvisni od mikrostrukturnih, mehanskih in površinskih lastnosti SF. V nekaterih raziskavah so pokazali, da 3D struktura SF omogoča boljšo proliferacijo in diferenciacijo MSC kot 2D filmi SF, najverjetneje zaradi boljšega posnemanja naravnega celičnega okolja, ki ga nudi 3D oblika [147, 148]. Na boljšo proliferacijo in diferencijo MSC, gojenih na SF, je pomembno vplivalo tudi kombiniranje SF z drugimi elementi, kot so grafenov oksid [147] ali zaporedje aminokislin arginin–glicin–aspartat (RGD), vezavna domena fibronektina zunajceličnega matriksa [149]. Hondrogeno diferenciacijo MSC, gojenih na SF, so spodbudili z dodajanjem hialuronske kisline [150], askorbinske kisline [151] ali krvno plazmo, obogateno s trombociti (angl., Platelet rich plasma, PRP) [151, 152], osteogeno diferenciacijo pa z dodatkom kombinacije polikaprolaktona, deksametazona in askorbinske kisline [153]. Nadalje na proliferacijski in diferenciacijski potencial MSC na SF vpliva tudi spreminjanje mehanskih in površinskih lastnosti SF [154], stopnja mineralizacije [145] in različna poroznost 3D nosilca SF [146]. Površinske, kemijske, mehanske in mikrostrukturne lastnosti SF so tako odločilnega pomena za vodenje celične usode *in vitro* in posledično njihov regeneracijski potencial *in vivo.*

1.1.10 Idealni celični nosilec

Ne glede na vrsto nosilca obstajajo ključni dejavniki, ki jih je potrebno obravnavati pri odločanju o primernosti nosilca za uporabo v tkivnem inženirstvu. 1) Nosilci morajo biti biokompatibilni: omogočati morajo celično adhezijo, normalno celično funkcijo, migracijo celic skozi nosilec, pred izločanjem novega matriksa pa morajo biti celice na nosilcu sposobne proliferacije. Po presaditvi v telo nosilec ne sme izzvati imunskega odziva, ki bi sicer lahko vodil v zmanjšano celjenje ali zavrnitveno reakcijo. 2) Nosilec mora biti razgradljiv in tako omogočiti telesu lastnim celicam, da nosilec nadomestijo in začno izločati lastni zunajcelični matriks. Produkti razgradnje nosilca morajo biti netoksični in sposobni eliminacije iz telesa, ne

da bi pri tem škodovali drugim organom. 3) Nosilec mora imeti mehanske lastnosti, ki se skladajo z anatomskim mestom, kamor bo presajen. Hkrati mora biti dovolj močan, da prenese kirurško manipulacijo med presajanjem. Ravnotežje med mehanskimi lastnostmi in porozno strukturo, ki omogoča celično infiltracijo in vaskularizacijo, je ključnega pomena za uspešno rabo nosilca. 4) Struktura nosilca mora biti porozna, da lahko zagotovi infiltracijo celic, zadostno difuzijo hranilnih snovi in odstranjevanje odvečnih produktov ter da celicam omogoča izločanje zunajceličnega matriksa [3]. Meja najustreznejše velikosti por naj bi segala od 85 do 325 µm, odvisno od tkiva in vrste uporabljenih celic [155]. 5) Da bi bila uporaba nosilca v klinčni medicini izvedljiva, mora biti proizvodnja nosilca stroškovno sprejemljiva, hkrati pa mora omogočati izdelavo večih nosilcev naenkrat. Razvoj proizvodnega postopka v okviru standardov dobre proizvodne prakse je ključnega pomena za zagotovitev uspešnega prenosa tkivnega inženirstva iz laboratorija v kliniko [3].

1.2 NAMEN DELA IN HIPOTEZE

Prvi namen našega dela je bil ugotoviti, kako vrsta, spol živali ter pozne pasaže vplivajo na proliferacijski in diferenciacijski potencial AMSC psov in mačk, gojenih na običajni podlagi iz plastike:

1. Gojenje celic *in vitro* omogoča preiskovanje celic in fizioloških ter bolezenskih procesov tkiva izven oganizma. Običajna podlaga za gojenje matičnih celic je 2D osnova iz polistirena. Ker je za namen zdravljenja bolezni z MSC potrebna obsežna namnožitev celic in transport celic od laboratorija do pacienta in ker različne lastnosti MSC v pogojih *in vitro* lahko vodijo v potencialno različen terapevtski učinek, so za namen presoje zdravljenja z MSC v veterinarski klinični medicini potrebne raziskave, ki temeljijo na odkrivanju vplivov različnih dejavnikov na osnovne značilnosti MSC *in vitro*. Med njimi sta vpliv vrste in vpliv spola v veterinarski regenerativni medicini redko proučevana.

Drugi namen našega dela je bil gojiti celice na celičnih nosilcih iz SF in ugotoviti, kako svilni fibroin vpliva na AMSC:

2. Poskusi, izvedeni v 2D celičnih modelih, nam omogočajo razumevanje osnove celičnega delovanja in povezavo celic z mikrookoljem, v katerem se nahajajo. Vendar se zaradi težnje k posnemanju naravnega celičnega okolja, stremenja po možnosti nadzora, vodenja usode MSC *in vitro* ter možnosti uvajanja novih načinov vnosa celic v telo za gojenje MSC vedno bolj poudarja pomen uporabe celičnih nosilcev. Običajne plastične podlage za gojenje celic tako nadomeščajo 3D načini gojenja celic. Zaradi svojih mehanskih lastnosti, elastičnosti, biokompatibilnosti in nadzorovane razgradnje v ospredje številnih raziskav s področja biomaterialov stopa SF, naravni polimer, ki v različnih oblikah, kot so filmi, vlakna, mreža vlaken ali 3D nosilec, omogoča proliferacijo in diferenciacijo MSC.

Zastavili smo si štiri delovne hipoteze:

1 Vrsta živali vpliva na proliferacijo in diferenciacijo AMSC
2 Spol živali vpliva na proliferacijo in diferenciacijo AMSC psov in mačk
3 Pozne pasaže negativno vplivajo na proliferacijo in diferenciacijo AMSC psov in mačk
4 Svilni fibroin omogoča adhezijo, proliferacijo in diferenciacijo AMSC

2 OBJAVLJENA ZNANSTVENA DELA

2.1 MATIČNE CELICE V VETERINARSKI MEDICINI: TRENUTNO STANJE IN MOŽNOSTI ZDRAVLJENJA

Stem cells in veterinary medicine: current state and treatment options

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Izvleček

V zadnji letih se močno razvija regenerativna medicina - veja medicine, ki razvija metode za rast, popravilo ali nadomeščanje poškodovanih, obolelih ali odmrlih celic, organov in tkiv. Matične celice so nediferencirane celice z zmožnostjo samoobnavljanja in diferenciacije v specializirane celic tkiv. Namen zdravljenja z matičnimi celicami je premostitev telesne nezmožnosti za regeneracijo poškodovanih tkiv ali metabolnih procesov po akutni ali kronični poškodbi. Koncept zdravljenja z matičnimi celicami je bil prvič predstavljen leta 1991 s predpostavko, da je množično diferenciacijo celic v celice kateregakoli tkiva mogoče doseči z izolacijo, gojenjem in namnožitvijo matičnih celic v pogojih *in vitro*. Med različnimi vrstami matičnih celic so mezenhimske matične celice (MSC) prepoznane kot najustreznejše za uporabo v terapevtske namene zaradi enostavne izolacije in gojenja ter odsotnosti etičnih zadržkov pri njihovi uporabi. Zaradi izjemnih imunomodulatornih sposobnosti MSC in omejitev obstoječih možnosti zdravljenja se vedno bolj razvija tudi veterinarska regenarativna medicina. MSC predstavljajo možnost zdravljenja različnih belozni živali, kot so ortopedske in orodentalne bolezni, bolezni prebavnega trakta, bolezni jeter, ledvic, srca, kože in dihal, živčnomišična obolenja, očesne bolezni in bolezni reprodukcijskega sistema. Kljub napredku v znanju in razumevanju delovanja MSC pa še vedno ostajajo odprta številna vprašanja v zvezi z rabo MSC v terapevtske namene. Namen tega pregleda je bil povzeti trenutno stanje in izpostaviti ključne izzive na področju rabe MSC za terapevtske namene v veterinarski regenerativni medicini ter predstaviti rezultate klinične uporabe MSC pri veterinarskih pacientih.

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Stem Cells in Veterinary Medicine—Current State and Treatment Options

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Regenerative medicine is a branch of medicine that develops methods to grow, repair, or replace damaged or diseased cells, organs or tissues. It has gained significant momentum in recent years. Stem cells are undifferentiated cells with the capability to self—renew and differentiate into tissue cells with specialized functions. Stem cell therapies are therefore used to overcome the body's inability to regenerate damaged tissues and metabolic processes after acute or chronic insult. The concept of stem cell therapy was first introduced in 1991 by Caplan, who proposed that massive differentiation of cells into the desired tissue could be achieved by isolation, cultivation, and expansion of stem cells in *in vitro* conditions. Among different stem cell types, mesenchymal stem cells (MSC) currently seem to be the most suitable for therapeutic purposes, based on their simple isolation and culturing techniques, and lack of ethical issues regarding their usage. Because of their remarkable immunomodulatory abilities, MSCs are increasingly gaining recognition in veterinary medicine. Developments are primarily driven by the limitations of current treatment options for various medical problems in different animal species. MSCs represent a possible therapeutic option for many animal diseases, such as orthopedic, orodental and digestive tract diseases, liver, renal, cardiac, respiratory, neuromuscular, dermal, olfactory, and reproductive system diseases. Although we are progressively gaining an understanding of MSC behavior and their mechanisms of action, some of the issues considering their use for therapy are yet to be resolved. The aim of this review is first to summarize the current knowledge and stress out major issues in stem cell based therapies in veterinary medicine and, secondly, to present results of clinical usage of stem cells in veterinary patients.

Keywords: stem cells, clinical veterinary medicine, regenerative medicine, dogs, cats, horses

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TYPES OF STEM CELLS

By definition, stem cells are undifferentiated cells capable of self—renewal and transformation into different specialized cells (1). They are classified by their source as (a) embryonic (ESC), (b) adult, and (c) induced pluripotent stem cells (IPSC) (2, 3). Considering their phase of development and differentiation, they are further classified as totipotent, pluripotent, or multipotent cells (4).

Totipotent stem cells are present only in a very early embryo during the morula stage before gastrulation starts. They are capable of developing into all embryonic and extra-embryonic tissues. Subsequent divisions of cells during early embryonic development lead to the emergence

of the blastocyst with pluripotent ESC being present in the inner cell mass. ESC can give rise to all tissue cells in the body, with the exception of extra-embryonic tissues and germ cells (2, 5). With further cell development, pluripotent ESC gradually lose their pluripotency and become multipotent. The multipotent stage is characterized by the ability of cells to differentiate into limited types of specific cells, often depending on their germ layer origin (6).

The first isolation of human ESC was reported in 1998 (7). This triggered numerous studies about gene expression and function during embryonic development and cell differentiation processes, as well as attempts to identify gene targets for new drugs that might be useful in tissue regeneration therapies. However, broad-spectrum therapeutic capabilities of human ESC collided with ethical, moral, and cultural dilemmas because their harvesting is associated with the destruction of human embryos. Other sources of stem cells, therefore, had to be explored to continue the research into stem cell—based therapies. One alternative was developed in 2006 by Takahashi and Yamanaka, who reprogrammed adult mouse fibroblasts into pluripotent stem cells by retroviral transduction of four specific genes: OCT4, c-Myc, SOX2, and KLF4. These cells were termed IPSC and are similar to the ESC in their morphology, growth properties, and in the expression of ESC marker genes. Although the discovery of IPSC was remarkable progress in stem cell therapy, retroviral transductions can create chromosomal alterations, which increase the risk of tumorigenesis, raising questions about the safety of IPSC for regenerative medicine (3).

Another alternative to ESCs presents the stem cells which are present in the adult organism. Bone marrow and umbilical cord blood contain hematopoietic stem cells (HSCs) and non-hematopoietic or mesenchymal stem cells (MSC), the latter residing also in numerous other tissues. These cells are multipotent because they can differentiate into specific body cell types. HSCs can differentiate into different cells of the immune system, erythrocytes and platelets, and MSCs into cells of bone, cartilage, ligaments, tendons, fat, skin, muscle, and connective tissue. MSCs are activated endogenously when needed to replace dead, injured, or diseased tissue cells (8). The first mention of adult multipotent cells/MSC dates to 1968 when the osteogenic population of cells with fibroblast-like morphology was isolated from the bone marrow (9). Early studies showed that multipotent stem cells are capable of differentiating into osteoblasts, chondroblasts, and adipocytes (10). This leads to the belief that MSCs show their therapeutic potential through differentiation into tissue cells (11, 12). However, numerous subsequent studies have questioned this and today it is believed that the primary mechanism of MSC regenerative abilities stems from their immunomodulatory and tissue repair mechanisms. It is presumed that perivascular localization of MSC in various tissues plays an essential role in enabling these cells to detect local or distant tissue damage and respond to it by directed migration to the site of injury and participation in the healing process (13). Based on this, Caplan proposed that the term "mesenchymal stem cells" should be changed into "medicinal signaling cell" (MSC) (14).

Compared to other stem cell types, MSCs are recognized as the most promising stem cell type for stem cell therapy due to the simple procedures needed for their harvest, isolation, high cell yield upon their harvesting, and the lack of ethical restraint when in use. To prevent the confusion in the field of adult stem cells research, The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) proposed a set of standards to define human MSC for both laboratory-based scientific investigations and pre-clinical studies (15). In essence: (1) MSC must be plastic-adherent when maintained in standard culture conditions using tissue culture flasks, (2) 95% of the MSC population must express CD105, CD73, and CD90 and lack the expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA class II, and (3) MSCs must be able to differentiate into osteoblasts, adipocytes, and chondroblasts under standard *in vitro* differentiating conditions.

MSC SOURCES

Tissue Origin of MSC

To date, MSCs were successfully isolated from various tissues, and based on the source they have different properties, which should be considered when choosing the optimal stem cell therapy approach aiming at the tissue healing. In dogs, horses and cats, the most common companion veterinary patients, MSCs have been isolated from bone marrow (16–23), adipose tissue (16, 17, 19–21, 23, 24), synovium (16), synovial fluid (17, 21, 25, 26), synovial membrane (26), infrapatellar fat pad (16), umbilical cord (27–29), umbilical cord blood (19, 30, 31), Wharton's Jelly (19, 31), muscle and periosteum (20, 32), gingiva and periodontal ligament (33), peripheral blood (34–37), endometrium (38), and placenta (31). In mice, MSCs were also isolated from the brain, spleen, liver, kidney, lung, muscle, thymus, and pancreas (39). Currently, the most commonly used sources of MSC for stem cell therapies are bone marrow and adipose tissue because they offer larger number of MSCs than other tissues. Among the two, the adipose tissue is a particularly attractive source of MSCs due to the minimally invasive procedure needed to obtain cells. Although MSCs isolated from bone marrow and adipose tissue have similar surface immunophenotyping and trilineage differentiation (16, 17, 40), there are important differences in terms of proliferation and differentiation capacity, and their secretory profiles. In some studies, canine adipose tissue derived MSC (ADMSC) were shown to have higher proliferative potential (17, 19, 40, 41), whereas bone marrow derived MSC (BMMSC) exhibited a higher secretory production of soluble factors and exosomes (19, 41). Canine ADMSCs were reported to have superior chondrogenic (17) and osteogenic potential (19) in comparison to BMMSCs, whereas in horses, chondrogenic and osteogenic potential seem to be higher in BMMSC (42, 43). Equine BMMSCs also seem to have a higher migration capacity (21) than ADMSCs. Another potential source of stem cells with high chondrogenic potential might be synovium derived MSCs, as some studies have shown that they are expanding more rapidly than ADMSC in horses (21) and have a greater chondrogenic potential than ADMSC and BMMSC in dogs (16, 17). When choosing adipose tissue as a source of MSCs, anatomical site of

harvesting is also important. Guercio et al. (44) reported that subcutaneous ADMSCs have better proliferation potential than ADMSCs derived from visceral fat depots, and Yaneselli et al. (45) reported that subcutaneous ADMSCs remain multipotential in cell culture for a longer time and have higher osteogenic potential. Bahamondes et al. (46) also reported that visceral adipose tissue yields a higher number of MSCs in comparison to subcutaneous adipose tissue.

Since differences in stem cell properties might lead to differences in the success of stem cell therapy, they will have to be explored more closely in the future. Currently, there is no evidence that would generally suggest the preferential tissue source of MSC. This is at least partially due to variability in donors' species, donors' age, and donors' health conditions in different studies. Moreover, lack of standardization for the isolation, culture, and characterization of animal MSC considerably hinders the comparison of results between studies, and the variety of tissue sources are causing problems to set the criteria to define MSC. To date, there are no minimal established criteria for the identification of MSC in animals like criteria in humans (15). While all animal MSC show plastic adherence and differentiation potential, not all express the same panel of surface antigens that has been described for human MSC. Most non-human MSC express CD29 and CD44. However, the expression of CD73, CD90, and CD105 varies depending on the species and strain (47).

Autologous and Allogeneic MSC

Based on the donor–recipient relationship, stem cells can be classified as autologous, allogeneic, or xenogeneic stem cells. Autologous stem cells are collected from and administered to the same individual, allogeneic stem cells are collected from a donor and used in a recipient of the same species, whereas xenogeneic stem cells are those that are transplanted across species (48). When aiming to choose the most appropriate type of cells for particular stem cell therapy, choosing between autologous vs. allogeneic sources may prove challenging, and advantages and disadvantages for one over the other option should be considered. The isolation and expansion of autologous stem cells are time-consuming and associated with the costly procedure. Moreover, the potency of autologous MSC could be affected by patient age (44, 49–53) and existing disease (54). The need for allogeneic off-the-shelf stem cell products derived from young and healthy donors is, therefore, on the rise.

The main concern with allogeneic stem cell therapy is the possibility that MHC I surface molecules on allogeneic MSCs are recognized by recipient CD8+T cells, leading to direct cytotoxicity of foreign cells. In addition, MHC II molecules can be recognized by recipient CD4+ T cells, leading to either cytotoxic or humoral immune response. MHC molecules could also be subjected to indirect recognition by antigen presenting cells, leading to alloantibody production in B cells (55). Despite promising results regarding the safety of allogeneic MSC, several studies conducted *in vitro* (56, 57) and *in vivo* not only in rodents (58, 59) but also in horses (60–62) and dogs (63), showed immunogenic responses provoked by allogeneic MSC. This has raised some concerns about their presumed immunoprivileged characteristics. Joswig et al. (60), Bertoni et al. (64), and Cabon et al. (63) reported local side effects when the application of allogeneic cells was repeated and proposed that adverse reactions are most likely due to recipient's immune recognition of cells after re-exposure. However, when the effects of single and repeated applications of allogeneic cells for osteoarthritis treatment were compared in horses, no clinically relevant differences were observed in the outcome (65).

In line with contradictory clinical outcomes concerning to the immunogenicity of MSC, conflicting results have also been reported in terms of MHC expression depending on their state, tissue origin, breeds, individual donor, and culture conditions. For example, Menard et al. (66) showed that ADMSCs possess an increased capacity to modulate immune cells and that their phenotypic and transcriptomic profile is consistent with lower immunogenicity in comparison to BMMSC.

Regardless of many positive results of the studies encouraging the use of allogeneic MSC, several studies have confirmed that immunosuppressive properties of MSC do not exclude their immunogenicity. Further research is therefore needed to determine potential mechanisms to regulate MHC expression on MSC and to reach an agreement on the issue of MSC immunogenicity. Autologous stem cells, therefore, remain the most commonly used stem cell source in contemporary veterinary medicine.

THERAPEUTIC POTENTIALS OF MSC

Although stem cells were initially thought to be the source of cells that would differentiate and replace damaged or diseased tissues, it has become evident that the therapeutic properties of MSC are achieved mainly through their immunomodulatory functions, which operate in the interaction with the immune system cells. Complex immunomodulation activity of MSC includes their paracrine action, secretion of extracellular vesicles (ECV), apoptosis mediated immunomodulation, and mitochondrial transfer of membrane vesicles and organelles.

Paracrine Effects

Increasing evidence suggests that the primary mechanism of action of MSC relies on paracrine signaling which results in functional changes in the immune cells, such as monocytes/macrophages (67), dendritic cells (68), T-cells (69), B-cells (70), and natural killer cells (71). Several factors have been reported to contribute to the immunomodulatory effects of MSC. Among them are well-established effectors such as transforming growth factor-beta (TGF-β), indolamine-2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), interleukin 10 (IL-10), and tumor necrosis factor- (TNF) stimulated gene-6 (TSG-6).

TGF-β is involved in many biological processes such as proliferation and differentiation of cells, embryonic development, wound healing, and angiogenesis (72). It affects migration and homing of MSC (73, 74) and their proliferation and differentiation. TGF-β was shown to induce a switch from inflammatory (M1) to antiinflammatory/regulatory (M2) state of macrophages and thus importantly participates in the induction of the regulatory T-cells (Tregs) (75–79). IDO, a

metabolic enzyme, is another soluble factor that is secreted by MSC in an inflammatory environment (70). IDO catalyzes reactions leading to T- and B-cell cycle arrest (80), inhibition of T-cell proliferation and induction of Tregs generation (81), inhibition of B-cells (80), and NK cells (82), and is correlated with bystander differentiation of M2 macrophages (83). PGE2, the major prostaglandin, modulates chemokine production, inhibits the attraction of proinflammatory cells, and enhances differentiation of regulatory cells (84). It is a crucial mediator in NK-cell inhibition (82) and has a role in macrophage polarization toward the M2 phenotype (85). Also, its role was recently demonstrated in the clearance of apoptotic cells by MSC (86). IL-10 is an antiinflammatory cytokine that limits Th1 and Th2 response and accessory functions of macrophage and dendritic cells while inhibiting T-cell expansion (87) and driving the production of Tregs (88). MSCs' secretion of IL-10 is stimulated by an inflammatory environment and contact with T-cells (68, 89). TSG-6 is an inflammation-associated protein with anti-inflammatory and protective properties (90). MSC constitutively express TSG-6, which affects their morphology, the size of ECV, proliferation rate, differentiation potential, survival, and colony-forming unit capacity and is, therefore, crucial in maintaining MSC stemness (91). It was shown that TSG-6 induces the switch from M1 to M2 phenotype and increases the number of Tregs, resulting in relieving the symptoms of inflammatory conditions in experimental models of many diseases (92–95).

MSCs are, therefore, capable of altering the course and consequences of a particular disease through the paracrine effects on an individual's immune response.

Secretion of Extracellular Vesicles (ECV)

The paracrine action of MSCs is not limited solely to the secretion of soluble factors since MSCs have the capability to transfer various molecules through the extracellular vesicles (ECV). ECVs are vesicles arising from the plasma membrane by outward or inward budding (96). They are carriers of miRNA, mRNA, proteins, and mitochondria that are protected by the membrane. This enables ECVs to move long distances inside the body (97, 98). ECV include exosomes, which are 30–150 nm large plasma membrane coated vesicles of endocytotic origin, microvesicles, which are 100–1000 nm large vesicles of non-endocytotic origin, and apoptotic bodies, 50 nm−5 μm large vesicles released during membrane blebbing of apoptotic cells (99).

MSCs were shown to secrete exosomes and at least three other similarly sized types of ECV (100).

Regarding ECVs mechanisms of action, they seem to be similar to those exhibited by MSC themselves. The study by Hyvarinen et al. (101) demonstrated that ECVs enhance M2 macrophages in the same way as MSC, via PGE2 activation. Further, results from a recent study suggest that ECVs suppress T-cells through TGF-β and adenosine signaling (102). The primary role in increasing Tregs was attributed to TSG-6 from canine ADMSC-derived ECVs used for therapy of induced colitis in mice (92). ECVs were also shown to upregulate the IL-10 production when used to treat a mouse model of sepsis (103). In the pig, mouse, and rat animal models, MSC derived ECVs were reported to be beneficial in the respiratory (104), renal (105), and liver diseases (106), and also in the treatment of osteoarthritis (107), spinal cord injury (108), cerebral ischemia (109), and myocardial infarction (110). ECVs were also used in dogs and horses. Kornicka-Garbowska et al. (111) reported improved angiogenesis and elasticity of damaged tendon in a stallion after treatment with ADMSC-derived microvesicles. In dogs, ECVs were reported to promote vascularization, collagen synthesis, and cutaneous wound healing with better effects than their originator cells (112).

ECVs represent the potential to exploit MSC effects in a cell-free manner, with the main advantage being the avoidance of possible MSC side effects such as immune response and pulmonary embolism upon intravenous (IV) application of MSC (97, 98). Yet, cell-to-cell contact is believed to be important for some MSC immunomodulatory properties (68, 70, 113). When heat-inactivated MSCs without secretome but with the intact membrane integrity were infused IV, they did modulate monocyte function in the same way as control cells, increasing IL-10 levels and reducing IFN-γ levels. The results of this study suggest that immune response after MSC administration is not dependent on their active immunomodulatory activity (114), and contact with MSCs alone is sufficient for some immunomodulatory effects.

However, the lack of standardized techniques for isolation and purification of the ECVs remains the major limitation in ECV research. The most commonly used methods for exosome isolation are ultracentrifugation, ultrafiltration, tangential flow filtration, precipitation and size exclusion chromatography and immunoaffinity based methods. For example, key markers of exosomes are associated with endocytosis (115) and include caveolins, clathrins, transferrin receptors, tetraspanins (CD81, CD63, CD9), Alix and TSG101 (116). Ligands and cargo differ between ECV types leading to the presumption that each type of ECVs has a different function. Lack of standardized methods for exosome isolation leads to the incapacity to separate exosomes from other similarly sized ECV. Moreover, there is presently no standard measurement for ECV purity. Inconsistencies in describing ECVs are, therefore, present in the literature (116, 117). Guidelines of the International Society for Extracellular Vesicles appeal to the researchers to use the generic term "extracellular vesicle" rather than a designation of a specific subtype, which should be carefully defined if used. Furthermore, guidelines suggest that isolation and preparation procedure should be described in detail to allow the replication. Confirmation of ECV function requires demonstration that the effect of ECVs occurs without cell-cell contact, and is not achieved with the soluble, non-ECV associated secreted factors (118).

Apoptosis-Mediated Immunomodulation

Apoptosis might also play an important role in the immunomodulatory effect of MSC. Phagocytic clearance of dying cells (efferocytosis) takes part not only in resolving inflammation and restoring the function of damaged tissue but also in the adaptive and immune responses in inflamed

tissues (119). In a study conducted by Luk et al. (114), heat-inactivated MSC modulated monocyte function in the same way as control cells, resulting in increasing IL-10 levels and reducing IFN-γ levels. The results of this study suggest that immune response after MSC administration is not dependent on their active immunomodulatory activity but is derived from other cells, triggered by MSC presence. Recent evidence also shows that innate immune system cells are determinant in mediating the MSC effect. In particular, it was demonstrated by Galleu et al. that MSCs undergo apoptosis in the presence of cytotoxic cells, namely CD56+ NK cells and CD8+ T-cells, after being IV infused. MSC apoptosis induced by cytotoxic cells is MHC-independent and requires physical contact between MSC and cytotoxic cells. Apoptotic MSCs are then phagocytosed by macrophages that ultimately deliver immunosuppressive activity by producing IDO (120). Similar results were obtained by Cheung et al. (121), where monocytes, engulfed with apoptotic MSC, enhanced the inhibition of T-cell proliferation by producing PGE2. Mechanism of apoptosis derived immunosuppression can be, therefore, predictive in clinical therapies where patients displaying high cytotoxicity would be more responsive to MSC (120, 121). de Witte et al. (122) also showed that MSCs were rapidly phagocytosed in the lungs by monocytes and neutrophils after IV administration in mice. Phagocytosis of MSC induces expression of regulatory phenotype in monocytes and induces their polarization, which in turn modulates an adaptive immune system by inducing T-reg cells.

Mitochondrial Transfer

The mitochondrial transfer has been proposed as another mechanism of MSC action. In addition to transferring molecules via ECVs, MSCs seem to be capable of intercellular transfer of organelles via tunneling nanotubes. In 2006 the first mitochondria transfer between MSC and somatic cells was observed (123). This study revealed that active transfer of mitochondria from adult stem cells to somatic cells can rescue aerobic respiration in mammalian cells with non-functional mitochondria. In a mouse model of pneumonia, human BMMSC could transfer their mitochondria through the tunneling nanotubes to alveolar macrophages, which led to the enhanced phagocytosis of macrophages and antimicrobial effect of MSC (124). Mitochondria transfer was also demonstrated *in vivo* from systemically administered BMMSC to diabetic nephropathy mice model (125). Since mitochondrial transfer is associated with various physiological and pathological activities, the mitochondrial transfer could be potentially useful for future treatments of many pathological conditions.

MSC HOMING

Besides their complex mechanisms of immunomodulation, one of the key advantages of MSC-based therapies is their ability to home the damaged tissue. MSC homing is tightly correlated with chemical factors such as chemokines, cytokines, and growth factors. One of the main chemical factors involved in MSC migration is a stromal cell derived factor 1 (SDF-1), a chemokine released from damaged tissue, sending chemo-attractive signals for cells expressing CXCR4 receptors on the outer membrane (126). However, CXCR4 in non-activated MSCs is present only at low levels on the cell surface but at higher levels intracellularly. Upon activation, MSCs can quickly translocate CXCR4 molecules to the cell surface, which enables them to follow the migration cues (127). Besides CXCR4, other chemokine receptors have been identified on MSC membranes that are involved in MSC migration, such as CCR6, CCR9, CXCR3, and CXCR6 (128). Another chemical agent upregulated in injured tissues and inflammation is osteopontin (OPN). This cytokine recruits MSC to the sites of injury through ligation to the integrin β1 that is expressed on MSC upon induction by OPN (129). Among growth factors, fibroblast growth factor (130), vascular endothelial growth factor (131), hepatocyte growth factor (132), insulin-like growth factor-1 (133), and TGF-β1 (134) have been shown to affect MSC homing importantly. Mechanical factors such as mechanical strain, shear stress, matrix stiffness, and microgravity are also importantly involved in MSC homing (135). In stem cell therapies, local transplantation of MSC is a desirable method for cell administration. In some instances, however, intraparenchymal injection of MSC may not be possible due to potential invasiveness (136). Systemic applications, of which IV route is the least invasive, are therefore preferred. The homing of MSC after IV application is faced with various obstacles. Firstly, systemically transplanted MSC must first exit the circulation and then migrate to the site of the injury (137). Secondly, the MSCs after IV transplantation are often sequestrated and then cleared from the lungs (122, 138–142). MSC are relatively large cells, with the average size of 30 μm in suspension. In comparison, pulmonary capillaries are, on average, only 14 μm in diameter, which causes the mechanical entrapment of MSC in the lungs (138). In addition to their size, molecular interactions of MSC with the pulmonary endothelium may be another reason for their accumulation in the lungs. Wang et al. (143) were the first to show that the critical cause of MSC entrapment in lung tissue is the excessive expression and activation of integrins. Their study demonstrated that the blockade of integrins resulted in substantially reduced lung entrapment of MSCs in mice, increased levels of circulating MSCs in the blood, and enhanced homing of MSCs toward the target tissues. Monitoring MSCs after systemic infusion also demonstrated that MSCs are short lived and often disappear 24 h after infusion (122, 139). The long-term beneficial effects of MSCs are thus somehow contradictory to their short lifetime (144). Interestingly, their therapeutic effect may not be correlated with cells' viability, as it was shown by de Witte et al. (122) that despite the accumulation of cells in the lungs and short viability after IV administration, MSC exhibit long term effect through the apoptosis and phagocytosis by immune cells.

To avoid problems with IV administration, other routes have been tested, such as intraarterial (IA) and intraperitoneal (IP) administration. IA administration may reduce the accumulation of MSC in the filtering organs and is thus a promising way for stem cell treatment of ischemic injuries (98). IA injection of MSC may allow better distribution of cells. Centralized IA administration of MSC via the femoral artery in an intact

porcine model showed increased uptake of MSC in various organs, especially in the liver (98). The downside of IA administration is that it is technically a more challenging procedure than IV injection (145) and there is a risk of a possible intravascular occlusion (145, 146). Sole et al. (145) observed arterial thrombosis in horses when the intraarterial application of allogeneic BMMSC was performed via IA regional limb perfusion. Interestingly, the complication of thrombosis was not detected when performing IA injection without using a tourniquet, indicating that a thrombosis is a consequence of blood stasis and not the MSC application (147). Similarly, IA injection of MSC was proven feasible with allogeneic equine BMMSC injected into the cranial tibial artery in horses, also without a tourniquet (148). Nishimura et al. (149) also proved the safety and efficacy of the IA application of MSCs by administering autologous BMMSCs via the hepatic artery in a canine model of liver fibrosis. IP administration of MSCs is rarely used, but carries the potential to reach intraabdominal sites and appears relatively safe when used in cats (150). IP administration of MSCs was shown to be beneficial also in the treatment of bladder detrusor deterioration in rats (151) and in inflammatory bowel disease in mice (93). Moreover, the IP approach was used to inject Neo-islets, aggregates of ADMSCs and pancreatic islet cells in an FDA guided pilot study in insulin-dependent diabetes mellitus in pet dogs. Neo-islets appear to engraft, redifferentiate, produce insulin, and do not trigger auto- or alloimmune response (152).

MSC PRECONDITIONING WITH PROINFLAMMATORY CYTOKINES

Since many patients treated with MSCs suffer from acute or chronic inflammatory diseases, the inflammatory environment is likely to be present *in vivo* when MSCs are being administered. Priming MSCs with IFN-γ before treatment, therefore, imitates the environment in which MSCs will be present in the body. It was proposed that inflammatory conditions enhance the interaction between MSCs and B-cells. Luk et al. (70) showed that MSCs cultured under inflammatory enviroment significantly reduced B-cell proliferation and IgG production by B-cells via induction of indolamine-2,3-dioxygenase activity (IDO), whereas MSCs cultured under non-inflammatory conditions increased the percentage B-regs, but did not influence their proliferation. Tissue origin should also be considered when deciding about priming of MSCs with IFN-γ as, for example, canine MSCs from Wharton jelly are not influenced by IFN-γ (102). In correlation with preconditioning MSCs to improve their therapeutic potential, preconditioning of ECVs has also been shown to be beneficial for their therapeutic effectiveness. Recently it was reported that ECVs from canine MSCs, preconditioned with antiinflammatory cytokines, enhanced macrophage polarization and generation of Tregs in murine colitis (92). However, some studies also reported adverse effects of preconditioning MSC with proinflammatory cytokines. IFN-γ pretreatment enhanced the immunogenicity of MSC with the upregulation of MHC (71) and MHC II expression (153–155). IFN-γ pretreatment also importantly upregulates the expression of genes involved in apoptosis, reflecting negative influence on MSC (156). It was also shown that treatment with both IFN-γ and TNF-α induced apoptosis in mice MSCs. Apoptosis was stimulated by the expression of inducible nitric oxide synthase (iNOS) and the generation of nitric oxide, required for apoptosis (157). To avoid adverse effects of preconditioning with IFN-γ with simultaneous enchancement of their immunosuppressive abilities, pretreatment of MSCs with IL-17A was proposed as an alternative (156). A study by Brandt et al. (158) demonstrated that equine ADMSCs are compromised in an inflammatory environment. High concentrations of proinflammatory cytokines TNF-α and IL-1β and the presence of leukocytes increased ADMSCs proliferation potential and osteogenic differentiation, but negatively affected cells' viability, engraftment, chondrogenic and adipogenic differentiation potential, and expression of the musculoskeletal markers. Conflicting results from various studies about preconditioning MSC with proinflammatory cytokines, therefore, suggest that, although there is a potential beneficial effect of such pretreatments, these should be considered very carefully, and further studies will be needed to clarify potential positive effects of such preconditioning.

CLINICAL USE OF MSC IN VETERINARY MEDICINE

To date, stem cells have been used, mostly experimentally, for treatments of a variety of diseases in different animal species. The initial focus of regenerative veterinary medicine was directed to the orthopedic diseases, but the focus is now rapidly expanding to other areas such as orodental and digestive tract diseases, liver, renal, cardiac, respiratory, neuromuscular, dermal, olfactory, and reproductive system diseases. Stem cell treatments were most often used in dogs and horses for various diseases of various organ systems, and in cats for renal, respiratory, and inflammatory diseases.

Musculoskeletal System Diseases

Tendons and Ligaments Diseases

Traumatic and stress injuries of tendons and ligaments naturally heal with the formation of a scar tissue, which is functionally deficient in comparison to the healthy tissue. While the initial injury causes a reduction in structural stiffness, fibrosis obliterates the physiological architecture, and function of the tendon or ligament (159). This results in compromised locomotor function prone to re-injury (160). The optimal treatment should, therefore, aim at the restoring normal structure and function of the tissue. Traditional therapies for tendon injuries in horses are based on cooling (161), bandage, and rehabilitation period with controlled exercise. Pharmacological treatments include the use of systemic and local corticosteroids or other anti-inflammatory drugs (162), but surgical treatment is often required (163, 164). These conservative techniques do not allow for complete tissue healing, reinjury is common, and often animals aren't able to return to the preinjury performance level (162). Ideal treatment should, therefore, aim to regenerate normal tendon matrix. The use of MSC has been introduced as

an alternative to the traditional approach because it represents a potential tool for better tissue regeneration (165, 166). Regenerative cell-based therapy aims toward healing with the proper formation of collagen fibers and successful regaining of normal tendon activity with a lesser risk for reoccurrences. It is predicted that MSC isolated from the same tissue that needs treatment would be the most adequate source of MSC for stem cell therapy. The best source of stem cells for tendinopathies would, therefore, be tendon-derived stem cells (167), but the isolation of stem cells from tendon tissue is very challenging, and no standard induction protocol for tendonogenesis exists (168). Stem cells from other sources, mainly from adipose tissue and bone marrow, were therefore used for tendon regeneration. Autologous BMMSC implantation into the horse superficial digital flexor tendon was first reported in 2003 (165). After cells were injected into 11 racehorses with superficial digital flexor tendon lesions, significant clinical recovery was reported (169). Similarly, in a cohort study including 141 racehorses with naturally occurred superficial digital flexor tendon injury, intralesional injection of autologous BMMSC resulted in <28% of reinjuries in all horses with 2 years follow up (170). Results showed a significant reduction in reinjury rate compared to those from a similar study of the same type of injury and follow-up, where horses were treated with intralesional injection of hyaluronan, beta aminopropionitrile fumarate or polysulfated glycosaminoglycans (160). It was demonstrated by Smith et al. (171) that autologous BMMSC treatment of naturally occurring tendinopathies induces the formation of tissue resembling a normal tendon matrix rather than a fibrous tissue that is formed during the natural healing process. In addition to the autologous MSC therapy, promising results were also reported with allogeneic MSC therapy for tendon and ligament disorders such as tendinitis of superficial and deep digital flexor tendons and desmitis of the suspensory and inferior check ligaments (172). However, in surgically induced lesions of the equine superficial digital flexor tendons, autologous BM- or ADMSC therapy rendered no or very small improvement in comparison to other treatments like platelet-rich plasma (PRP) (173, 174).

Similar to horses, dogs were also subjected to experimental MSC treatments. A common injury in dogs is a tear of a cranial crucial ligament in the stifle joint (175). Its rupture is associated with stifle osteoarthritis and is the most common cause of lameness in adult dogs (176). Currently, the recommended therapy is a surgical correction (177).

Positive treatment results from several studies highlighted the value of MSC use in this condition. It was demonstrated that the level of post-operative lameness and pain after single intra-articular injection of allogeneic BMMSC could be a valuable alternative to 1 month course of oral administration of non-steroidal anti-inflammatory drugs (NSAIDs) in dogs requiring tibial plateau leveling osteotomy (TPLO) (178). It was shown that intraarticularly injected autologous BMMSCs engraft to the site of the injured cranial crucial ligament (179) and have an anti-inflammatory effect. Post-operatively intraarticular or IV injection of autologous MSC in dogs with the same condition resulted in a decreased level of CD8+ T-cells, decreased serum and synovia CRP, and decreased synovial IFN-γ levels that persisted over 8 weeks after BMMSC injection (180). In cases of partial tears with no destabilization of the stifle joint, where surgery is not the optimal solution, promising results were collected from the retrospective study, where autologous BMMSC treatment in combination with PRP prevented progression of further degenerative changes in the joint and contralateral ligament rupture in dogs (181).

Joint Diseases

Because of the relative hypocellularity and avascularization, cartilage tissue has a limited capacity of self-repair. In horses, it is further affected by the enormous loading forces and mechanical stress that are placed on the articular surfaces during the performance (182). One of the most common reasons for equine athletic career-ending and chronic lameness are joint diseases, with osteoarthritis being the most prevalent (183). Conventional treatment of musculoskeletal injuries, involving the damage to the articular cartilage, ligaments, and menisci is often associated with poor prognosis for the athletic performance of horses (184, 185). The *in vivo* effectiveness of intra-articular MSC treatment of bone, meniscal, and cartilage conditions in horses has been reported. The most studied and described locomotive system disorder in horses is bone spavin, a degenerative joint disease in which conventional treatment is based on the application of anti-inflammatory corticosteroids for decreasing pain and inflammation. Results obtained from the study in which 16 horses with bone spavin were treated intraarticularly with autologous ADMSC suggest the positive and long-lasting effect of MSC therapy. No signs of lameness were observed 180 days after treatment in the treated horses in comparison to the untreated control group. This was confirmed by scintigraphic examination, revealing no signs of inflammation process in tarsal joints of treated horses when compared to the control group where inflammation was still present (186). MSC treatment is also very promising in horses with meniscal damage. Horses treated with intraarticular administration of autologous BMMSC returned to work in a higher percentage than those treated with arthroscopy alone (187). In one study, 80 horses with osteoarthritis were treated with allogeneic ADMSC, and a significant reduction in the lameness was observed during 90 days follow-up period, suggesting the beneficial effect of allogeneic cells (188). Similarly, allogeneic umbilical cord derived MSC for the treatment of osteoarthritis of metacarpophalangeal/metatarsophalangeal joint in horses resulted in a significantly improved lameness over 6 months, but no clinical differences were observed with either single or repeated MSC injection (65). Some studies, however, did report adverse clinical responses after repeated intraarticular injections of allogeneic MSCs in horses with osteoarthritis (60). Even single injections of allogeneic MSCs have been reported to induce mild to moderate local inflammatory signs (64). Several studies in dogs demonstrated that MSC administration into the arthritic joints decrease the patients' discomfort and increase their functional ability. A significant improvement in lameness was confirmed in dogs with stifle osteoarthritis (189) demonstrated by the significantly delayed progression of osteoarthritis in autologous ADMSC treated joints compared to placebo-treated joints. Similar results were reported by Black

et al. (190) and Vilar et al. (191) in dogs with hip osteoarthritis. The effect of intraarticular injection of autologous ADMSCs in treating canine osteoarthritis of different joints seems to be long-lasting, as shown in a study with up to 4-year follow-up (192). Significant improvement of MSC therapy for treating osteoarthritis has also been shown with the use of allogeneic ADMSCs. In 74 dogs treated with allogeneic ADMSCs in a prospective, randomized, masked, and placebo-controlled study, no adverse effects were reported, and efficacy in reducing clinical signs was shown in comparison to the placebo group (193). In another extensive study performed on 203 dogs with severe osteoarthritis, causing severe chronic pain, and lameness, results showed excellent improvement in 90% of young dogs and good improvement in 60% of older dogs 10 weeks after the treatment (194). In a dog model of osteoarthritis treated with allogeneic umbilical cord derived MSCs, cartilage repair was demonstrated in the form of cartilage neogenesis, decreased joint fluid content, reduced inflammatory response, and improved healing of the surrounding tissues in comparison to the control untreated group (27). Contrary to study in horses, repeated allogeneic MSC therapy was shown to be safe with only mild and self-limiting inflammatory reactions without adverse effects even 2 years after intraarticular MSC injection (63). MSC therapy of canine osteoarthritis, either autologous or allogeneic, was also tested and proved to be beneficial in combination with PRP or hyaluronic acid (191, 195, 196). In the comparison of ADMSC and PRP treatments in dogs' osteoarthritis, MSC therapy had stronger and more beneficial effects (197).

MSC therapy in treating musculoskeletal disorders has proven remarkably effective, especially in horses with tendon injuries, bone spavin, and meniscal damages, and in dogs with osteoarthritic conditions. Such positive outcomes of MSC therapies are thus decreasing the need for prolonged local or systemic use of anti-inflammatory drugs with their known toxic side effects. However, additional studies are needed to broaden our knowledge on mechanisms of action of MSCs, and especially allogeneic MSCs, as not all studies provided positive results on their safety when used in the therapy. MSC derived ECVs might represent a promising alternative to the allogeneic MSC therapy as they mimic several biological actions of MSCs. ECV therapy has already been tested for treating suspensory ligament injury in a stallion, rendering positive results shown as increased lesion filling, improved angiogenesis, and elasticity of the damaged tendon (111).

Orodental Diseases

Oral pain and mastication problems can have a major impact on the quality of the animal's life. Many oral diseases can also lead to systemic problems (198). Oral diseases such as dental caries, periodontal disease, permanent tooth loss, oral mucosal lesions, oropharyngeal cancer, and dental trauma are also one of the major public health problems worldwide (199). With the expanding development of regenerative cell therapy, stem cells have attracted interest in the healing of orodental tissues. Studies focus on MSCs immunomodulatory effects to induce regeneration of dental and periodontal tissues, and differentiation potential of MSCs to improve implant strength and bone tissue repair in the alveolar defects. In addition to usual sources of stem cells such as bone marrow and adipose tissue, cells derived from local tissues such as dental pulp stem cells (DPSC) (200–202) or periodontal ligament stem cells (PDLSC) (203, 204) are studied as a therapeutic option in orodental diseases. In experimental dog models, autologous BMMSCs or xenogeneic periodontal ligament MSCs have proven beneficial in periodontal ligament reconstruction, when combined with the growth factors (205), fibrin glue, PRP (206), ephrinB2—a membrane protein regulating bone homeostasis (204) or with a construct of porous biphasic calcium phosphate (203). Allogeneic ADMSCs alone are also capable of inducing periodontal tissue regeneration in the mini pig periodontal defect model (207). For dental pulp regeneration, autologous (200) and allogeneic (201) stem cells from dental pulp or autologous BMMSCs (208) were efficient in dental pulp regeneration in canine models. Although these studies show promise in orodental tissue regeneration, others have reported no beneficial effect of stem cells in dental conditions, such as defects associated with dental implants (209).

Studies conducted on animal models do indeed represent a basis and reference for the use of stem cells and tissue engineering in promoting orodental tissue regeneration. However, extensive research is still needed to prove the efficacy and usefulness of stem cell treatments for orodental problems on actual patients with naturally occurring diseases.

However, very encouraging results are emerging from the MSC treatment of feline chronic gingivostomatitis (FCGS), a painful and debilitating oral condition in cats, characterized by chronic inflammation of gingiva extending to the buccal and caudal oral mucosa. Cats suffering from FCGS are presented with anorexia, oral pain, weight loss, ptyalism, halitosis, and lack of grooming (210). Current treatment options include medications such as corticosteroids (211), cyclosporin (212), and surgical extraction of teeth (213) and have variable response rate and several possible adverse effects (214). Arzi et al. (215) showed that IV treatment with autologous ADMSC resulted in complete clinical and histological resolution or reduction in clinical disease severity in most cats. Immunomodulation of MSC was demonstrated by the normalization of immune cell subsets, serum protein, and cytokine levels. The results of the study also suggested the absence of CD8Io cells as a biomarker to predict the response to MSC therapy (215). Interestingly, allogeneic ADMSCs seem to have lower clinical efficacy in comparison to autologous MSC in treating FCGS (216). The clinical, histologic and systemic response was demonstrated in 70% of cats with FCGS treated with IV administration of allogeneic ADMSC (217).

Digestive tract Diseases

Inflammatory bowel disease (IBD) is an autoimmune condition with chronic hypersensitivity reaction in the intestinal mucosa of unknown etiology (218). Some dogs are refractory to the traditional lifelong treatments using cyclosporine or steroids (219). Single IV infusion of allogeneic ADMSCs resulted in clinical remission in 9 out of 11 dogs with severe IBD 6 weeks after the treatment together with a significant increase in albumin, cobalamin, and folate levels in the blood (219).

IBD is also relatively common in cats with chronic vomiting and diarrhea. In a placebo-controlled blinded study, cats with IBD were treated with allogeneic ADMSC. The owners reported significant improvement or complete resolution of clinical signs in 5 out of 7 cats. In contrast, in cats receiving placebo, no change, or even worsening of the clinical symptoms were reported (220).

Due to their immunomodulatory and anti-inflammatory effects, MSCs seem to be a suitable alternative therapy for dogs and cats with IBD. Results of preliminary studies are promising, but significant follow-up studies and further research is needed to establish MSC treatment as a safe and effective method for treating IBD in animals.

Liver Diseases

Several studies focused on stem cell treatments of liver disease in dogs. Yan et al. (142) examined the effect of IV administration of autologous ADMSC for artificially induced acute hepatic injury in dogs. ADMSC homed to the liver, levels of liver enzymes in the peripheral blood were reduced, and liver tissue structure was restored after the therapy, indicating a potential for MSC use in liver diseases in pets. MSC were also used in a canine model of liver cirrhosis. IV application of autologous BMMSC significantly decreased the area of the liver fibrosis and improved liver function in the group receiving cells without any adverse side effects (221). Similarly to the IV, IA administration of BMMSC in a canine model of liver fibrosis was shown to be safe, but, interestingly, the effect on reducing levels of the liver enzymes in peripheral blood lasted longer with IA application of MSC (149). Autologous ADMSCs were also used repeatedly IV to treat 10 dogs with degenerative hepatopathy. All animals exhibited significantly improved liver function concerning the decline in hepatic biomarkers after each application in comparison to the control group (222). A clinical case of hepatocutaneous syndrome treated with MSC was also reported. Allogeneic ADMSCs were administered repeatedly either into the liver parenchyma or IV. The dog survival with regressed or limited clinical signs was longer than expected for this disease (223).

Since IV administration of MSCs results in the accumulation of cells in the liver after being cleared from the lungs (122, 142), IV route of the administration seem to be logical for treating liver diseases that are responsive to the MSC therapy in animals. Yet conclusions on the best administration route and also on the MSC efficacy and safety of allogeneic MSCs in treating liver diseases is limited by a low number of studies conducted on actual patients. Therefore, further studies are needed to address these issues.

Renal Diseases

Chronic kidney disease (CKD) is a common medical condition in geriatric cats and is characterized by chronic tubulointerstitial nephritis, tubular atrophy, and interstitial fibrosis. Currently, renal transplantation is the only therapy that may restore renal function (224).

Stem cell based therapies may, therefore, present less aggressive treatment options. Due to severe side effects and anesthesia associated risks of intrarenal stem cell inoculation, IV application of stem cells is the preferred choice of cell delivery (225, 226). However, IV administration of allogeneic ADMSC in cats with kidney disease was not associated with any side effects, but neither were any short-term improvements in the renal function reported (227, 228). However, in a study conducted by Vidane et al. (226) cats with spontaneous CKD were repeatedly injected IV with allogeneic MSC derived from the feline amniotic membranes, and after the second administration of MSC, significant improvement in the renal function was observed. Specifically, serum creatinine and urine protein concentrations decreased, and urine specific gravity increased. Considerable improvement was also reported in the overall clinical condition of cats, including food intake and social behavior.

Contradictory results from a few studies hinder the conclusion on the suitability of MSC therapy in cats with CKD. Further studies are necessary to determine the possible influence of different factors that might affect the results of MSC therapy in cats with CKD, such as tissue source of MSC, single or repeated administrations of MSCs, and time of application in regard to the stage of the disease. Additionally, too few studies have been conducted with regard to the safety of allogeneic cells in cats, and this will have to be further explored.

Cardiac Diseases

In human medicine, cardiac stem cell therapies directed toward myocardial repair following the acute or chronic myocardial infarction are being used for several years (229). Primary myocardial infarction is rarely observed in the companion animals (230). However, in large and giant dog breeds, dilated cardiomyopathy is a fairly common disease. Inevitable progression of this disorder leads to the refractory congestive heart failure and death (231). An experimental treatment for this condition was performed in Dobermans with retrograde coronary venous allogeneic ADMSC delivery. Although the treatment was safe, no beneficial effects of stem cell therapy were observed (231). Similarly, treatment of dilated cardiomyopathy with allogeneic cardiosphere-derived cells did not have any beneficial effects after cells were transplanted into the coronary vessels (232). In smaller dog breeds, the most common cardiac disease is the degenerative valvular disease, which is often complicated by ventricular dilation and dysfunction (233). Petchdee and Sompeewong investigated the effect of IV administration of puppy deciduous teeth derived stem cells on the degenerative valvular disease (234). Their results showed an improvement in the left ventricular ejection fraction, but this was a small study, and more studies will be needed to establish any potential positive effects.

Respiratory Diseases

Respiratory diseases are a common problem also in veterinary medicine. Especially in horses, asthma, comprised of several diseases such as recurrent airway obstruction (RAO) or inflammatory airway disease, is a severe medical condition for which there is no successful treatment available. The disease develops in the presence of moldy hay, dusty straw, and pollens. Horses suffer from frequent coughing, increased respiratory effort at rest, and exercise intolerance. Clinical signs can be controlled by the administration of corticosteroids,

bronchodilators, or changing environment. Medications may have adverse side effects, and new therapy options are needed. Barussi et al. (235) studied the effect of the intratracheal application of bone marrow derived mononuclear cells on the course of the respiratory inflammation in horses affected by RAO. Comparison of treatment with single intratracheal administration of autologous cells and oral therapy with dexamethasone showed that bone marrow-derived mononuclear cells improved clinical signs and the inflammatory response in horses suffering from RAO. Levels of IL-10 increased after the cell treatment and were significantly higher than in the control group treated with dexamethasone. The results of this study correlate with positive results of experimental studies with induced respiratory conditions in dogs (236) and cats (237).

Neuromuscular Diseases and Injuries

One of the most common neuromuscular injuries in both humans and animals are spinal cord injuries (SCI), which often result in a lifelong disabilities (238). In dogs, spinal cord injury could be induced by trauma or herniated vertebral disc. In both pathologies, stem cell treatments were tested with beneficial results. Autologous BMMSC therapy was tested for spontaneous injury of the spinal cord due to spinal trauma in dogs with locally administered cells through hemilaminectomy. Mild to moderate improvements in gait, nociception, and proprioception were observed in some of the animals (238, 239). In another study, allogeneic BMMSCs were combined with the standard medication therapy and this combination induced significantly better improvement in the functional recovery of the patients with traumatic spinal cord injury in comparison to the conventional medication alone (240). Similarly, as in MSC therapy of dogs with traumatic spinal cord injury, positive results of MSC therapy were also observed in acute disc herniation in dogs. Dogs with acute paraplegia had faster locomotor recovery after the epidural application of ADMSCs in comparison to dogs treated with surgical decompression alone (241). However, in dogs with naturally occurring degenerative intervertebral disc disease, transplantation of autologous BMMSC did not affect clinical outcomes, and no regenerative effects were detected in any of the three dog patients (242).

Results of the studies using MSC for the treatment of the traumatic spinal cord injuries and disc herniation in dogs did show some positive effects, but future studies are necessary to find a way to augment currently observed therapeutic effects of the MSC therapies. One possibility is a tissue engineering approach. In one study, constructed canine MSC-derived neural network tissue was transplanted into the spinal cord and resulted in the gradual restoration of paralyzed limb motor function (243). More studies and further developments are therefore needed to establish whether cell therapy and tissue engineering approaches are beneficial for spinal cord injuries, especially in patients with spontaneous injuries where the progress of the disease is often very different from the experimentally induced pathologies.

Skin Diseases and Wound Healing

Unsuccessful wound healing is often the consequence of a variety of inadequate cellular and molecular mechanisms. It often leads to the persistent, chronic wound, accompanied by the discomfort for the patient. Therefore, for the treatment of chronic wounds with severe inflammation and hyper-plastic response, MSCs might be potentially a viable treating option due to their anti-inflammatory and regenerative potential (244). Several studies in animal models have shown the beneficial effect of MSC treatment in wound healing in goats (245), sheep (246), horses (247), and dogs (248). Significantly improved cutaneous wound healing was also achieved using MSC derived ECV injected locally to treat circular wounds created in dogs (112). In addition to animal models, significant improvement of naturally occurring wounds has been documented in several studies using stem cell therapy. In four horses with naturally occurring infected wounds unresponsive to conventional therapies, peripheral blood stem cells were injected locally and systemically. In all four cases, the positive outcome of the treatment was seen as crusts formation and small scars in the center of the wound, leading to the tissue overgrowth within 4 weeks after treatment (36). Complete healing of the non-healing skin wound was also observed in a filly after repeated local application of heterologous Wharton/s Jelly derived MSC with the use of carboxymethylcellulose gel. The wound healed completely in 5 days (249).

In addition to wound healing, MSCs were also used for the treatment of atopic dermatitis, one of the most common skin diseases in dogs. Contradictory results of two studies using a similar number of IV administrated ADMSC in dogs with atopic dermatitis have been reported. In the first one, no significant improvement of clinical signs or pruritus was observed (250). The second study included 22 dogs with atopic dermatitis, non-responsive to conventional therapy. Pruritus decreased significantly after 1 week and cadesi-04 scores after 1 month after IV administration of allogeneic ADMSC. Remission of clinical signs lasted for at least 6 months, with no adverse side events observed (251).

Several studies in both laboratory animals and clinical veterinary patients suggest that stem cells might be interesting novel therapy to promote chronic wound healing. But as with other diseases, numerous questions remain unanswered and will have to be addressed in future studies before such treatments will enter general clinical practice. Regarding AD in dogs, the data are very limited, and, therefore, it is impossible to predict at the moment whether stem cell treatments might prove to be beneficial in the future.

Eye Diseases

Stem cell therapy is also investigated in the ophthalmology. Some eye diseases, for example, corneal ulcers are incurable with available methods. Autologous peripheral blood stem cells were used for the treatment of three clinical cases of chronic corneal ulcers and one case of retinal detachment in the horse, all non-responsive to the conventional therapy. Cells were applied either IV or locally into the ophthalmic artery, by subconjunctival injection or in the form of eye drop formulation. All

four patients showed significant improvement after treatment, with the restoration of the epithelial surface as well as a decrease in inflammation (37). Subconjunctival administration of autologous BMMSC also led to the improvement of immune mediated keratitis in 3 out of 4 horses, seen as increased corneal clarity, reduced neovascularization of the area, and decreased surface irregularities (252). Immunomodulatory effects of MSC could potentially change the course of equine recurrent uveitis (ERU), as increased expression of IFN-γ by cd4+ T cells from horses with ERU decreased after incubation with ADMSC *in vitro*. Activation of CD4+- T cells was shown to decrease via contact dependent mechanism and PGE2 signaling (253). In cats, MSC therapy was proposed for the treatment of feline eosinophilic keratitis (FEK), as allogeneic ADMSC implanted subconjunctivally showed promising results seen as an effective decrease in the clinical signs of FEK throughout the study (254). In dogs, MSC therapy has been shown as an effective therapeutic alternative for treating *keratoconjunctivitis sicca* (KCS) or dry eye disease. Allogeneic ADMSC implanted locally around the lacrimal gland of both eyes significantly reduced clinical signs with a sustained effect during a study period (255). Similarly, the study by Sgrignoli et al. (256) demonstrated that the expression of KCS markers CD4, IL-6, IL-1, and TNF-α in dogs was decreased significantly 6 months after repeated topical administration of allogeneic ADMSC into the conjunctival sac.

Based on the published studies, MSC therapy holds a great promise in regenerative eye medicine and presents innovative solutions for several eye diseases in animals, such as corneal ulcers, immune mediated and eosinophilic keratitis, recurrent uveitis, and dry keratoconjunctivitis. However, additional blinded prospective studies, especially for recurrent uveitis in horses, are needed to assess the *in vivo* effect of MSC administration more accurately. Continuous scientific research is undoubtedly needed to fully understand the complexity and severity of specific diseases and regenerative effects of stem cells in the eye therapies, which would contribute to bring stem cell therapy closer to translation into clinics.

Reproductive System Diseases

Many studies are attempting to find treatments for fertility improvement, both for commercial purposes in farm animals and for the translation into human medicine. The goal of restoring fertility with an intraovarian injection of BMMSC was, however, not accomplished, and ovarian function could not be improved or restored in aged mares (257). Similarly, no changes in sperm parameters or fertility rates were observed after intratesticular administration of allogeneic BMMSC in stallions. However, the safety of the procedure at least suggests that such an approach could be theoretically exploited to treat degenerative testicular conditions (258). Interestingly, dog sperms seem to be susceptible to the treatment with ADMSC derived ECV during cryopreservation, as the addition of ECVs reduced the number of damaged sperms decrease of ROS in thawed semen (259). Based on the results from the treatment of other inflammatory conditions, there is a hope that pathologies of reproductive organs will also be susceptible to the MSC treatment. In mares, for example, endometriosis is an incurable degenerative disease of the uterus and is causing substantial economic losses in the equine industry (260). To exploit MSCs immunomodulatory properties in uterine pathologies, endometrial MSCs were investigated and isolated from sows (261), cows (262), ewes (263), goats (264), and mares (265). MSCs delivery to the uterus of mares with endometriosis has already been proposed by Mambelli et al. (266), who demonstrated that MSCs remain in the uterus up to 21 days after intrauterine application. Still, additional studies are needed to assess potential immunomodulatory and anti-inflammatory properties of endometrial MSC and their potential in the treatment of endometriosis (260). In addition to the reproductive system pathologies, MSC therapy is also investigated for the potential treatment of mastitis in farm animals, showing an antiproliferative effect against *Staphylococcus aureus* mediated mastitis in cows (267) and reparative and antifibrotic effect in goat chronic mastitis (268).

SAFETY AND REGULATORY ASPECTS OF STEM CELL THERAPIES IN VETERINARY MEDICINE

The European Medicines Agency's (EMA) Committee for Medicinal Products for Veterinary Use (CVMP) has proposed some basic guidelines for stem cell based medications for veterinary use. Strict microbiological monitoring during the entire manufacturing process from the sourcing of materials to the finished product is essential. Since the use of allogeneic MSC in dogs and horses is increasing, so are the raising questions for manufacturers, authorities, and users. Currently, no specific guidance is available. Safety aspects of extraneous agents concerning veterinary medicinal products are included in the guidelines for the production and control of immunological veterinary medicinal products. In these guidelines is a list of viruses and bacteria for horses and dogs that should not be present in the medicinal products, and this should be adhered to also with the allogeneic MSC. Furthermore, investigations for protozoa may be relevant for dogs and horses depending on the animal region of origin, a prevalent epidemiological situation in their region of origin, and the travel history of the animal donors. Furthermore, as a general guideline, it is recommended that cell donors are always clinically healthy. If cells from newborn animals or placental tissues are used, it is advisable to test mothers for the presence of any infectious agents. To demonstrate the absence of disease-causing agents. A combination of donor screening using anamnesis and clinical information, donor testing for the presence of specific disease agents and product (cells ready for therapy) testing should be applied. All material with biological origin needed for collection, selection, culture, and modification of cells should also be clearly specified and evaluated for the absence of any potentially harmful agents. Furthermore, aseptic manufacturing is necessary for reducing the presence of extraneous agents (269). Within the EU, there is currently no central legislation about stem cell therapies in veterinary medicine, so currently, each EU member state regulates the field independently. However, this is expected

to change in the near future with EMA issuing guidelines and legislative rules for regenerative veterinary medicine.

In 2015 the USA Food and Drug Administration (FDA) published recommendations for the use of cell-based products in animals. According to this, cell-based products, including animal stem cell based products (ASCP) that are intended for use in the diagnosis, mitigation, treatment, or prevention of diseases, are regulated as new animal medicines and require a premarket review to be legally marketed. The requirements for approval include the demonstration of safety, effectiveness, and manufacturing quality. Evaluation of tumorigenicity, immunogenicity, donor selection criteria, the transmission of infectious agents, long term safety, cell survival, biodistribution, and ectopic tissue formation are required (48). In the future, additional regulatory guidelines can be expected. It is unclear whether these new regulations will significantly affect the advancement of stem cell trials and the development of novel therapies (230), but any new regulations should be prepared and approved by experts from various fields, from cell biology to clinical veterinary medicine. Currently, no animal cell based treatments are FDA-approved. Considering a great promise that veterinary regenerative medicine holds for the future, FDA started the Veterinary Innovation Program or VIP to help manufacturers/providers of stem cell therapies with obtaining high-quality data from well-conducted, well-controlled, and well-designed scientific studies (270).

SUMMARY

Veterinary regenerative medicine is an active area of research. Significant advances in developing safe and effective stem cell therapies have been made in recent years. Notable outcomes of MSC therapies have been reported, especially for orthopedic conditions in dogs and horses, but important advancements in MSC therapy have also been made in treating other conditions such as FCGS, IBD, and wound healing. Positive outcomes of many studies suggest a great promise for the future of stem cell therapies for various animal diseases, but numerous issues need to be addressed. One of them is the optimal source for MSC isolation. Adipose and bone marrow derived MSC were used in the majority of the studies, but mostly because they are easily obtainable and easy to work with. Therefore, other stem cells from different tissues might prove in the future to be more suitable for the treatments of certain diseases.

Furthermore, the effect of age and potentially sex on the medicinal properties of MSC will have to be established in future studies. Time with regard to the disease progression, dosage of cells, and mode of MSC application also vary widely between studies. There are no standard protocols established that would suggest the optimal treatment protocols for specific diseases. IV application of MSCs has been often used for treating various animal diseases, despite some suggestions about short viability and rapid clearance of cells. However, some studies suggest that healing immunomodulatory processes in the body are induced by apoptosis-mediated immunomodulation through the immune cells, and this could prolong the action of MSCs. Lung entrapment of MSC after IV application is also an important issue in the field of systemic stem cell therapies. Although other routes of administration have been considered to avoid lung entrapment, the main alternative to IV administration of MSC might be systemic administration of ECV. Early studies suggest that ECVs could be a promising, cell-free stem cell therapy that would prevent lung entrapment and avoid possible pulmonary embolism caused by IV application of MSC, but further studies are needed about both efficacy and safety of ECVs. Another unresolved question is the use of autologous or allogeneic cells. Autologous cells are certainly safer, but their use is more complicated and expensive for the animal owners. Allogeneic cells from healthy donors are, therefore, a possible alternative, but there are still unresolved questions about their immunogenicity and potential to trigger an immune response in the recipient of cells.

Despite considerable advancements in veterinary regenerative medicine in recent years, this field is still in its infancy and much more work is needed to resolve many questions before proven, standardized therapies could be offered to the clinical patients. We live in exciting times as new regenerative therapies are on the rise. One can be hopeful that the continuous research in this area will lead us to the point when the stem cell treatments for many currently untreatable diseases will not be a mere possibility but a realistic and accessible choice for the patients in both veterinary and human medicine.

AUTHOR CONTRIBUTIONS

MVo and NA drafted the manuscript. MVe and GM edited the draft. All authors contributed to the final manuscript.

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Conflict of Interest: GM is partial owner of Animacel ltd.

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

2.2 PRIMERJAVA IZRAŽANJA POVRŠINSKIH OZNAČEVALCEV, VIABILNOSTI TER PROLIFERACIJSKEGA IN DIFERENCIACIJSKEGA POTENCIALA MEZENHIMSKIH MATIČNIH CELIC / MEDICINSKIH SIGNALIZIRAJOČIH CELIC IZ MAŠČOBNEGA TKIVA PSOV IN MAČK

Comparison of canine and feline adipose – derived mesenchymal stem cells / medicinal signaling cells with regard to cell surface marker expression, viability, proliferation and differentiation potential

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Izvleček

Izjemne imunomodulatorne sposobnosti mezenhimskih matičnih celic, imenovanih tudi medicinske signalizirajoče celice (MSC) so v zadnjih letih omogočile izrazit napredek veterinarske regenerativne medicine. Kljub pozitivnim učinkom zdravljenja različnih bolezni živali z MSC, pa razlike v lastnostih MSC med posameznimi živalskimi vrstami niso dobro poznane. V raziskavi smo primerjali izražanje površinskih označevalcev, viabilnost ter proliferacijski in diferenciacijski potencial MSC iz maščobnega tkiva psov in mačk. Za izolacijo, karakterizacijo in gojenje celic psov in mačk smo uporabili isti medij in metode. Maščobno tkivo smo odvzeli 11 psom in 8 mačkam obeh spolov. Izražanje površinskih označevalcev smo določili z metodo pretočne citometrije. Viabilnost MSC, merjeno s hemocitometrom neposredno po tripsinizaciji celic in pretočno citometrijo po predhodnem shranjevanju celic čez noč smo primerjali med seboj. Proliferacijski potencial MSC smo analizirali med drugo in osmo pasažo z določanjem števila celičnih podvojitev in časa, potrebnega za podvojitev števila celic. Diferenciacijski potencial MSC smo določali pri zgodnji in pozni pasaži z diferenciranjem celic v kostne, hrustančne in maščobne celice. Rezultati raziskave so pokazali, da MSC psov v večji meri izražajo izbrane površinske označevalce, izkazujejo večjo viabilnost v manj optimalnih pogojih in izkazujejo večji proliferacijski in diferenciacijski potencial kot MSC mačk.

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Comparison of Canine and Feline Adipose-Derived Mesenchymal Stem Cells/Medicinal Signaling Cells With Regard to Cell Surface Marker Expression, Viability, Proliferation, and Differentiation Potential

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Remarkable immunomodulatory abilities of mesenchymal stem cells, also called multipotent mesenchymal stromal cells or medicinal signaling cells (MSCs), have entailed significant advances in veterinary regenerative medicine in recent years. Despite positive outcomes from MSC therapies in various diseases in dogs and cats, differences in MSC characteristics between small animal veterinary patients are not well-known. We performed a comparative study of cells' surface marker expression, viability, proliferation, and differentiation capacity of adipose-derived MSCs (ADMSCs) from dogs and domestic cats. The same growth media and methods were used to isolate, characterize, and culture canine and feline ADMSCs. Adipose tissue was collected from 11 dogs and 8 cats of both sexes. The expression of surface markers CD44, CD90, and CD34 was detected by flow cytometry. Viability at passage 3 was measured with the hemocytometer and compared to the viability measured by flow cytometry after 1 day of handling. The proliferation potential of MSCs was measured by calculating cell doubling and cell doubling time from second to eighth passage. Differentiation potential was determined at early and late passages by inducing cells toward adipogenic, osteogenic, and chondrogenic differentiation using commercial media. Our study shows that the percentage of $CD44^{+}CD90^{+}$ and $CD34^{-/-}$ cells is higher in cells from dogs than in cells from cats. The viability of cells measured by two different methods at passage 3 differed between the species, and finally, canine ADMSCs possess greater proliferation and differentiation potential in comparison to the feline ADMSCs.

Keywords: mesenchymal stem cells, dog, cat, comparison, proliferation, differentiation, cell surface marker, viability

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INTRODUCTION

In recent years, significant interest for stem cell therapies has arisen in veterinary medicine. It has become evident that mesenchymal stromal cells' or medicinal signaling cells' (MSCs') therapeutic actions are the result of their complex immunomodulatory abilities, including paracrine action (1), secretion of extracellular vesicles (2–4), apoptosis mediated immunomodulation (5), and

mitochondrial transfer of membrane vesicles and organelles (6, 7). In dogs and cats, most common veterinary patients, notable positive outcomes have been reported from MSC therapies of various diseases such as orthopedic diseases (8–12), feline chronic gingivostomatitis (13–15), inflammatory bowel disease (16, 17), and skin diseases (18). MSCs have been isolated from various tissues such as adipose tissue (19–23), bone marrow (20, 21, 23–25), synovium (20), synovial fluid (19, 26), umbilical cord (27), Wharton's jelly (28), peripheral blood (29), muscle, and periosteum (30). Adipose tissue is generally considered the most attractive source of MSCs because of a large MSC yield and minimally invasive procedure needed for cell harvesting (31). After their isolation, MSCs are characterized to prove their mesenchymal nature. In addition to plastic adherence, minimal criteria to define human MSCs, set by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy, include their ability to differentiate into osteoblasts, adipocytes, and chondroblasts. Also, MSCs must express CD105, CD73, and CD90 and lack the expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA class II (32) surface markers.

While all animal MSCs are plastic-adherent and show trilineage differentiation potential, not all express the same panel of surface antigens. Previous MSC studies showed that canine (19, 33–35) and feline MSCs (22, 36) consistently express CD44 CD90 and lack CD34 expression, while the expression of other markers varies. Standards to define animal MSCs are therefore not yet established. Clinical administration of MSCs usually entails expanding cells *in vitro* to obtain a sufficient number of cells. It is well-known that MSC populations are intrinsically heterogeneous what can significantly impact their therapeutic potency (37). Besides different factors, such as MSC source (19, 21, 23, 38), tissue collection site (39–41), animal age (39, 42–44), and the number of passages (45–48) that have been demonstrated to affect MSC characteristics *ex vivo*, animal species could likely also influence MSC potency. Only a few studies compared MSCs between species directly (49–51), while it is difficult to draw any comparisons between different studies using cells from different species because of the lack of standardization of methods for the isolation, culture, and characterization of animal MSCs. As differences in stem cell properties between different animal species might lead to the differences in the stem cell therapy's success, they should be therefore explored. In addition to the importance of studying interspecies differences, investigating sex differences is another critical aspect of scientific research, although it is often neglected in preclinical studies (52). It is well-established that the sex of a patient can affect the risk for both disease susceptibility and progression (53). Sex differences have also been found in stem cell biology and therapeutic efficacy in different species (54–57). Studying both males and females is therefore necessary in different studies including cells, animals, and humans, as it may lead to novel targets for disease modifiers (53). The aim of our study was to determine differences in adipose-derived MSCs (ADMSCs) properties from dogs and cats of both sexes in terms of their surface marker expression, viability, and proliferation and differentiation capacity. Although no sex differences were found between the species in the study, the results of our study show important differences in MSC proliferation and differentiation potential between dogs and cats. The results of our study show that species as a factor must be considered when planning the preparation of canine and feline ADMSCs for clinical applications.

MATERIALS AND METHODS

Animals and Adipose Tissue Collection

Adipose tissue was individually collected from 11 dogs (aged 6 months to 2 years, four males and seven females) and eight cats (aged 3 months to 1 year, four males and four females) during routine castration or ovariohysterectomy at the Small Animal Clinic of the Faculty of Veterinary medicine in Ljubljana and from the Veterinary Clinic of Biotechnology educational center in Ljubljana. All animals were healthy, with no known comorbidities. In all animals, adipose tissue was obtained from the abdominal subcutaneous region. All animals were client-owned, and all owners agreed to the collection of tissue and signed informed consent. As the study was conducted on client-owned animals undergoing a routine clinical procedure with the owner's approval to collect a small piece of adipose tissue, no other approval of the ethical committee was needed according to Slovenian legislation and official opinion from the Administration of the Republic of Slovenia for Food Safety, Veterinary and Plant protection responsible for issuing ethical permits for animal experiments.

Isolation of ADMSCs

Adipose tissue was washed with Dulbecco phosphate-buffered saline (DPBS, Gibco, USA) and cut with a scalpel into small pieces. Adipose tissue was then incubated overnight at 37°C in Dulbecco modified eagle medium (DMEM, Gibco, USA) containing 0.1% collagenase type II (Sigma–Aldrich, DE). The digested tissue was centrifuged at 240 g for 4 min, and the supernatant was discarded. The pellet of cells was resuspended in a cell culture medium containing DMEM and 10% fetal bovine serum (Gibco, USA). The cell suspension was plated into six-well plates (TPP, Switzerland) as passage 0 (P0) and cultured at 37°C in a 5% CO_2 incubator. The cell culture medium was changed every 2–3 days. After 70–90% confluency was reached, cells were trypsinized and further processed for flow cytometry, proliferation, and differentiation assays.

Flow Cytometry (Fluorescence-Activated Cell Sorting) Analysis

For fluorescence-activated cell sorting (FACS) analysis, 1×10^6 cells from passage 3 were used to detect the expression of cell surface markers CD44, CD90, and CD34. Following trypsinization, cells were counted, centrifuged (240 g for 4 min), and washed twice with DPBS. Cells were kept in suspension in DPBS at 4°C overnight. The following day, cells were transferred to the Blood Transfusion Center of Slovenia. Cells were stained with the following antibodies: APC conjugated against CD44 (antibody clone IM7, 103012, Biolegend, USA) and fluorescein isothiocyanate conjugated against CD34 (antibody clone 581, 60013FI, Stemcell Technologies, USA) for both canine

and feline ADMSCs, PE conjugated against CD90 (antibody clone YKIX337.217, 12-5900-42, eBioscience, USA) for canine ADMSCs, and PE conjugated against CD90 (antibody clone 5E10, 555596, BD bioscience, USA) for feline ADMSCs. For antibody titration, 1, 2, 3, 4, 5, and 10 μL of each antiserum per 100 μL of 1×10^6 cells were used. Appropriate dilutions of antibodies used for staining are shown in **Table 1**. Cells were then vortexed, incubated at room temperature in the dark for 10 min, washed twice with DPBS, vortexed, and centrifuged again (500 g for 5 min). The supernatant was decanted. Finally, cells were resuspended in 300 μL of DPBS for FACS analysis. The exclusion of non-viable cells was performed by staining cells with a 7-amino-actinomycin D solution (Miltenyi Biotec, USA). Experimental settings were set up using unstained cells, single color stain, and Fluorescence Minus One controls. A minimum of 20,000 events was recorded. Cells were analyzed with a BD FACSAria flow cytometer (BD Bioscience). FACSDiva 8.0.1 software (BD Bioscience) was used for FACS data analysis.

Proliferation Potential Assay

After 70–90% confluency was reached in each passage, cells were trypsinized to the single cell and seeded at the density of $10e^4$ cells per cm^2 as the next passage into a new T25 cell culture flask (TPP). Cells in the cell culture were maintained up to the eighth passage (P2–P8). At each passage from second to eighth, the number of cells at seeding and harvesting was determined with a hemocytometer, and cell viability was assessed using trypan blue dye. Cell doubling (CD) and cell doubling time (CDT) were calculated using the following formulas:

$$CD = log\ (Nt/N0) \times 3.32$$
$$CDT = T/CD$$
$$C(CD) = CD(P1)+CD(P2)+....+CD(P10)$$

where *Nt* is the number of cells at harvesting, *N0* is the number of cells at seeding, *T* is the time of cell culture for each passage, *CD* is the number of cells' doublings at one passage, *C(CD)* is the cumulative CD of all passages, and CDT is the time needed for a cell number to double (58).

Cell Viability

Cell viability was measured by two methods. During proliferation potential assay, viability was measured with hemocytometer immediately after cell trypsinization using trypan blue dye, at each passage from second to eighth. At passage 3, viability was also measured during FACS analysis using 7-amino-actinomycin D solution to exclude non-viable cells from the surface marker expression analysis and to compare the effect of additional manipulation and overnight storage on cells from both species.

Differentiation Potential Assay

Differentiation potential was assessed by inducing cells into adipocytes, osteocytes, and chondrocytes. Differentiation potential was assessed at early (P2) and late (P8 for canine ADMSCs and P6 for feline ADMSCs) passages. For the adipogenic differentiation, 4×10^4 cells were seeded in 12-well plates. The day after seeding, the cell culture medium was removed. Adipogenic (StemPro Adipogenesis Differentiation Kit, Gibco, USA) medium was added and changed every 2–3 days. The cell culture medium was added to the wells that served as negative controls. Adipogenic differentiation was analyzed with oil-red-O staining (Sigma–Aldrich, DE) after 14 days of culturing, following standard procedure. For the osteogenic differentiation, 4×10^4 cells were seeded in 12-well plates. After 90–100% confluency was reached, the cell culture medium was removed. Osteogenic (StemPro Osteogenesis Differentiation Kit, Gibco, USA) medium was added and changed every 2–3 days. Osteogenic differentiation was analyzed with alizarin red S staining (Sigma–Aldrich, DE) following standard procedure after 14 days of culturing. For the chondrogenic differentiation, micromass cultures were generated by seeding 5-μL droplets of 4×10^4 cells in the center wells of the 12-well plate. After cultivating micromass cultures for 6 h under high humidity conditions, a chondrogenic medium (StemPro Chondrogenesis Differentiation Kit, Gibco, USA) was added to culture vessels. The cell culture medium was added to the wells that served as negative controls. Micromass cultures were incubated at 37°C in an incubator with 5% CO_2 and a humid atmosphere. The medium was changed every 2–3 days. Chondrogenic differentiation was analyzed with Alcian blue staining (Sigma–Aldrich, DE) following standard procedure after 14 days of culturing. Differentiated cells were then visualized under light microscope.

Light Microscopy and Analysis

For analysis of multilineage differentiation potential of canine and feline ADMSCs, an inverted microscope (Nikon Eclipse TS100, Nikon, Japan) with Nikon Digital Sight DS-U2 camera was used. Images were captured in NIS-Elements D3.2 Live quality program. Images of adipogenic differentiation were captured at 400× magnification and qualitatively analyzed. Images of osteogenic and chondrogenic differentiation were captured at 40× magnification. Seven view fields in one well of a 12-well plate were randomly selected and quantitatively analyzed by measuring the area of differentiated cells. In the ImageJ program (59), images were converted to binary type and then segmented using DynamicThreshold_1d.class plugin (60), displaying (max + min)/2 images. The area of particles larger than 100 μm^2 was measured in each field view, and the total area covered by differentiated cells was calculated.

Statistical Analysis

All statistical analyses were performed with the NCSS software package (Kaysville, UT, USA). The normality of the data was checked by the Kolmogorov–Smirnov test for normality. One-way analysis of variance (ANOVA) was used to determine differences in the cell surface marker expression and the viability in the third passage with species and sex as independent variables. Proliferation capacity and viability throughout the experiment were analyzed by repeated-measures ANOVA with species and sex as independent variables and passage as within factor. Differences in differentiation were determined by repeated-measures ANOVA with sex and species as independent variable and optic field as within factor. Differences in differentiation were determined separately for early and late passages. Additionally, the *post-hoc* Tukey–Kramer multiple-comparisons test was used

TABLE 1 | Data on antibodies and their dilutions used for FACS analysis in the study.

Cell surface marker	Conjugation	Antibody clone	Isotype	Target species	Catalog number	Source	Antibody dilution (canine ADMSCs)	Antibody dilution (feline ADMSCs)
CD34	FITC	581	Mouse IgG1	Human	60013FI	Stemcell Technologies, USA	1: 20	1: 20
CD44	APC	IM7	Rat IgG2b	Mouse, human	103012	Biolegend, USA	1: 66	1: 400
CD90	PE	YKIX337.217	Rat IgG2b	Dog	12-5900-42	eBioscience, USA	1: 20	/
CD90	PE	5E10	Mouse IgG1	Human	555596	BD bioscience, USA	/	1: 66
CD90	PE	5E10	Mouse IgG1	Human	60045PE	Stemcell technologies, USA	/	/
CD105	PE-Cy7	SN6	Mouse IgG1	Human	25-1057-42	eBioscience, USA	/	/

CD, cluster of differentiation; FITC, fluorescein isothiocyanate; PE, phycoerythrin; APC, allophycoerythrin; PE-CY7, phycoerythrin–cyanine 7; Slash (/), non-reactive.

to clarify the differences between particular pairs further. Statistical significance was determined with $p < 0.05$.

RESULTS

Cell Culturing and Proliferation Potential of MSCs

The adipose tissue was successfully collected from all animals. Under the light microscope, cells from both species appeared spindle-shaped with the fibroblast-like morphology (**Figure 1**). Cells were maintained up to the eighth passage. We attempted to grow cells for longer, but after the eighth passage, most cells stopped proliferating or proliferated very slowly. Therefore, all analyses were performed with cells up to the eighth passage. At each passage from second to eighth, CD and CDT were determined. Cumulative CD [(C)CD)] of canine ADMSCs was significantly higher than (C)CD of feline ADMSCs ($p < 0.01$; **Figure 2A**), and average CDT was significantly shorter for canine ADMSCs than for feline ADMSCs ($p < 0.05$; **Figure 2B**). An increase in CDT from the second to the eighth passage was relatively gradual in canine ADMSCs in comparison to CDT of feline ADMSCs, which increased unevenly. Interestingly, CDT increased significantly in passage eight in cats only and was different from all other feline and canine passages ($p < 0.001$; **Figure 2C**). There were no statistically significant differences in CD or CDT between the sexes of both species.

Flow Cytometry Analysis

Undifferentiated ADMSCs at passage 3 were evaluated for the expression of cell surface markers CD44, CD90, and CD34 (**Table 1**). We also tested antibodies against CD105 (**Table 1**); however, these antibodies did not work with our cells and were therefore not included in the analyses.

FACS analysis revealed that the most canine and feline ADMSCs were positive for CD44 and CD90 and negative for CD34 (**Figure 3**). Percentage of live $CD44^{+}CD90^{+}$ ADMSCs was statistically significantly higher in canine than in feline cells ($p < 0.01$). The percentage of live $CD34^{-/-}$ ADMSCs was also statistically significantly higher in canine than in feline cells ($p < 0.05$; **Figure 4**). No differences in cell marker expression between sexes were observed in either of the species.

Cell Viability

MSC viability was determined with the hemocytometer using trypan blue at each passage during the proliferation potential assay. Additionally, viability was determined at passage 3 with flow cytometry using the 7-amino-actinomycin D staining solution to exclude non-viable cells from the surface marker expression analysis and to compare the effect of additional manipulation and overnight storage on the cells from both species. Mean viability of ADMSCs measured with the hemocytometer during proliferation ranged from 93 to 96% in the second passage and from 82 to 88% in the eighth passage (**Table 2**), with no statistically significant difference between canine and feline cells, although there was a trend toward statistical significance with better viability of canine ADMSCs. Also, no differences in viability between the sexes of both species were observed. Contrary to the viability measured during proliferation assay, flow cytometry results showed a statistically significant difference in the viability between canine and feline ADMSCs in passage 3, with cells from dogs showing higher viability than cells from cats ($p < 0.01$). The mean viability of canine and feline ADMSCs in passage 3 was 90.44 and 79.67%, respectively (**Figure 5**).

Differentiation Potential of MSCs

Canine and feline ADMSCs were induced toward trilineage differentiation at early (P2) and late (P8 for canine and P6 for feline ADMSCs) passage. Late passage for feline ADMSCs was determined as passage 6, as these cells were able to differentiate at passage 6 but not at later passages. Although late passages are different and therefore not directly comparable,

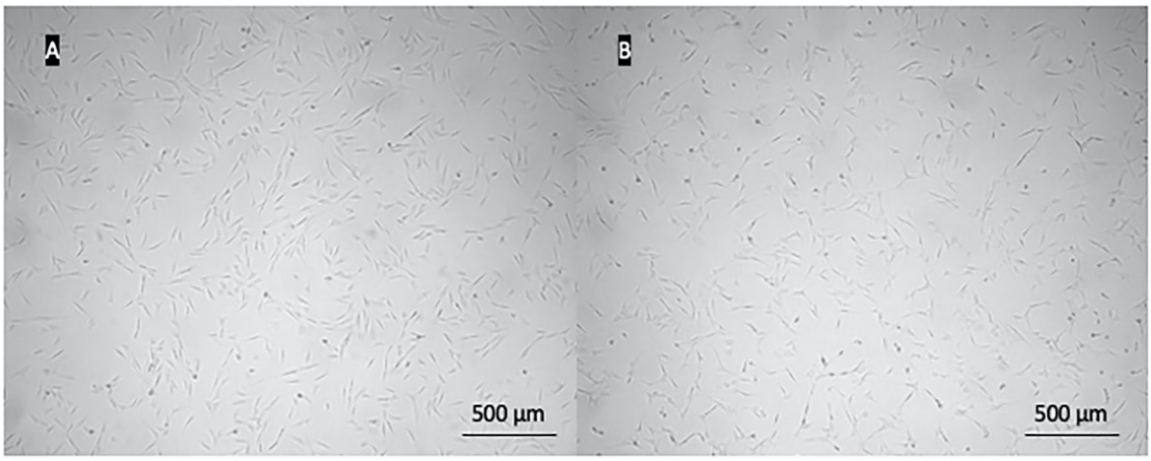

FIGURE 1 | Images of canine **(A)** and feline **(B)** ADMSCs. Cells from both species exhibited similar spindle-shaped fibroblast-like morphology.

our aim was to determine differentiation capacity toward the end of the proliferative capacity of ADMSCs, and as these were different between dogs and cats, we chose different passages for late differentiation.

Osteogenic differentiation led to mineral deposits in the extracellular matrix staining red with alizarin-red-S (**Figures 6A,B**). After chondrogenic differentiation, proteoglycans in the extracellular matrix of layered cell clusters stained positive with Alcian blue (**Figures 6C,D**). Adipogenic differentiation resulted in the formation of intracellular lipid droplets staining red with oil-red-O (**Figures 6E,F**). Both canine and feline ADMSCs differentiated into adipocytes, osteocytes, and chondrocytes at early (P2) and late passages (P8 for canine cells and P6 for feline cells). Adipogenic differentiation was assessed qualitatively as quantification of adipogenic differentiation was thwarted because of the very small size of lipid droplets that needed to be visualized under the large magnification. No apparent differences in the adipogenic differentiation based on the qualitative assessment between canine and feline cells were observed. Osteogenic and chondrogenic differentiated cells were analyzed with the ImageJ program wherein the area of particles larger than 100 μm^2 was measured and compared between canine and feline ADMSCs. Results showed that the total positively stained area in chondrogenic differentiation was statistically significantly more extensive in canine ADMSCs than in feline ADMSCs at passage 2 (early passage; $p < 0.05$, **Figure 7**), and there was a statistical trend for difference between species in osteogenic differentiation at passage 2 (early passage; $p = 0.07$). Interestingly, there was a statistically significant difference between sexes in osteogenic potential in passage 2 (early passage) with cells from female dogs and cats showing larger osteogenic potential than cells from males of both species ($p < 0.01$, **Figure 7**). In late passages, we found statistically significant difference in the osteogenic potential between species, although curiously *post-hoc* test revealed that this difference was only present in males with male dogs showing higher osteogenic potential than male cats ($p < 0.01$, **Figure 7**).

DISCUSSION

Despite substantial progress in veterinary regenerative therapy in recent years, understanding MSC behavior and their mechanisms of action is an ongoing process. MSCs differ in various characteristics with regard to the tissue source (19, 21, 23, 38), anatomical location (39–41), animal age (39, 42–44), and their characteristics change with the number of passages (45–48). There are very limited data about differences between MSCs from different species. However, one mode of cell therapy used in certain species could not be necessarily directly applicable to another species. In the present study, we examined characteristics of adipose-derived MSCs from two species, both common veterinary patients, cats and dogs.

We performed a comparative study of surface marker expression, viability, proliferation, and differentiation capacity between canine and feline ADMSCs, with the same media and methods used for the isolation, cell culture, and characterization of ADMSCs of both species. Both canine and feline ADMSCs exhibited similar spindle-shaped fibroblasts–like morphology. Although animal MSCs are plastic adherent and able to differentiate not only into adipocytes, chondrocytes, and osteocytes but also into other lineages such as neuronal lineage (61, 62), there are no minimal criteria set to define animal MSCs based on the surface antigens as are for human MSCs (32). Previous MSC studies mostly described ADMSCs from dogs (19, 33–35) and cats (22, 36) as consistently CD34-negative and -positive for CD44 and CD90. Expression of some other markers, essential to define human MSCs, varies in animal MSCs. The expression of CD105 and CD73 in canine and feline MSCs varies depending on the tissue of origin (19) or is expressed only in dogs (38, 63) but not in cats (36).

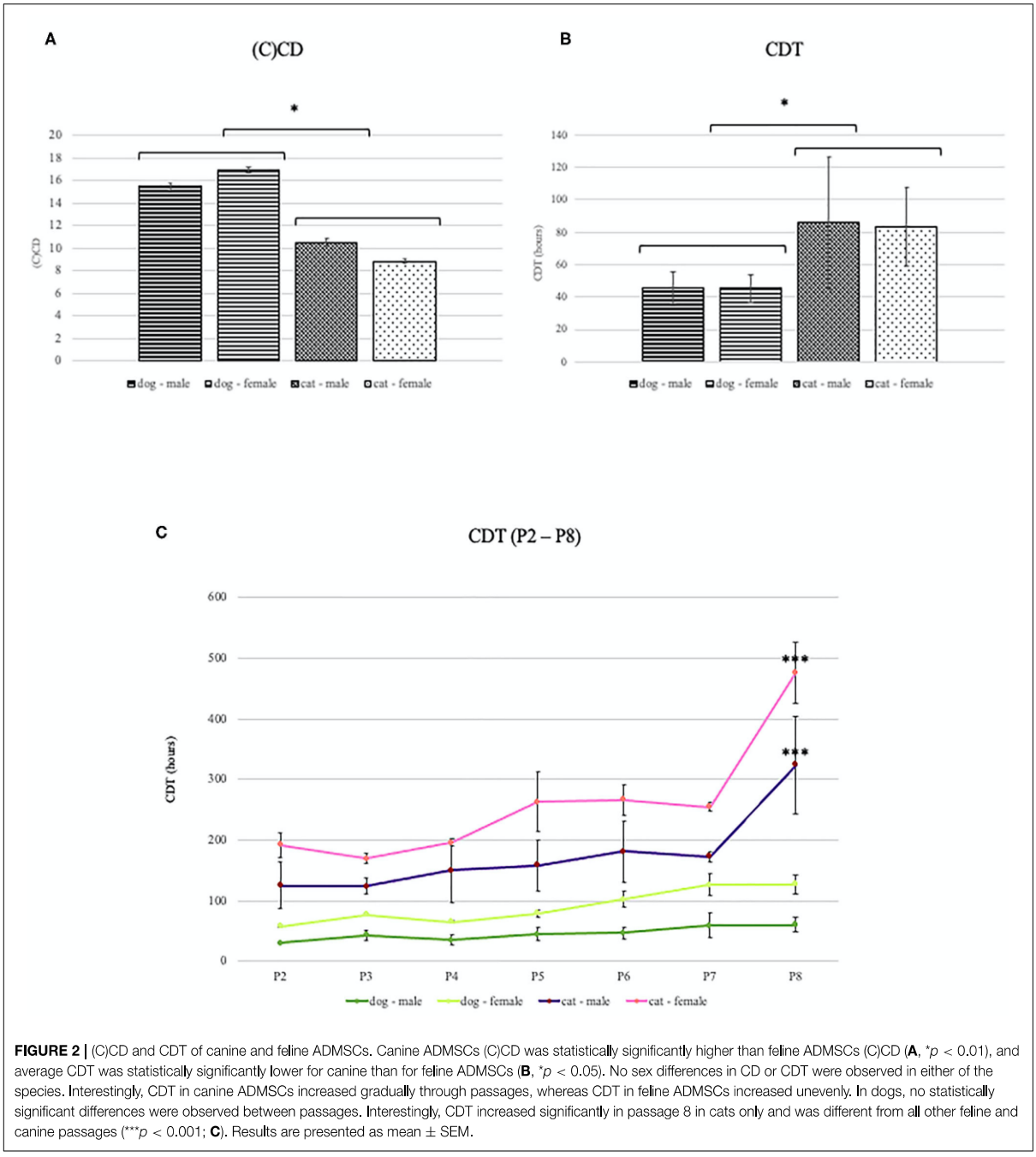

FIGURE 2 | (C)CD and CDT of canine and feline ADMSCs. Canine ADMSCs (C)CD was statistically significantly higher than feline ADMSCs (C)CD (**A**, $*p < 0.01$), and average CDT was statistically significantly lower for canine than for feline ADMSCs (**B**, $*p < 0.05$). No sex differences in CD or CDT were observed in either of the species. Interestingly, CDT in canine ADMSCs increased gradually through passages, whereas CDT in feline ADMSCs increased unevenly. In dogs, no statistically significant differences were observed between passages. Interestingly, CDT increased significantly in passage 8 in cats only and was different from all other feline and canine passages ($***p < 0.001$; **C**). Results are presented as mean ± SEM.

The aim of our study was to compare the expression of surface markers that are known to be consistently expressed on MSCs from both species. Therefore, we used markers CD34, CD44, and CD90. We also considered two other markers (CD105 and CD73) that define human MSCs (32), but were not included in the final study. Marker CD105 was not used as we were unable to find commercially available anti-canine or anti-feline CD105 antisera. However, we tested antibodies against CD105 that were reportedly used in one previous study (64), but we could not obtain positive signal despite extensive optimization with our canine or feline cells. Marker CD73 was not included in the study as it was shown before that it is not expressed in feline cells and was therefore not considered as a marker consistently expressed in both species.

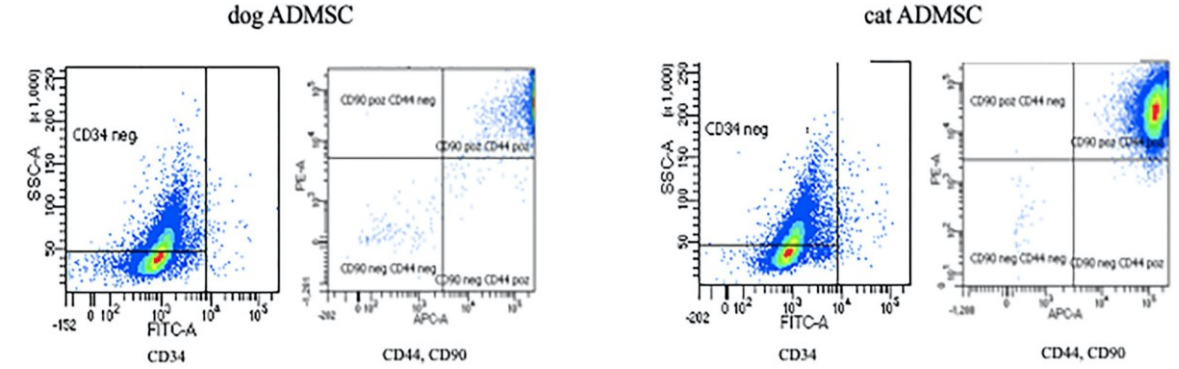

FIGURE 3 | Expression of canine and feline ADMSC surface markers analyzed by FACS at passage 3. Both canine and feline ADMSCs expressed CD44 and CD90 but did not express CD34.

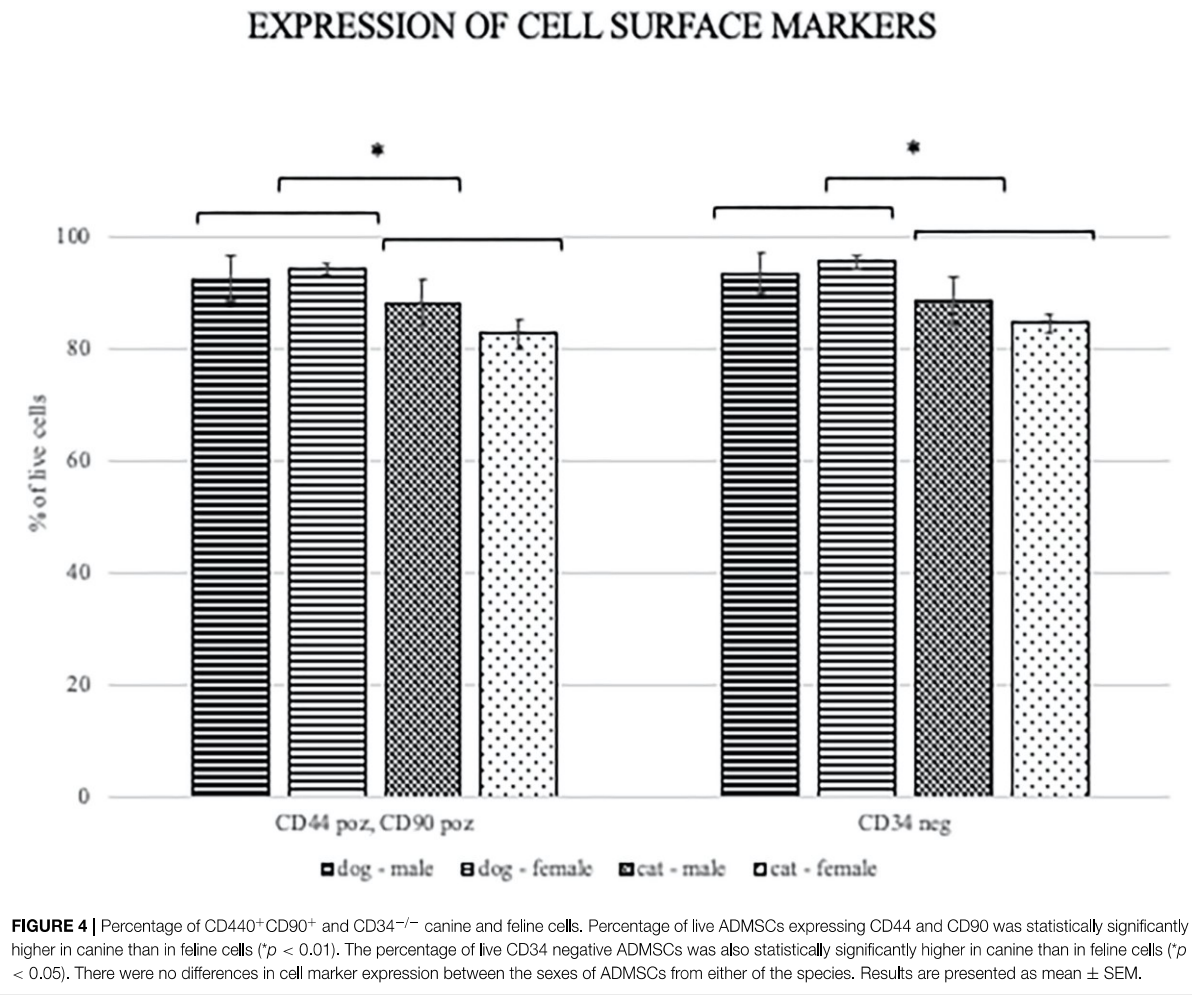

FIGURE 4 | Percentage of $CD440^{+}CD90^{+}$ and $CD34^{-/-}$ canine and feline cells. Percentage of live ADMSCs expressing CD44 and CD90 was statistically significantly higher in canine than in feline cells (*$p < 0.01$). The percentage of live CD34 negative ADMSCs was also statistically significantly higher in canine than in feline cells (*$p < 0.05$). There were no differences in cell marker expression between the sexes of ADMSCs from either of the species. Results are presented as mean ± SEM.

However, as there are no reports on the comparisons of relative marker expression between species, future studies should also consider more thorough investigations of MSC surface marker expression differences between various animals, especially when species-specific MSC markers will hopefully become available in the future.

TABLE 2 | Average viability (%) of canine and feline ADMSCs measured by hemocytometer during proliferation potential assay at each passage (P2–P8).

Passage	P2	P3	P4	P5	P6	P7	P8
Dog cells	96	93	94	92	91	86	88
Cat cells	93	91	91	92	89	76	82

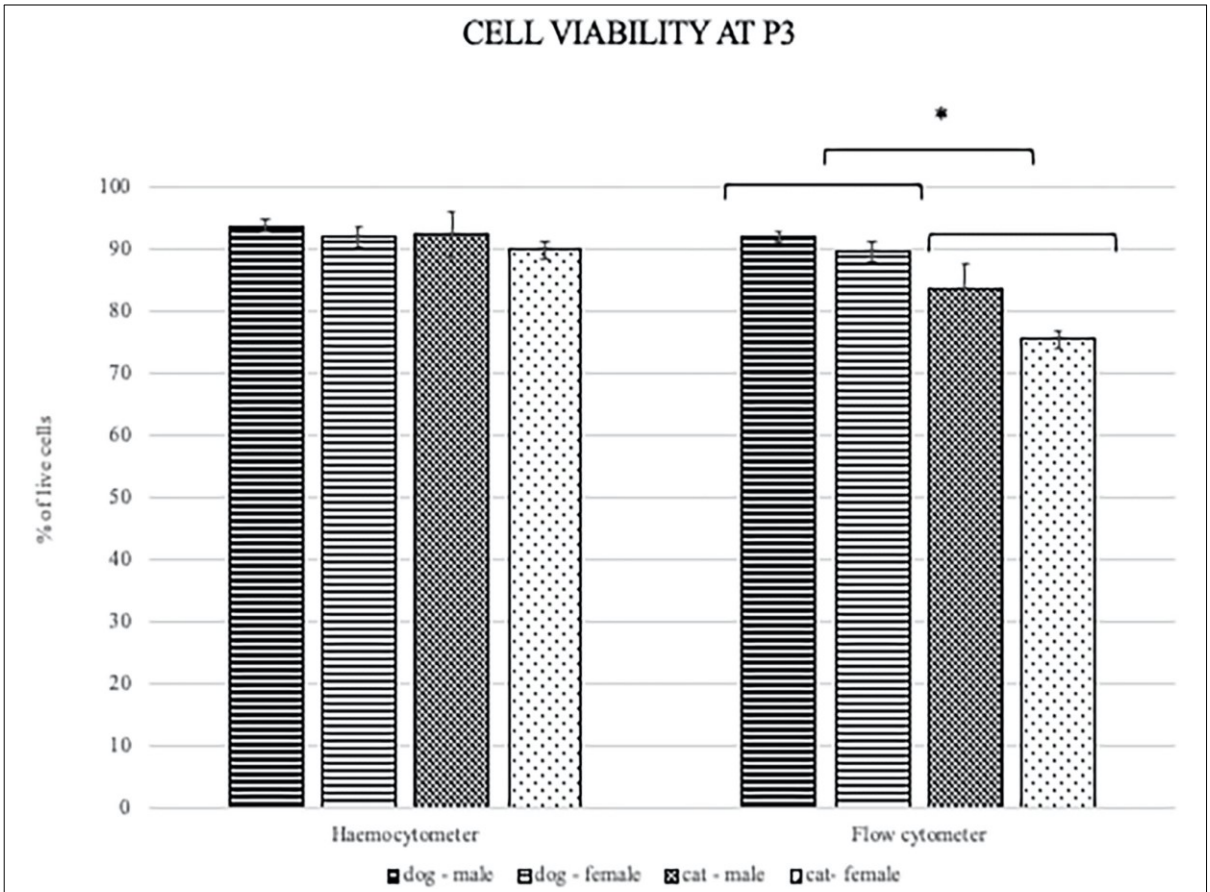

FIGURE 5 | Viability of canine and feline ADMSCs measured by hemocytometer (left) and flow cytometry (right) at passage 3. When measured by hemocytometer, the viability of ADMSCs was similar between both species. When measured by flow cytometry, the viability of feline ADMSCs was statistically significantly lower than that of canine ADMSCs (*$p < 0.01$). No differences in cell viability between sexes from either species were present, regardless of the method. Results are presented as mean ± SEM.

In line with the results of previous studies, we showed that most of the canine and feline ADMSCs express MSC surface markers CD44 and CD90 and lack the expression of hematopoietic marker CD34. Interestingly, the percentage of cells expressing CD44 and CD90 and not expressing CD34 was statistically significantly higher in canine ADMSCs than in feline ADMSCs. It was previously described that MSCs exhibit donor-to-donor and intrapopulation heterogeneity and that MSC populations consist of distinct subpopulations, so the properties of MSC populations cannot be ascribed to single cells (37). One possible reason for the difference in relative surface marker expression between canine and feline cells in our study might be that feline cell populations are more intrinsically heterogeneous than canine cells.

We measured the viability of cells by two different methods, hemocytometer during proliferation potential assay immediately after trypsinization at each passage and by flow cytometer during surface marker expression analysis at passage 3 after 1 day of handling. The cell viability measured by two methods was used to compare the effect of additional manipulation and overnight storage on the cells from both species. The viability of canine ADMSCs in passage 3, measured by flow cytometry, was statistically significantly higher than the viability of feline ADMSCs. However, the viability of both canine and feline

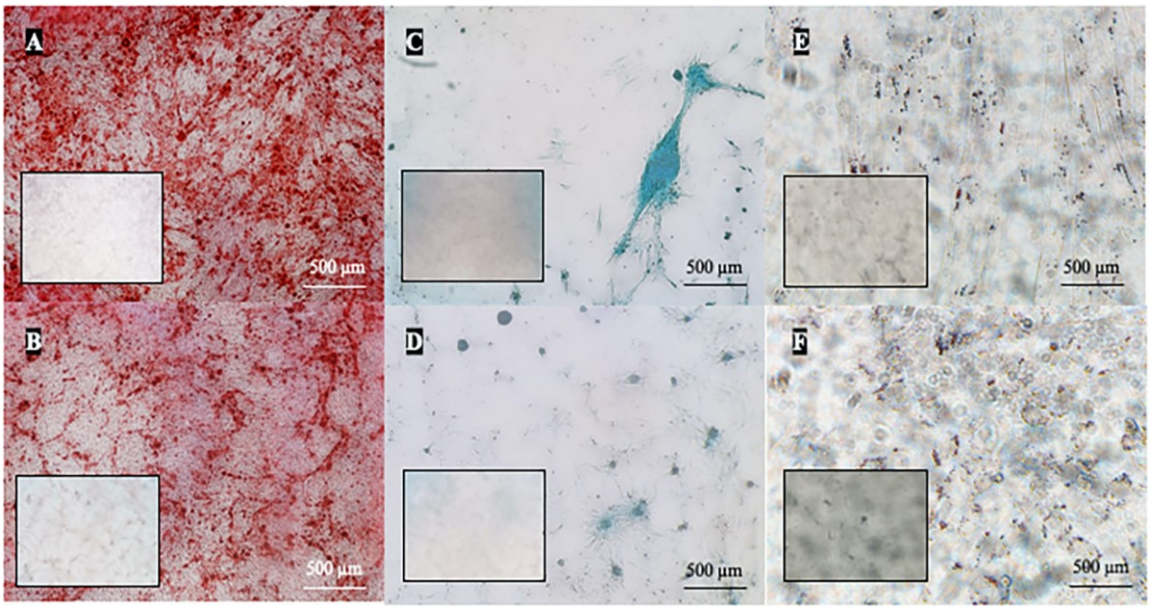

FIGURE 6 | Multilineage differentiation of cells in passage 2. ADMSCs from cats and dogs successfully underwent osteogenic **(A,B)**, chondrogenic **(C,D)**, and adipogenic differentiation **(E,F)**. In osteogenic differentiation, mineral deposits in the extracellular matrix were stained red by alizarin-red-S (dog **A**, cat **B**). Chondrogenic differentiation is indicated by the formation of chondrogenic nodules that stain blue with Alcian blue (dog **C**, cat **D**). Red intracellular lipid droplets stained with oil-red-O are indicative of adipogenic differentiation (dog **E**, cat **F**). Respective negative controls are shown as inserts in each photomicrograph.

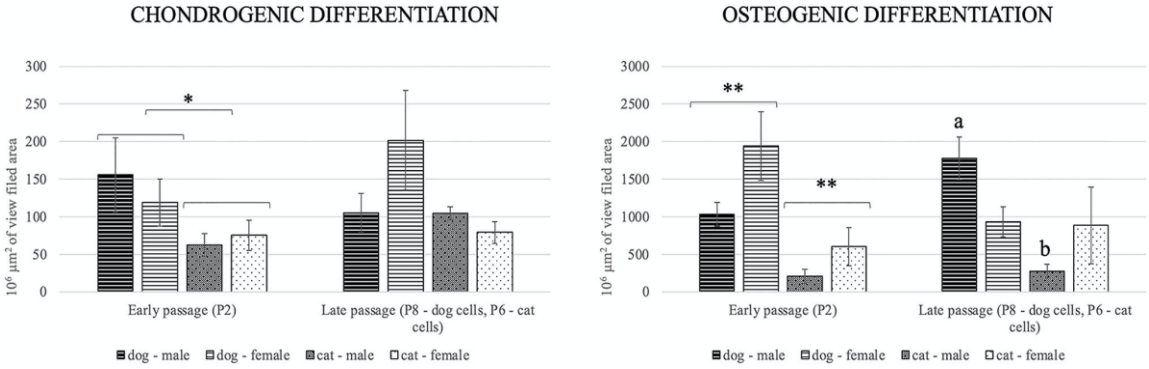

FIGURE 7 | Chondrogenic and osteogenic differentiation at early (P2) and late (P8 for canine cells and P6 for feline cells) passages. Positively stained area in chondrogenic differentiation was statistically significantly more extensive in canine ADMSCs than in feline ADMSCs at passage 2 (*$p < 0.05$). In osteogenic differentiation, there was a statistically significant difference between the sexes of both species (**$p < 0.01$) but not between species at passage 2, although there was a statistical trend for difference between species ($p = 0.07$). In late passages, there was statistically significant difference in osteogenic differentiation between the species, but only in males (a different from b, $p < 0.01$).

cells, measured with trypan blue through the passages, was not significantly different at any passage. Several studies showed that viability determined by microscopy and flow cytometry contributes to similar results and that correlation data are in good agreement with both methods (65–67). Therefore, it is unlikely that this difference was caused by the difference in methods, but rather by differences in handling the cells. While cells for trypan blue staining were counted almost immediately after trypsinization, cells for flow cytometry underwent much longer manipulation, including overnight storage at 4°C in DPBS suspension and additional manipulation of cells prior to flow cytometry analysis. Although these differences will have to be confirmed in further studies, the results suggest that feline cells might be more sensitive to handling and storage in suboptimal conditions. This could have important implications for potential clinical use of MSCs in veterinary medicine, as any such use

inadvertently involves the transport of live cells. If feline cells are more sensitive to handling and storage, this will have to be considered.

The life span of MSCs cultured *ex vivo* is limited, and it has been demonstrated in several studies that serial passages alter their multipotent properties (45–48). The ability of MSCs to self-renew is thus an important feature to be analyzed *in vitro* and can be done by calculating CD and CDT—number of cells' doublings in one passage and time needed for a cell number to double. It has been shown in several studies that CD decreases and CDT increases with passages in cells from various species (36, 38, 45, 68). Similarly, the results of our study showed that the proliferation capacity of both canine and feline ADMSCs decreased with passages. Interestingly, the proliferation capacity of canine ADMSCs seems to decrease very gradually with passages, whereas the proliferation capacity of feline ADMSCs was much more varied between passages. This variation between passages was observed in all feline samples and therefore suggests a real effect, although it is difficult to explain what might cause such non-linear difference between passages.

In previous studies, proliferation potential was shown to depend on various factors, including tissue of origin and anatomical site of the tissue collection. There might also be a difference between species regarding MSC proliferation and differentiation potential, depending on a tissue source. For example, canine ADMSCs have higher differentiation potential than MSCs from bone marrow, umbilical cord, amniotic membrane, or placenta (21). In contrast, in horses, chondrogenic (69) and osteogenic potentials (70) seem higher in bone marrow MSCs than in ADMSCs. These results indicate the possibility that MSCs from different animals have different properties indeed. The results of our study confirmed that there is a difference in proliferation potential between species. Canine ADMSCs exhibited higher proliferation potential than feline ADMSCs as cells from dogs had significantly higher (C)CD and significantly shorter CDT than cells from cats.

Similarly, as in proliferation potential, there was a difference in the differentiation potential between canine and feline ADMSCs. While MSCs from both species were able to differentiate into adipocytes, chondrocytes, and osteocytes at early passages, canine cells were able to differentiate also at passage 8, whereas feline cells were able to differentiate at passage 6, but not at later passages. Canine ADMSCs also seemed to possess greater chondrogenic and osteogenic potential than feline cells, as seen after quantification of differentiation images. Adipogenic differentiation was assessed only qualitatively as lipid droplets were very small, and large magnification was required to visualize these droplets. No apparent difference in qualitatively assessed adipogenic differentiation between canine and feline ADMSCs was observed. The small size of lipid droplets formed in canine ADMSCs is in line with the results from other studies, where lipid droplets from differentiated canine bone marrow–derived MSCs were shown to be much smaller than those of human MSCs (50). Also, lipid droplets formed during adipogenesis were reported to be smaller in adipose and bone marrow–derived MSCs than in MSCs from synovium or infrapatellar fat pad (20).

Taken together, lower proliferation and differentiation potential and lower relative cell surface marker expression in feline cells in comparison to canine cells could be explained, at least in part, by the assumption that the feline ADMSC population is more heterogeneous than canine ADMSCs. These findings should be considered in stem cell therapies as population heterogeneity combined with the requirement for the large-scale cell expansion needed for stem cell therapy may significantly impact the *in vitro* characteristics of cells and possibly therapeutic potency of MSCs.

One of the aims of our study was to examine potential sex differences in the viability, proliferation, and differentiation potential in cells from dogs and cats. Sex-related differences have been previously reported in regard to the neurogenic potential (54, 55), immunomodulation (56), and therapeutic efficacy (57) of stem cells in humans and animal models. In our study, the only differences between sexes were observed in osteogenic potential, which was different with cells from dogs and cats at passage 2. This suggests that cells do not differ majorly between sexes in basic parameters such as proliferation and expression of cell surface markers. However, more subtle sex-related differences in animal MSC characteristics might still be present and must not be neglected when studying cell characteristics, especially their therapeutic potentials. Furthermore, sex difference in osteogenic capacity is interesting as it might suggest different properties of cells in regard to sex, and this might reflect in differences in regenerative potential of ADMSCs from different sexes. Therefore, this difference must be further explored in the future study.

In conclusion, our study indicates that animal donor species play an important role with regard to the MSCs' characteristics *in vitro.* ADMSCs from dogs have a higher proliferation rate and better differentiation capacity than cells from cats. Feline cells also seem to be more sensitive to the handling, as viability after 1 day of handling was lower in feline than in canine cells. Furthermore, the percentage of cells expressing stem cell markers was lower in cells derived from cats than in canine cells. However, sex differences were observed only in osteogenic potential in the early passage. Therefore, the results of this study suggest that species should be taken into account when working with MSCs, and protocols for cell isolation, culturing, or therapeutic use cannot be translated from one species to another.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because all animals were client-owned, and all owners agreed to the collection of tissue and signed informed consent. Since the study was conducted on client-owned animals undergoing

a routine clinical procedure with the owner's approval to collect a small piece of adipose tissue, no other approval of the ethical committee was needed according to Slovenian legislation and official opinion from the Administration of the Republic of Slovenia for Food Safety, Veterinary and Plant protection responsible for issuing ethical permits for animal experiments. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

MV performed the experiments. GM planned the experiments and analyzed the data together with MV. MV and GM wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: GM is a partial owner of Animacel Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

2.3 SVILNI FIBROIN SPROŽA HONDROGENO DIFERENCIACIJO MULTIPOTENTNIH MEZENHIMSKIH STROMALNIH CELIC / MEZENHIMSKIH MATIČNIH CELIC IZ MAŠČOBNEGA TKIVA PSOV

Silk fibroin induces chondrogenic differentiation of canine adipose – derived multipotent mesenchymal stromal cells / mesenchymal stem cells

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Izvleček

Pod ustreznimi pogoji *in vitro* so se mezenhimske matične celice (MSC) sposobne diferencirati v različne specializirane celice. Z namenom vodenja celične usode se razvijajo različni biomateriali, katerih namen je posnemanje naravnega okolja - niše MSC. V naši raziskavi smo kot celični nosilec uporabili biomaterial iz svilnega fibroina in proučevali učinek nosilca na hondrogeno diferenciacijo MSC iz maščobnega tkiva psov. Maščobno tkivo smo odvzeli devetim lastniškim psom. Celice smo gojili na dvodimenzionalnih fibroinskih filmih in trodimenzionalnih fibroinskih poroznih nosilcih v običajnem celičnem gojišču. Morfologijo celic smo analizirali z uporabo vrstičnega elektronskega mikroskopa. Hondrogeno diferenicacijo celic smo analizirali z barvanjem celic z alcian modrim in s kvantifikacijo izražanja genov kolagen tipa 1, kolagen tipa 2, SOX9 in agrekan. Celice, gojene na fibroinskih filmih in poroznih nosilcih, so se obarvale modro z alcian modrim. Na slikah, posnetimi z elektronskim mikroskopom, so bili vidni vezikli in vlakna zunajceličnega matriksa ob skupkih celic, podobnimi hrustančnim celičnim skupkom. Izražanje genov SOX9 in agrekan je bilo statistično značilno večje v celicah, gojenih na fibroinskih filmih, v primerjavi s celicami negativne kontrole. Rezultati raziskave nakazujejo, da je hondrogeno diferenciacijo MSC iz maščobnega tkiva psov mogoče doseči z gojenjem MSC na fibroinskih nosilcih v običajnem celičnem gojišču brez uporabe specifičnih pogojev, ki so sicer potrebni za sprožitev hondrogene diferenciacije na standardni plastični podlagi.

Original Article

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Silk fibroin induces chondrogenic differentiation of canine adipose–derived multipotent mesenchymal stromal cells/mesenchymal stem cells

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Abstract

Under appropriate culture conditions, mesenchymal stem cells (MSC), also called more properly multipotent mesenchymal stromal cells (MMSC), can be induced toward differentiation into different cell lineages. In order to guide stem cell fate within an environment resembling the stem cell niche, different biomaterials are being developed. In the present study, we used silk fibroin (SF) as a biomaterial supporting the growth of MMSC and studied its effect on chondrogenesis of canine adipose–derived MMSC (cADMMSC). Adipose tissue was collected from nine privately owned dogs. MMSC were cultured on SF films and SF scaffolds in a standard cell culture medium. Cell morphology was evaluated by scanning electron microscopy (SEM). Chondrogenic differentiation was evaluated by alcian blue staining and mRNA expression of collagen type 1, collagen type 2, Sox9, and Aggrecan genes. cADMMSC cultured on SF films and SF scaffolds stained positive using alcian blue. SEM images revealed nodule-like structures with matrix vesicles and fibers resembling chondrogenic nodules. Gene expression of chondrogenic markers Sox9 and Aggrecan were statistically significantly upregulated in cADMMSC cultured on SF films in comparison to negative control cADMMSC. This result suggests that chondrogenesis of cADMMSC could occur when cells were grown on SF films in a standard cell culture medium without specific culture conditions, which were previously considered necessary for induction of chondrogenic differentiation.

Keywords

Mesenchymal stem cells, multipotent mesenchymal stromal cells, dog, silk fibroin, chondrogenic differentiation

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Introduction

Mesenchymal stem cells (MSC), more properly called multipotent mesenchymal stromal cells (MMSC), have received significant interest for their potential use in regenerative therapy in human and veterinary medicine due to their immunosuppressive and multilineage differentiation capabilities.[1] There are several established protocols for induction of MMSC differentiation in vitro, which are optimized for conventional culturing of MMSC in two-dimensional (2D) cell culture system with polystyrene vessels. Under appropriate culture conditions, differentiation of MMSC can be induced toward adipocytic, osteocytic, and chondrocytic lineages.[2,3] Chondrogenesis can be induced with specific culture conditions such as high cell density, or by induction with different hormones and growth factors, particularly TFG-β.[4,5] However, there are also several reports describing spontaneous differentiation of MMSC toward different lineages.[6–9]

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The key factors responsible for MMSC differentiation are not yet known. Current stem cell research is trying to understand the mechanisms needed for guiding stem cell fate in a desired manner.[10] Traditional culturing of MMSC on 2D surfaces, such as cell culture polystyrene is thus being replaced by three-dimensional (3D) cell culture techniques. In vivo cells reside in a very complex 3D environment, which plays an essential role in determining stem cell fate and regulating their self-renewal and differentiation. In vitro, however, the culture conditions are very different, especially in classical 2D systems, and this raises the questions how much the cells cultured in vitro resemble cells inside the organisms. Therefore, there is a need for the development of novel biomaterials, which could provide appropriate physiologically relevant biochemical and mechanical signals in order to guide stem cell fate[10] and to restore tissue functions.[11] It has been demonstrated that mechanical cues,[12–14] surface chemistry,[15–17] and physical properties[18–20] of biomaterials play key roles in the regulation of stem cell differentiation.

Different biomaterials such as polyetherurethane and poly(ether imide),[21] polyethylene glycol-linked multiwalled carbon nanotube films,[22] reduced graphene oxide-coated hydroxyapatite composites,[23] poly-lactic-co-glycolic acid nano-fiber scaffold,[24] and electrospun nanofibrous scaffolds[25] have been shown to promote differentiation of MMSC into adipogenic, myogenic, osteogenic, and chondrogenic lineages. However, the mechanisms regulating these differentiation processes are not yet understood. Until now, there is no universal biomaterial that could meet all the requirements for different tissues with specific physical and mechanical properties. One of the promising biomaterials to be used in tissue engineering is silk fibroin (SF), derived from the silkworm *Bombyx mori*. It is biocompatible, has suitable mechanical properties, and is produced in bulk in the textile industry.[26] In comparison to other polymers used for tissue engineering, SF provides a remarkable combination of strength, toughness, and elasticity that are assigned to its crystallinity, hydrogen bonding, and numerous small β-sheet crystals.[27] Moreover, as a natural biopolymer, it degrades to non-toxic and neutral degradation products (amino acids and peptides), whereas the degradation is slow and controllable. Although it is a natural biopolymer, it could be sterilized using common sterilization techniques, it has high thermal stability, and the processing of materials from silk can be aqueous-based.[28] Furthermore, SF can be fabricated into different materials such as hydrogels, tubes, sponges, composites, fibers, microspheres, and films that could be used in tissue engineering.[29] Various modifications of SF properties can aid in stem cell proliferation and differentiation potential,[30–35] although it has not been reported before that SF promotes differentiation of MMSC. In this study, we demonstrate that SF guide canine adipose–derived MMSC (cADMMSC) toward presumed chondrogenic differentiation.

Materials and methods

Adipose tissue collection

Subcutaneous adipose tissue was collected from nine privately owned dogs. Adipose tissue was individually collected during routine clinically indicated surgery at Small Animal Clinic of the Faculty of Veterinary Medicine in Ljubljana. All samples were randomly assigned to different experimental groups. All owners agreed with the collection of tissue and signed an informed consent. Since study was conducted on client-owned animals undergoing routine clinical procedure with owner's approval to collect small piece of adipose tissue, no approval of ethical committee was needed according to Slovenian legislation and official opinion from The Administration of Republic of Slovenia for Food Safety, Veterinary and Plant protection, responsible for issuing ethical permits for animal experiments.

Isolation and culture of cADMMSC

Immediately after collection, adipose tissue was washed with Dulbecco's Phosphate Buffered Saline (DPBS, Gibco, USA) and cut with a scalpel into small pieces. Adipose tissue was then incubated overnight at 37°C in Dulbecco-modified Eagle medium (DMEM, Gibco, USA) containing 0.1% collagenase type II (Sigma-Aldrich, DE). The digested tissue was centrifuged at 1600 r/min for 4 minutes, and the supernatant was discarded. Pellet of cells was resuspended in cell culture medium containing DMEM and 10% Fetal Bovine Serum (FBS, Gibco, USA). The cell suspension was plated into 6-well plates (TPP, Switzerland) and cultured at 37°C in a 5% CO_2 incubator. Cell culture medium was changed every 2–3 days. After 80%–90% confluency was reached, cells were trypsinized and multiplied by seeding 10^4 cells per cm^2 into a larger (T25) cell culture flask. Cells in cell culture were maintained up to the fourth passage. After a sufficient number of cells was reached, cells were used for assessing chondrogenic differentiation. All cells used in the experiments were from the second, third or fourth passage, and cells were cultured for 2 or 3 days in each passage. All experiments were repeated five times for positive and negative controls and six times for cells cultured on SF films (both 1 week and 2 weeks).

Multilineage differentiation potential of cADMMSC

Differentiation potential was assessed by inducing cADMMSC differentiation into adipocytes, osteocytes, and chondrocytes. For the adipogenic and osteogenic differentiation 4×10^4 cells were seeded in 12-well plates. When 90% confluency was reached, the cell culture medium was removed. Adipogenic (StemPro Adipogenesis Differentiation Kit, Gibco, USA) or osteogenic (StemPro

Osteogenesis Differentiation Kit, Gibco, USA) medium was added and changed every 2–3 days. Cell culture medium was added to the wells that served as negative controls. Adipogenic differentiation was analyzed with Oil-red-O staining (Sigma-Aldrich, DE) after 21 days of culturing. Osteogenic differentiation was analyzed with Alizarin Red S staining (Sigma-Aldrich, DE) after 14 days of culturing, which is shorter than recommended for human cells, but during our preliminary studies, we have established that with canine cells, 21 days of culture is too long period (cells start to detach) while majority of cells stain positive with Alizarine red already after 14 days of culturing. For the chondrogenic differentiation, micromass cultures were generated by seeding 5 µL droplets of 4×10^4 cells in the center wells of 12-well plate. After cultivating micromass cultures for 6 hours under high humidity conditions, chondrogenic medium (StemPro Chondrogenesis Differentiation Kit, Gibco, USA) was added to culture vessels. Regular cell culture medium was added to the wells that served as negative controls. Micromass cultures were incubated in 37°C incubator with 5% CO_2 and humid atmosphere. The medium was changed every 2–3 days. Chondrogenic differentiation was analyzed with alcian blue staining (Sigma-Aldrich, DE) after 14 days of culturing.

Preparation of SF films and scaffolds

SF films and scaffolds were prepared following the procedure described by Rockwood et al.[36] Briefly, *Bombyx mori* silk cocoons were cut in pieces and boiled for 30 minutes in 0.02 M solution of sodium carbonate (Na_2CO_3) to extract sericin. SF was rinsed in ultrapure water several times until the conductivity of water became constant and then dried overnight at 65°C. Degummed SF was dissolved in 9.3 M lithium bromide (LiBr) solution at 72°C for 3 hours and then subsequently dialyzed in a constant flow (0.4 L h^{-1}) of ultra pure water at 4°C until its conductivity fell below 0.5 µS. The molecular weight cut off of dialysis tubing cellulose membrane was 12–14 kDa. To eliminate impurities, the prepared solution was centrifuged at 20,000 r/min for 20 minutes. The concentration of SF solution was determined by Bradford assay protocol[37] based on the color change of Coomassie Brilliant Blue G-250 using Bio-Rad protein assay (Bio-Rad laboratories, Hercules, CA). The SF solution was added to the Bradford reagent and incubated for 5 minutes. The absorbance was measured at 595 nm. Two different concentrations of SF solution were used for the preparation of scaffolds and films. The concentration of the prepared solution was on average 8 mg/mL and was used for the preparation of scaffolds, whereas a higher concentration of SF solution, 12.5 mg/mL, was used for the preparation of films. Higher concentration was achieved using centrifugation through centrifugal filter units (Amicon Ultra–4 centrifugal filter unit, Merck, Cork, IE).

SF films were prepared by casting 300 µL of the SF solution (12.5 mg/mL) into the wells of 12-well plates with a subsequent overnight air-drying. Films were then incubated in 70% ethanol for 10 minutes. In the last step, films were thoroughly washed with PBS.

SF scaffolds were prepared by adding 300 µL of SF solution (8 mg/mL) into the wells of 48-well plate. SF solution in well plates was then frozen in liquid nitrogen and lyophilized at −50°C for 72 hours to sublimate water and thus form porous scaffolds. After lyophilization, SF scaffolds were soaked in absolute ethanol overnight and then dried in a desiccator. Finally, scaffolds were thoroughly washed with PBS to remove any remaining ethanol.

SF scaffold characterization

Porosity and the pore size distribution of the SF scaffolds were determined using a Pascal series mercury intrusion porosimeter (Thermo Scientific). The surface tension and the contact angle of the mercury were set to 0.485 and 140 mN/m, respectively.

Wettability of the SF film was evaluated by measuring water droplet contact areas of the curve fitted to the droplet image on a dry and wet SF film using the Contact Angle Instrument (First Ten Ångstroms, Inc., USA, FTA1000 series). The measurement system consisted of a sample stage, vertically fitted Hamilton micro-syringe to place the water droplet on the sample and the camera mount–TV lens camera with Extension tube set 40 mm (Edmund optics, Japan). Images were captured and analyzed for contact areas using the FTA32 Video 2.0 software.

Cultivation of cADMMSC on SF films and SF scaffolds

After a sufficient number of cells was obtained, cells were cultured in four different ways:

(1) On SF films in cell culture medium for 7 and 14 days: 10^4 cells per cm^2 were seeded onto 12-well plate with wells coated with SF films.
(2) On SF scaffolds in cell culture medium for 14 days: 9×5 µL droplets of 1×10^5 cells were seeded onto the bottom side of the SF scaffolds. During scaffold preparation, membrane-like portion of SF formed on the top of the scaffolds making the scaffold impassable for cells. Therefore, scaffolds were carefully lifted from the wells and turned upside down. Cells were then seeded onto the scaffolds.
(3) On a standard polystyrene surface in chondrogenic medium for 14 days: cells were cultured as described above for a multilineage differentiation potential.
(4) On a standard polystyrene surface in standard cell culture medium until 80%–90% confluency was reached.

Cell cultures were named accordingly (Table 1).

Table 1. Name of the cell cultures, cell seeding surfaces and densities, culture media, and culture conditions used in the study.

Name of the cell culture	Cell seeding surface	Cell seeding density	Culture medium	Days of culturing	Culture conditions
SF film cADMMSC	SF film	10^4 per cm^2	Cell culture medium	7 and 14	37°C, 5% CO_2
SF scaffold cADMMSC	SF scaffold	9×5 µL droplets of 5×10^5 cells per scaffold	Cell culture medium	14	37°C, 5% CO_2
Positive control cADMMSC	Standard polystyrene	5 µL droplets of 4×10^4 cells	Chondrogenic medium	14	37°C, 5% CO_2, high humidity
Negative control cADMMSC	Standard polystyrene	10^4 per cm^2	Cell culture medium	Until 80%–90% confluency	37°C, 5% CO_2

cADMMSC, canine adipose–derived multipotent mesenchymal stromal cells; SF, silk fibroin.

Alcian blue and DAPI staining

For alcian blue staining of SF film cADMMSC, positive control cADMMSC, and negative control cADMMSC, medium was removed from culture vessels. Wells were rinsed once with DPBS and cells were fixed with 4% paraformaldehyde solution for 1 hour. Following fixation, wells were rinsed twice with DPBS and incubated overnight in 1% alcian blue stain (pH 2.5), prepared in 0.1 N HCl. Next day wells were rinsed three times with 0.1 N HCl followed by DPBS to neutralize the acidity. Wells were examined under the light microscope. For comparison of alcian blue stained presumed chondrogenic nodules from positive control cADMMSC and SF film cADMMSC, some presumably chondrogenic nodules were immersed in tissue freezing medium (Leica Byosistems, Germany) and frozen in liquid nitrogen. The 2 µm thick cryosections were made with cryotome (Leica Byosistems), placed onto glass slides and visualized under the light microscope. For alcian blue staining of SF scaffold cADMMSC, the medium was removed from the wells. Whole SF scaffolds were immersed in tissue freezing medium (Leica Byosistems) and frozen in liquid nitrogen. The 18 µm thick cryosections were made with a cryotome (Leica Byosistems) and placed onto glass slides coated with 1% poly-L-lysine (Sigma-Aldrich, Germany). Slides were fixed in 4% paraformaldehyde solution for 10 minutes at 4°C, rinsed once with distilled water (dH_2O), incubated in alcian blue for 5 minutes at room temperature, rinsed again with dH_2O and dried at room temperature. Slides were then mounted with histology mounting medium containing DAPI (Sigma-Aldrich), covered with coverslips and visualized under light and fluorescent microscope.

Light and fluorescent microscopy

For analysis of multilineage differentiation potential of cADMMSC and alcian blue staining of SF film and SF scaffold cADMMSC, an inverted microscope (Nicon Eclipse TS100, Nikon, Japan) and fluorescent microscope (Nicon Eclipse 80i, Nikon) equipped with Nikon Digital Sight DS-U2 camera were used. For analysis of DAPI staining, ultraviolet (UV) fluorescence filter of wavelength 330–380 nm was used. Images were captured in NIS–Elements D3.2 Live quality program.

Scanning electron microscopy

Scanning electron microscope (FEG-SEM, JEOL JSM 7600 F, Japan) under low voltage imaging conditions was used to display adhesion and morphology of cADMMSC seeded onto SF films and scaffolds. Images of SF film and SF scaffold cADMMSC were compared to the images of positive and negative control cADMMSC. Before the examination, the samples were fixed following the protocol described elsewhere.[38,39] Briefly, the medium was removed from culture vessels. Wells were rinsed once with DPBS and fixed with 2.5% glutaraldehyde solution (Sigma) overnight. After fixation, the samples were rinsed with 0.1 M cacodylate buffer (Sigma) at pH 7.4 for 1 hour with three changes, rinsed with distilled water for 1 min and then dehydrated in 10 minute steps in a series of ascending ethanol baths (25%, 50%, 75%, 95%, and 100%). Dehydrated samples were then immersed into hexamethyldisilazane bath (5 minutes, 100% HMDS) (Sigma), air-dried, mounted, and sputtered with gold.

Scanning electron microscopy (SEM) was also used for the estimation of film thickness and for pore size evaluation in SF scaffolds. Film thickness was measured from the cross-section image of SF film. Pore size was estimated by measuring the pore diameter in two directions, parallel and perpendicular to the surface of the scaffold. Diameters of all pores that were not touching the edge of an image were measured by ImageJ software on three low magnification images, where approximately a third of the scaffold is visible.

RNA isolation

RNA was isolated from SF film cADMMSC, positive control cADMMSC, and negative control cADMMSC. SF film and positive control cADMMSC were detached from the SF film and polystyrene surfaces using cell scrapper. Negative control cADMMSC were detached by trypsinisation. The cell suspension was removed from the wells and

Table 2. Symbols, names, and assay identification numbers of genes of interest and their role in MSC chondrogenesis.

Gene symbol	Gene name	Assay ID	Gene role in chondrogenesis
Sox9	SRY (sex determining region Y)-box9	cf02625134_g1	The first transcription factor, essential for chondrocyte differentiation and cartilage formation[40]
Col1A1	Collagen type I, alpha 1	Cf01076765_m1	Most abundant in preaggregate cells[5]
Col2A1	Collagen type II, alpha 1	cf02622868_m1	Cartilage specific marker gene[40]
Acan	Aggrecan	Cf02674826_m1	Cartilage specific marker gene[41]
TBP	TATA box binding protein	cf02637231_m1	Reference gene

MSC, mesenchymal stem cells.

centrifuged at 1600 r/min for 4 minutes. Pellet of cells was resuspended in DPBS and centrifuged again. Pellet of cells was then homogenized in 150 µL Trizol (ThermoFisher, USA) with a homogenizer (IKA T10 basic, Germany). Total RNA extraction was carried out using Trizol according to the manufacturer's protocol. The amount of extracted total RNA was measured by UV spectrophotometer (ThermoFisher) at 260/280 nm wavelength.

Reverse transcription quantitative polymerase chain reaction

Two-step reverse transcription quantitative polymerase chain reaction (RT qPCR) for SF film cADMMSC, positive control cADMMSC, and negative control cADMMSC was performed. First, 1 µg of total RNA of each specimen was reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit with RNAse Inhibitor (ThermoFisher) according to the manufacturer's protocol. Negative reverse transcription controls were included in each PCR run. All reactions were conducted in a total volume of 20 µL. Conditions for reverse transcription were as suggested in the manufacturer's protocol: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes. In the second step, relative quantification was performed using TaqMan Universal PCR Master Mix with UNG (ThermoFisher) and TaqMan gene expression assays Sox9, Col1A1, Col2A1, and Acan. TBP was used as a reference gene (Table 2; ThermoFisher). All qPCR amplifications were conducted in triplicates in a total volume of 20 µL. A 20 ng cDNA was used as a template. The amplification was carried out in 96-well plates with a Light Cycler 96 (Roche Life Science) using the following program: 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles at 95°C for 15 seconds, 60°C for 60 seconds.

Statistical analyses

All statistical analyses were performed with NCSS software package (Kaysville, UT, USA).

Experiments with chondrogenic differentiation were repeated five times with cells from different dogs for negative and positive controls, and six times with cells from different dogs for cells growing on SF films. Three experiments with different cells were performed with SF scaffolds.

All RT qPCR experiments were run in triplicates. The efficiency corrected double delta Ct method was employed to normalize gene expression values.[42] The expression levels of Col1A1, Sox9, Col2A1, and Acan in positive control cADMMSC and SF film cADMMSC were compared to the expression levels of Col1A1, Sox9, Col2A1, and Acan in negative control cADMMSC and results were analyzed by Kruskal–Wallis non-parametric test. Statistical significance was determined with $p < 0.05$.

Results

Culturing and multilineage differentiation of negative control cADMMSC

Adipose tissue was successfully collected from all animals. Under the light microscopy, cells with fibroblast-like morphology were observed in all samples the day after plating. Using SEM, negative control cADMMSC appeared flat with spread morphology and wide extensions (Figure 1).

Cell culture was maintained up to the second through the fourth passage. cADMMSC were capable of differentiating into adipocytes, osteocytes, and chondrocytes when cultured in specific differentiating media. After adipogenic differentiation, intracellular lipid droplet stained red using Oil-red-O. After osteogenic differentiation, mineral deposits in extracellular matrix stained red using Alizarin-red-S and after chondrogenic differentiation, proteoglycans in the extracellular matrix of layered cell clusters stained positive with alcian blue (Figure 2).

Morphology and alcian blue analysis of positive SF film cADMMSC

SF film cADMMSC were cultured in normal culture medium on SF films. SF films prepared from SF solution with a concentration of 12.5 mg/mL were transparent, with diameter of 21 mm, and the approximate dry film thickness

of 80 µm. The surface of the film appears smooth and dense, some roughness is visible only on a submicron level caused by nanosized pores that are assumed to form during drying of the film (Figure 3). The contact angle of a water droplet on a dry SF film is 70°, indicating a hydrophobic surface. After washing the film with PBS, the surface becomes hydrophilic with a water contact angle of 0°.

SF film cADMMSC were cultured in normal cell culture media on SF films. SF films were transparent, with diameter of 21 mm, approximate thickness of 900 µm and average fibroin density of 12.5 mg/mL. Cells successfully adhered onto SF film surface. In comparison to negative control cADMMSC (Figure 1), SF film cADMMSC displayed less spindle-shaped morphology (Figure 4(a)). A tendency toward cell grouping was observed. The second day after seeding, nodule-like structures began to form (Figure 4(b)).

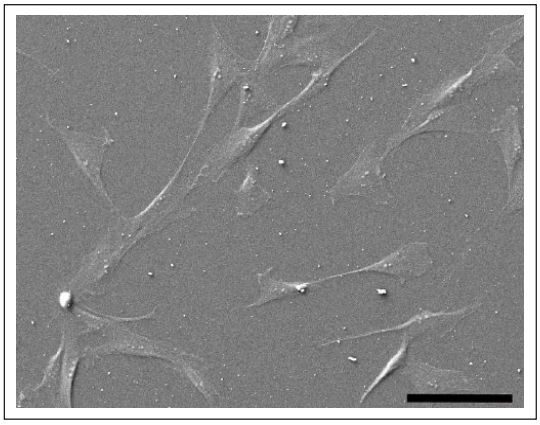

Figure 1. SEM image of negative control cADMMSC that appear flat with spread morphology and wide extensions.

Positive control cADMMSC were cultured in the chondrogenic medium on a standard polystyrene surface for 14 days. Cells changed their morphology from adherent monolayer spindle-shaped cells to layered nodule-like cell clusters. Nodules that morphologically resembled chondrogenic nodules tended to form connections among each other and stained positive with alcian blue (Figure 5(a)). SEM images of positive control cADMMSC confirmed chondrogenic nodule–like structures with many extracellular matrix vesicles (Figure 5(d)).

One to three days after culturing cADMMSC on SF films, chondrogenic-like nodules appeared (Figure 5(b) and (c)), similar to those formed in positive control cADMMSC (Figure 5(a)), with indistinguishable intensity of alcian blue staining. No apparent difference in alcian blue staining between SF film cADMMSC cultured for 7 and 14 days was detected (Figure 5(b) and (c)). SEM analysis of SF film cADMMSC revealed chondrogenic nodule–like structures (Figure 5(e) and (f)), comparable to those formed in positive control cADMMSC (5d). Many extracellular matrix vesicles were also observed. Nodule-like structures in SF film cADMMSC cultured for 14 days (Figure 5(f)) appeared to be more defined in comparison to those cultured for 7 days (Figure 5(e)).

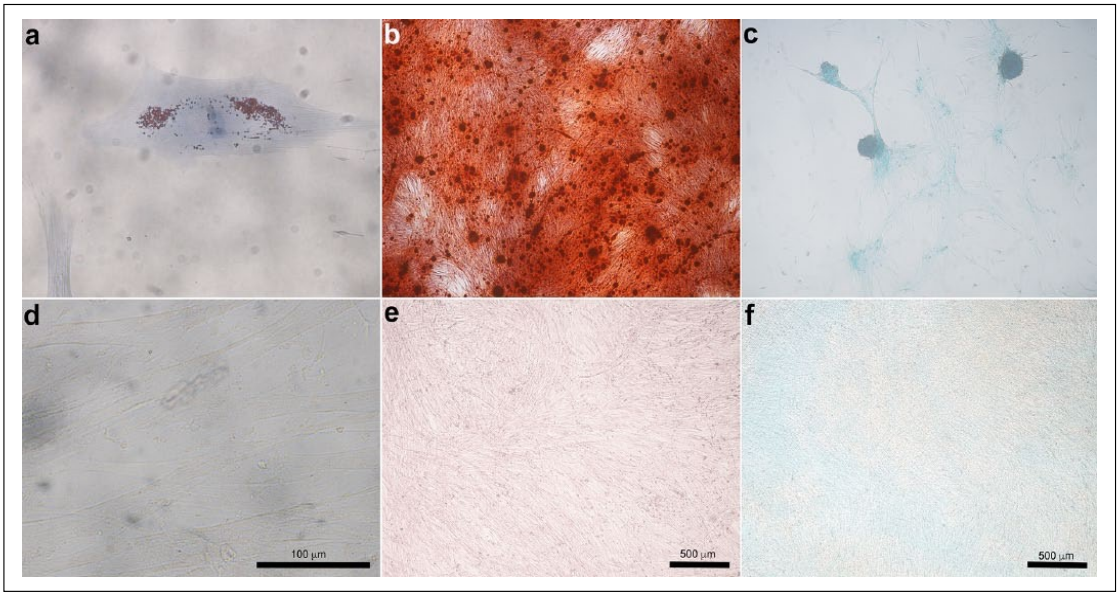

Figure 2. Differentiation potential of cADMMSC. Adipogenic potential of cADMMSC is indicated by red intracellular lipid droplets using Oil-red-O (a). In osteogenic differentiation, mineral deposits in extracellular matrix are stained red using Alizarin-red-S (b). Chondrogenic differentiation is indicated by the formation of chondrogenic nodules that stain blue with alcian blue (c). Respective negative controls are shown at the bottom (d, e, f).

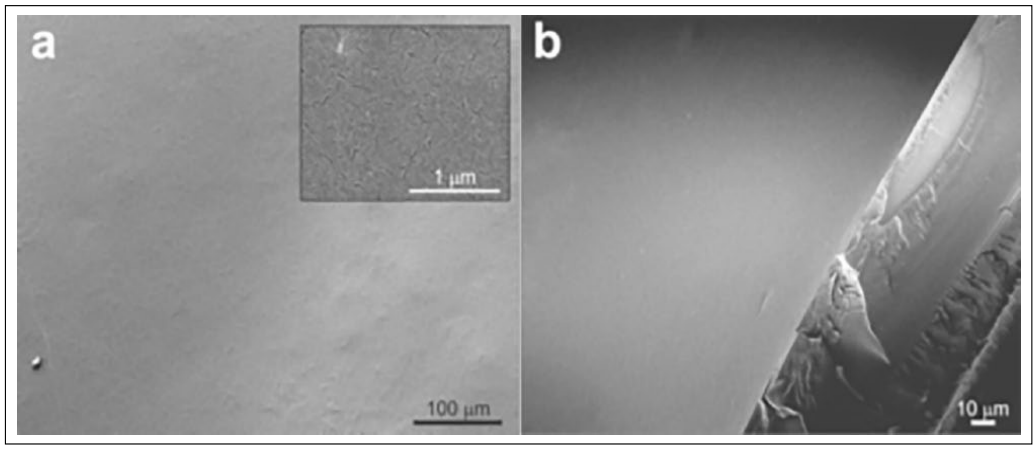

Figure 3. SEM images of SF film surface (a), the inset shows nanosized surface porosity at higher magnification, and cross-section of SF film (b).

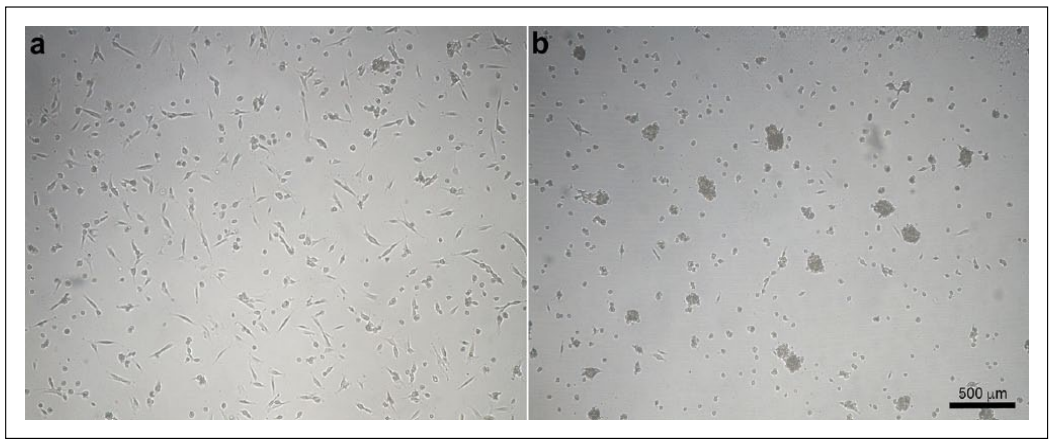

Figure 4. Morphology of SF film cADMMSC. The first day after seeding, SF film cADMMSC successfully attached to the SF film surface (a); Cells appeared less spindle-shaped compared to negative control cADMMSC. Many round-shaped cells are present and a tendency toward cell grouping was observed. On the second day after seeding, nodule-like structures began to form (b).

Cryosections of alcian blue stained chondrogenic-like nodules from positive control cADMMSC revealed the correct round shape of a nodule, enclosed within a capsule-like structure. Nodule fibers appeared thick and homogenously arranged (Figure 6(a)). Cryosections of alcian blue stained chondrogenic-like nodules from SF film cADMMSC revealed more irregularly shaped nodules without an apparent capsule compared to cryosections of positive control chondrogenic nodule. Nodule fibers were thinner and organized into smaller separate circles formed inside of a nodule (Figure 6(b)).

Gene expression of chondrogenic markers

The mRNA expression of Col1A1 was similar in all four samples (Figure 7(a), N=5 for positive and negative control, and 6 for SF films after 1 and 2 weeks). mRNA expression of Sox9 (Figure 7(c), N=5 for positive and negative control, and 6 for SF films after 1 and 2 weeks) and Acan (Figure 7(d), N=3 for positive control, 4 for negative control, and 6 for SF films after 1 and 2 weeks) was statistically significantly higher in positive control cADMMSC and SF film cADMMSC in comparison to negative control cADMMSC ($p < 0.05$ for Sox9 and $p < 0.01$ for Acan). The expression level of Col2A1 (N=4 for positive and negative control, and 6 for SF films after 1 and 2 weeks) was not statistically significantly different between negative and positive control cADMMSC and SF film cADMMSC (Figure 7(b)).

SF scaffold characteristics

The volume of the scaffolds was 0.3 mL with an approximate thickness of SF scaffolds 5.2 mm. Light microscopy images of unseeded cryosections of scaffold showed a porous matrix with interconnected elongated pores (Figure 8(a) and (b)). Scaffold porosity was 92.7%, of which

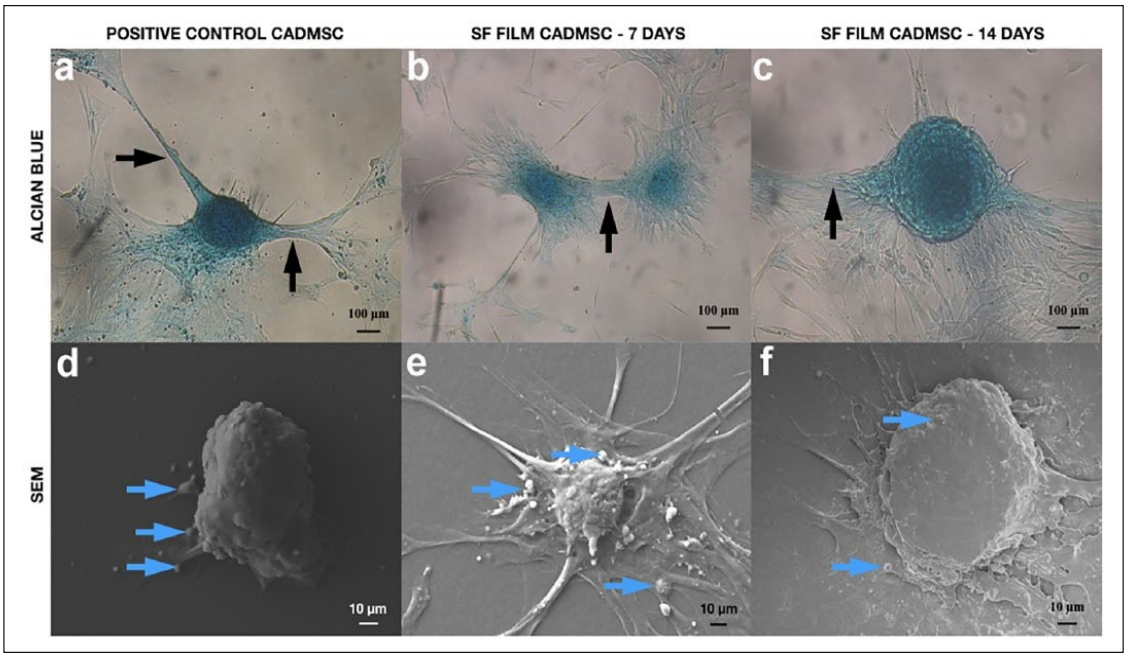

Figure 5. Morphology of positive control cADMMSC and SF film cADMMSC. In both, positive control cADMMSC (a, d) and SF film cADMMSC (b, e—7 days culture; c, f—14 days culture), clusters of cells reminding of a nodule were formed and stained blue with alcian blue. In both groups, interchondrogenic nodule connections were observed (black arrows). No apparent difference in shape or alcian blue staining intensity between presumed chondrogenic nodules in positive control cADMMSC (a) and SF film cADMMSC (b, c) were present. SEM analysis (d, e, f) confirmed the formation of chondrogenic-like nodules, which appeared more defined in positive control cADMMSC (d) and SF film cADMMSC cultured for 14 days (f) than in SF film cADMMSC cultured for 7 days (e). Many matrix vesicles are seen in positive control cADMMSC and SF film cADMMSC (blue arrows).

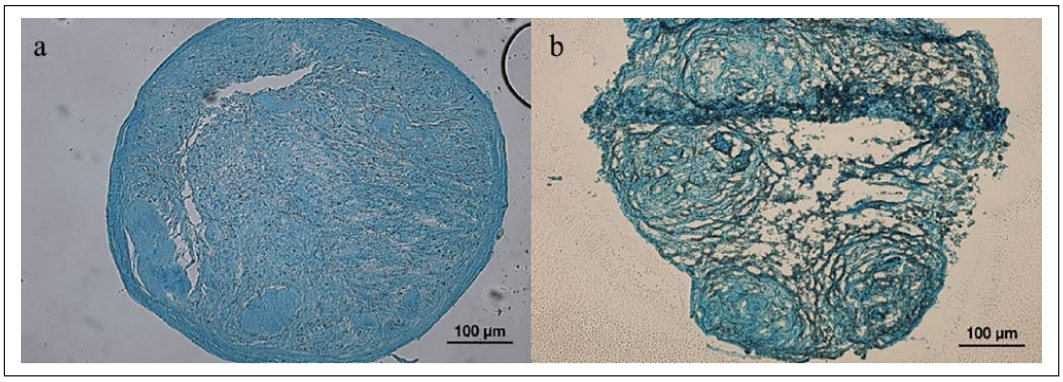

Figure 6. Morphology of cryosected chondrogenic nodules: (a) Cryosection of a chondrogenic nodule from positive control cADMMSC: chondrogenic nodule is roundly shaped surrounded with a capsule-like structure. Nodule fibers are thick and homogenously arranged; (b) Cryosection of a chondrogenic-like nodule from SF film cADMMSC: Nodule is more irregularly shaped without an apparent capsule. Nodule fibers are thin and arranged into individual smaller circles seen inside of a nodule.

84.6% represents open, and 8.1% closed porosity. The mean pore opening diameter measured as a function of pressure from the mercury intrusion measurements was 50 µm, with a minimum and maximum at 0.004 and 107 µm, respectively. SEM images of nonseeded SF scaffold revealed a porous matrix with a broad distribution of pore sizes, where the bottom side of a scaffold had larger pores (Figure 8(c)). Mean pore diameter measured on SEM images in the elongated direction was 314 ± 296 µm and 58 ± 55 µm in the direction parallel to the surface.

Morphology and alcian blue analysis of positive, SF scaffold cADMMSC

SF scaffold cADMMSC were cultured in culture medium in SF scaffolds. Cell migration into the scaffold was analyzed

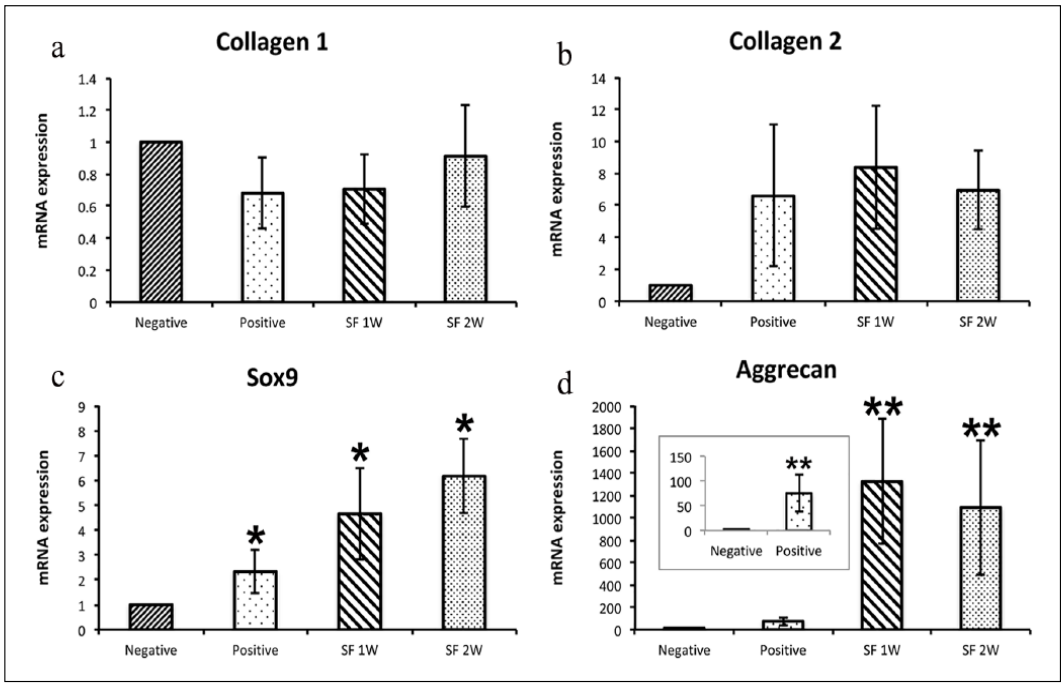

Figure 7. mRNA expression levels of Col1A1 (a) and ColL2A1 (b) were similar in all four groups (negative controls and positive controls (N = 5 for Col1A1 and N = 4 for Col2A1), and cells cultured for 1 week (SF 1W) or 2 weeks (SF 2W; N = 6 for all samples) on SF films. Expression levels of Sox9 (c, N = 5 for positive and negative controls and N = 6 for SF 1W and SF 2W) and Aggrecan (d, N = 3 for positive controls, N = 4 for negative controls and N = 6 for SF 1W and SF 2W) differed significantly between negative control and positive control groups, as well as between negative control and cells cultured for 1 or 2 weeks on SF films (*$p < 0.05$ for Sox9 and **$p < 0.01$ for Aggrecan). Results are presented as mean ± SEM and data were normalized to the expression of genes in negative control samples.

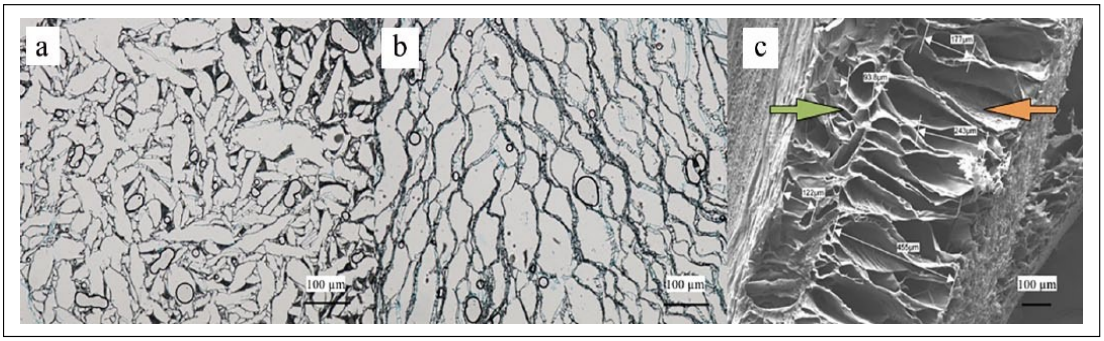

Figure 8. Morphology of a non-seeded SF scaffold: (a) Light microscopy image of a cryosection of an upper part of the scaffold with a pore size of about 100 µm; (b) light microscopy image of a cryosection of a bottom part of the scaffold with a pore size up to 455 µm; and (c) SEM image of the scaffold. The green arrow indicates the upper part of the scaffold. Orange arrow indicates the bottom part of the scaffold.

by a fluorescent microscope with DAPI staining (Figure 9(c) and (d)) and SEM (Figure 10). Based on the presence of the cells in all cryosections, it was concluded that SF scaffold structure allows penetration of cells inside the scaffold, although more cells remained on the surface of the scaffold (Figure 9). Nevertheless, some cells migrated throughout the scaffold and successfully attached to the pore walls. Positive alcian blue staining of cells was confirmed on the surface and within the scaffold (Figure 9(a) and (b)). Chondrogenic nodules were observed only occasionally close to the surface of the scaffold (Figure 9(b)). Mostly, amorphous layers of cells were observed, but these also stained positive with alcian blue (Figure 9(a)).

SEM analysis of SF scaffold cADMMSC confirmed homogeneous distribution of cells throughout the scaffold and their attachment to the pore walls. A dense network of

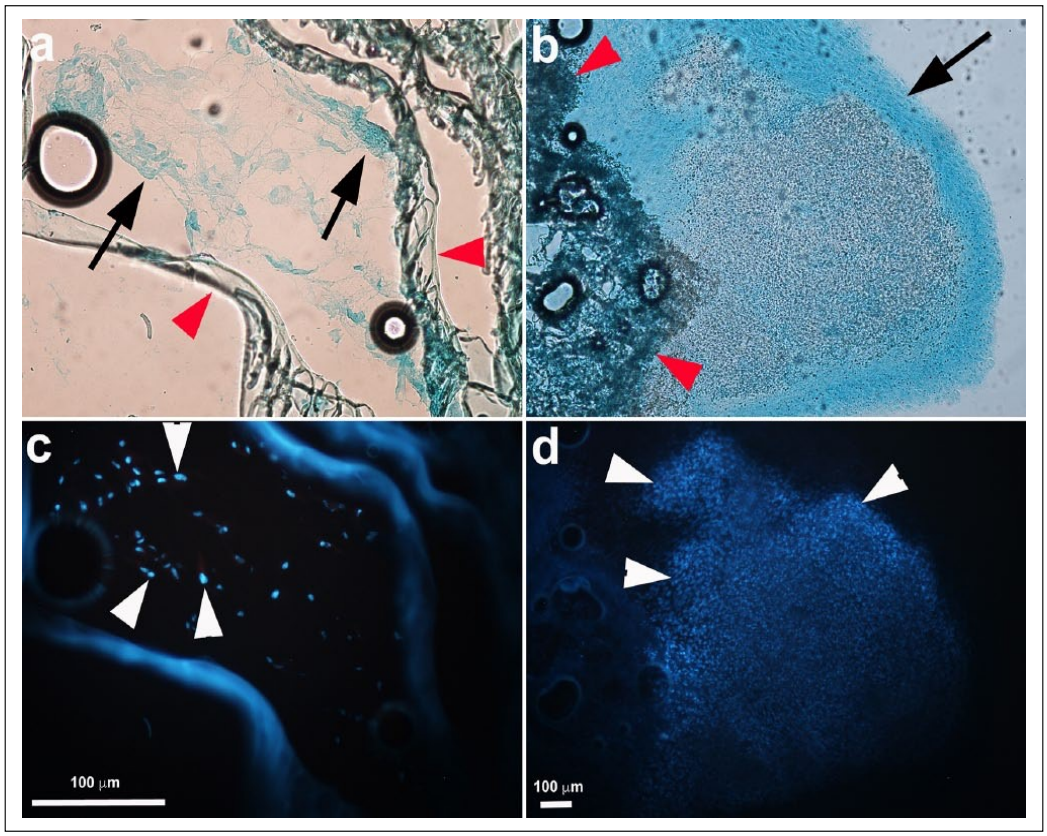

Figure 9. SF scaffold cADMMSC. In upper row, alcian blue staining of scaffold cryosections is shown. In lower rows, the same images are shown under fluorescence, where cell nuclei were stained with DAPI (white arrowheads). In parts of the scaffold surface, chondrogenic nodule–like structures (black arrow) were present (b) and cell density in these structures was very dense (white arrowheads—cell nuclei stained with DAPI in panel (d)). Inside the scaffold (panels a and c) cells were present, but at lower density in comparison to surface (white arrows—cell nuclei stained with DAPI in (c) and (d)) and they did not form chondrogenic nodules, although they stained positive with alcian blue (arrow, a). SF structure is marked with red arrowheads in panels a and b.

fibers was formed, and numerous extracellular matrix vesicles were observed (Figure 10(a) and (b)—scaffold with cells at different magnifications).

Discussion

In attempts to mimic the native extracellular matrix, numerous studies are focusing on culturing MMSC in a 3D culture system, which better imitates their natural environment. Although several biomaterials have been shown to promote differentiation of MMSC toward different lineages,[21–25] there are no reports about SF inducing differentiation of MMSC. In the present study, we demonstrate that cADMMSC presumably undergo chondrogenic differentiation when grown on SF films and SF scaffolds in a standard cell culture medium. cADMMSC appeared to follow chondrogenesis without chondrogenic-specific conditions such as high cell density or stimulation with TGF-β.[4] Morphology of cells observed under the microscope, positive alcian blue staining of the SF film cADMMSC and SF scaffold cADMMSC, and upregulation of Sox9 and Aggrecan mRNA expression in cADMMSC on SF films suggest that cells underwent chondrogenic differentiation. Chondrogenesis of cADMMSC cultured on SF films was also suggested by similar chondrogenic-like nodule morphology, and a tendency to form interchondrogenic nodule connections as it was observed in positive control cADMMSC. In comparison to cells cultured on SF films, cADMMSC cultured inside the SF scaffolds formed chondrogenic-like nodules only in parts of the scaffold. Elsewhere, amorphous layers of cells were present, but, interestingly, both nodules and amorphous layers of cells inside the SF scaffolds stained positive with alcian blue suggesting at least partial induction of chondrogenesis. Comparison of SEM images between positive control cADMMSC, SF film cADMMSC, and SF scaffold cADMMSC showed the presence of cell nodules that resembled chondrogenic nodules. In all three groups of cells, many extracellular matrix vesicles were present and abundant extracellular matrix formed which appeared as chondrogenic-like nodule structures. SEM images of SF scaffold cADMMSC

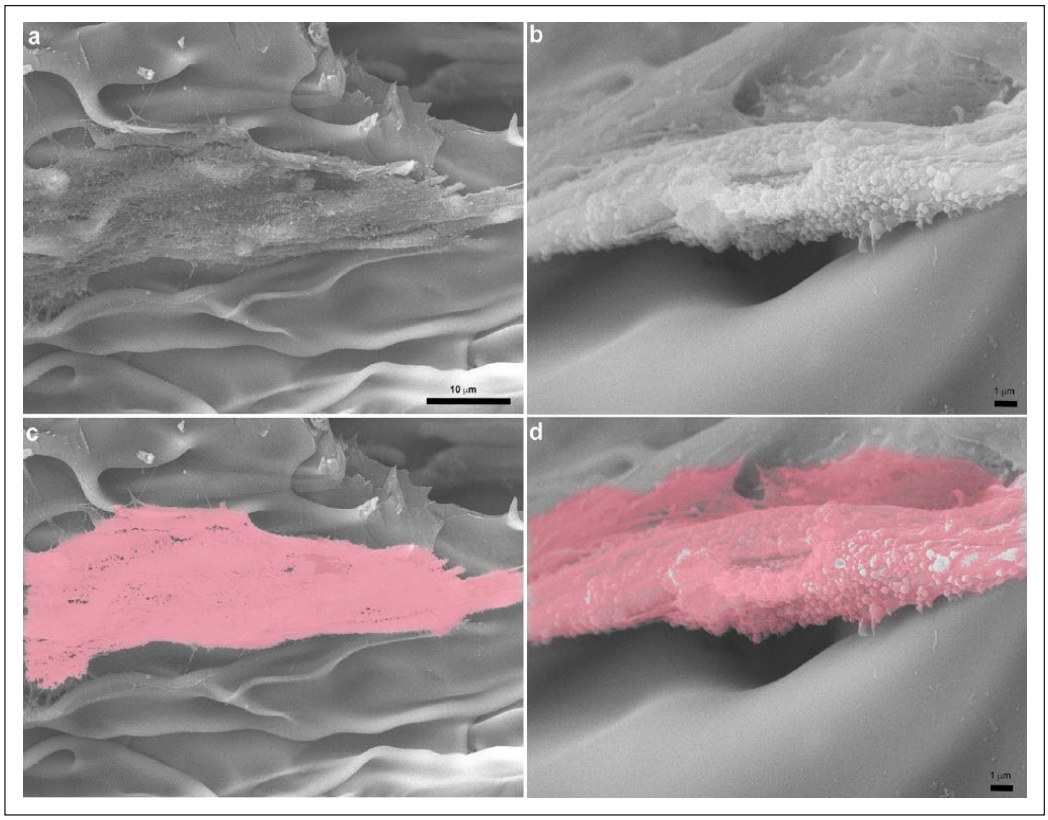

Figure 10. SEM images of cADMMSC grown on SF scaffold: (a) cells attached to the pore wall. Numerous matrix vesicles and fibers are seen on top of the cells; (b) cell inside the scaffold pore at higher magnification. Matrix vesicles are protruding from the cell; (c, d) Same cells marked with color.

also revealed numerous fibers formed by the cells, which suggest possible formation of collagen.

It has been previously demonstrated that in vitro initial cell aggregates, cultured on polystyrene surface under specific culture conditions, contain Col1A1. Within 2–3 weeks of chondrogenic differentiation, MMSC undergo chondrogenic differentiation and start to produce abundant extracellular matrix composed of Col2A1.[5] Differentiation of MMSC is characterized by a decrease in proliferation and upregulation of lineage-specific genes.[43] TGF-β is known to induce chondrogenesis by activating SMAD signaling pathway and upregulating chondrogenic genes such as Sox9.[1] The latter has been identified as the main transcriptional regulator of chondrogenic specific markers, namely, type II collagen.[40] Another marker of chondrogenic differentiation is Aggrecan, an essential component of mature cartilage, whose expression also increases during chondrogenic differentiation of MMSC.[44] In our study, the expression of Col1A1 and cartilage-specific markers Sox9, Aggrecan, and Col2A1 were determined by RT qPCR in cells grown on SF films and positive and negative controls. mRNA expression of Col1A1 and Col2A1 was not significantly different between positive and negative controls cADMMSC and SF film cADMMSC. However, both Aggrecan and Sox9 mRNA expression was statistically significantly upregulated in positive control cADMMSC and SF film cADMMSC. This suggests that chondrogenesis was indeed initiated also at the molecular level. Currently, it is difficult to speculate why we did not also detect increased expression of Col2A1. However, it has to be noted that mRNA expression of Col2A1 was very low and perhaps, the time of chondrogenesis was too short to induce also Col2A1, a marker of mature chondrocytes. The RT qPCR results of our study thus indicate that chondrogenic differentiation of SF film cADMMSC is comparable to positive control cADMMSC. Based on the upregulation of Sox9 and Aggrecan in both positive control and SF film cADMMSC, we concluded that the initial stages of chondrogenesis took place, but it might take longer than 14 days for Col2A1 to be significantly upregulated. Due to protein overload and lower number of cells when isolating RNA from cells with proteins from the scaffold, we were unable to purify enough RNA perform RT qPCR from SF scaffold cADMMSC to confirm mRNA expression results also with cells grown in SF scaffolds.

Mechanisms behind presumed chondrogenic differentiation of cADMMSC on SF in our study are not yet known. In line with the high cell density as one of the

conditions needed for MMSC differentiation, the study of Dudakovic et al.[8] showed that maintaining adipose-derived human MMSC in confluent cultures leads to post proliferative conveyance of more specialized cellular phenotypes expressing osteogenic, chondrogenic, and adipogenic biomarkers. Similarly, Bosnakovski et al.[6] showed that bovine bone marrow–derived MMSC cultured in a pellet culture system also differentiates into chondrocytic lineage. In the present study, the number of cells seeded on SF films was only 1×10^4 and did not reach confluency. Since cells started to differentiate the day after their attachment on SF films, high cell density was excluded as a possible reason for presumed chondrogenic differentiation of MMSC on SF films. High cell density, however, might have contributed to the chondrogenesis of SF scaffold cADMMSC, since cell seeding density was higher ($9 \times 5\,\mu L$ droplets of 5×10^5 cells per scaffold) due to the higher overall surface for cell attachment. Besides high cell density, culture medium could also play a role in chondrogenic MMSC differentiation even without specific factors added to the culture media. Fortier et al.[45] reported sporadic chondrogenesis of equine MMSC cultured with culture medium supplemented with 10% FBS. Since chondrogenesis did not occur in a serum-free medium in this study, the reason for chondrogenic differentiation was attributed to bioactive factors present in the FBS. In our study, FBS is unlikely to be the reason for chondrogenic differentiation of MMSC, as the chondrogenic differentiation of negative control cADMMSC, cultured on a standard polystyrene surface in a cell culture medium with FBS, was never observed.

Mechanical properties of SF are also unlikely the reason for presumed chondrogenic differentiation of cADMMSC since chondrogenesis seemed to occur not only on 2D SF films but also on architecturally different 3D SF scaffolds. Passage number was shown before to affect the differentiating behavior of cells. In human donors, spontaneous chondrogenesis was reported in early passages of human periosteum-derived cells, which are otherwise known to have bilineage potential.[7] In our study, cells from second to fourth passage were always used and therefore, an early passage might have contributed to the differentiation of cADMMSC grown on SF films. However, this was clearly not the main reason for presumed chondrogenic differentiation of cells grown on SF films as negative control cADMMSC cells did not differentiate into chondrocytes, despite being from the same passages. In addition, source of MMSC might also contribute to their spontaneous phenotypic changes. Naruse et al.[9] showed that rat cells derived from fetal circulating blood spontaneously differentiated into both chondrocytic and osteocytic cells on plastic culture dishes in a standard culture medium. However, rat bone marrow–derived MMSC did not undergo similar phenotypic changes under the same conditions. In our study, cADMMSC were used, which are rarely studied. There are no reports about spontaneous differentiation of cADMMSC. Therefore, it is not possible to speculate whether the tissue source of MMSC in our study contributed to the presumed chondrogenic differentiation of cADMMSC cultured on SF films without specific growth factors present. Surely, this cannot be the main reason as we never observed chondrogenic differentiation in negative controls even though the cells were obtained from the same donors. One possible explanation for presumably chondrogenic induction in our experiment would be hydrophobicity of SF films. Indeed, SF films are initially very hydrophobic, but when wetted, they form a hydrophilic hydrogel with a water contact angle of 0°. Therefore, when cells were seeded on SF films, the surface was hydrophilic and is unlikely to force cells into cell–cell adhesion. However, the surface of SF films has not been biochemically characterized and we do not know anything about cell adhesion processes on SF films. Therefore, in the future studies, it would be interesting to study cell adhesion molecules (such as integrins) and processes to determine how cells adhere to the SF and whether this contributes to the differentiation of cells on this biomaterial.

To find out whether there are differences between donor species regarding MMSC differentiation on SF films, a corresponding study of ADMMSC from different animal species should be conducted in the future. It is difficult to speculate about the reasons for cADMMSC differentiation in the absence of specific growth factors as the literature on this topic is sparse. Only a handful of studies reported a chondrogenic differentiation of MMSC either on a standard plastic surface or on biomaterials in the absence of special growth factors and/or high cell density seeding. Different parameters used in each study such as donor species, tissue source of MMSC, culture media composition, and culturing methods makes difficult any direct comparisons of these studies. Future studies are therefore necessary to determine which factors might contribute to the mechanisms lying behind chondrogenic differentiation of MMSC in the absence of specific growth factors.

Results of our study show that SF could be considered as a promising biomaterial not only for culturing cells but also for the induction of controlled chondrogenesis of MMSC. None of the previous studies using SF has shown its ability to guide MMSC toward chondrogenic differentiation, so this might be a species-specific effect, as our study was the first study to examine the growth of canine MMSC on SF. This effect of SF on MMSC in our study, therefore, represents a basis for further studies aiming to understand the key factors and mechanisms responsible for induction of differentiation of MMSC into chondrocytes.

Data availability

The raw data and processed data required to reproduce these findings are available from the authors upon request.

Declaration of conflicting interests

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3 RAZPRAVA

Matične celice predstavljajo potencial za zdravljenje številnih do zdaj neozdravljivih bolezni pri živalih in ljudeh. Izkoriščanje njihovih edinstvenih lastnosti se je v zadnjih letih izkazalo obetavno ne samo v humani, ampak tudi v veterinarski regenerativni medicini. Pri psih in mačkah, pogostih veterinarskih pacientih, po uspešnosti zdravljenja z MSC izstopajo predvsem ortopedske bolezni pri psih [72-77] in bolezni ustne votline pri mačkah [78-81]. Za klinično uporabo MSC je potrebna obsežna namnožitev celic in običajno tudi transport celic od laboratorija do pacienta. Ker znana intrinzična heterogenost MSC lahko znatno vpliva na njihov terapevtski potencial [156] in ker različne lastnosti MSC v pogojih *in vitro* lahko vodijo v različen terapevtski učinek, je proučevanje celic izjemnega pomena, saj nam omogoča vpogled v celično fiziologijo in procese tkiv izven organizma in posledično presojo uporabe MSC za zdravljenje. Za matične celice velja, da so sposobne adhezije na plastiko, zato se je za proučevanje MSC uveljavila 2D podlaga iz plastike. Matične celice definirajo stanje nediferenciranosti, sposobnost samoobnove in sposobnost diferenciacije v specializirane celice tkiv [2]. Dve pomembni lastnosti, ki ju lahko merimo v pogojih *in vitro*, sta tako njihov proliferacijski in diferenciacijski potencial.

V prvem delu naše raziskave smo AMSC iz psov in mačk gojili na standardni podlagi iz plastike in proučevali vpliv vrste, spola in poznih pasaž na proliferacijski in diferenciacijski potencial AMSC.

Življenjska doba MSC v pogojih *in vitro* je omejena, kar je bilo pokazano v večih raziskavah pri proučevanju vpliva poznih pasaž na njihov multipotentnost [104-107]. Določanje števila celičnih podvojitev (angl., Cell doubling, CD) in časa, potrebnega, da se število celic podvoji (angl., Cell doubling time, CDT), nam lahko omogoči vpogled v proliferacijski potencial MSC in olajša presojo zdravljenja z MSC. V predhodnih raziskavah so pokazali, da se z večanjem števila pasaž pri različnih živalskih vrstah vrednost CD zmanjšuje in vrednost CDT povečuje [79, 98, 104, 157]. Tudi v naši raziskavi smo pokazali, da se z večanjem števila pasaž proliferacijski potencial AMSC psov in mačk zmanjšuje, pri čemer je bilo podaljševanje CDT od druge do osme pasaže pri celicah psov razmeroma postopno, pri celicah mačk pa bolj neenakomerno. Na potentnost MSC poleg števila pasaž lahko vplivajo tudi drugi dejavniki, kot so tkivo izvora MSC [84, 86, 89, 98], anatomsko mesto tkiva [88, 99, 100] in starost živali [99,

101-103]. Glede na rezultate nekaterih raziskav bi lahko sklepali, da bi tudi vrsta živali lahko bila potencialni dejavnik vpliva na MSC. Pri MSC, izoliranih iz maščobnega tkiva psov, je bilo pokazano, da izkazujejo večji diferenciacijski potencial kot MSC, izoliranih iz drugih tkiv – kostnega mozga, popkovnice, amnijske membrane ali placente [86], medtem ko so raziskave pri konjih pokazale, da so MSC, izolirane iz kostnega mozga, izkazovale večji hondrogeni [158] in osteogeni [159] diferenciacijski potencial kot MSC iz maščobnega tkiva. Rezultati omenjenih raziskav nakazujejo možnost, da imajo MSC različnih živalskih vrst različne značilnosti. Tudi v naši raziskavi smo pokazali medvrstno različen proliferacijski potencial AMSC. AMSC psov so z ozirom na statistično značilno večji CD in statistično značilno krajši CDT izkazovale večji proliferacijski potencial kot AMSC mačk. Medvrstne razlike v proliferacijskem potencialu so se kazale tudi v poznih pasažah. V pasaži 8 pri AMSC mačk je bil CDT statistično značilno daljši kot v vseh ostalih pasažah pri obeh vrstah živali, kar nakazuje na to, da pozne pasaže bolj negativno vplivajo na proliferacijo AMSC mačk kot AMSC psov. Različen vpliv poznih pasaž med živalskima vrstama se je kazal tudi v diferenciacijskem potencialu AMSC psov in mačk. Medtem ko so bile celice obeh vrst živali sposobne diferenciacije v hrustančne, kostne in maščobne celice v zgodnji pasaži, so bile celice psov zmožne trilinijske diferenciacije tudi v pasaži 8, celice mačk pa v pasaži 6, ne pa tudi v poznejših pasažah. To kaže na bolj negativen vpliv poznih pasaž na diferenciacijski potencial AMSC pri mačkah kot pri psih. Celice psov so v primerjavi s celicami mačk izkazovale tudi večji hondrogeni in osteogeni diferenciacijski potencial v zgodnji pasaži, kar smo pokazali s kvantifikacijo pri majhni povečavi posnetih slik diferenciacije z uporabo programa ImageJ. Očitnih razlik med kvalitativno oceno maščobne diferenciacije med AMSC psov in mačk nismo zaznali. Adipogeno diferenciacijo smo analizirali zgolj kvalitativno zaradi majhnosti maščobnih vakuol, ki so v primerjavi z vakuolami, ki nastanejo pri maščobni diferenciaciji MSC ljudi, precej manjše [110]. Na velikost nastalih maščobnih vakuol morda vpliva tudi tkivo izvora MSC, kot je bilo pokazano v eni od raziskav, kjer so bile maščobne vakuole, nastale pri diferenciaciji MSC iz pokožnega maščobnega tkiva in kostnega mozga psov, manjše od vakuol maščobnih celic, pridobljenih iz MSC sinovije ali infrapatelarne maščobe [85].

Med analizo proliferacijskega in diferenciacijskega potenciala smo našli le manjše razlike med spoloma in sicer v zgodnji pasaži osteogene diferenciacije, kjer so samice obeh vrst živali izkazovale večji diferenciacijski potencial kot samci. Razlike med spoloma so v predkliničnih

raziskavah redko proučevane [160], čeprav je znano, da spol pacienta lahko vpliva na tveganje za dovzetnost in razvoj bolezni [161]. Razlike med spoloma so bile objavljene tudi na področju regenerativne medicine v povezavi z nevrogenim diferenciacijskim potencialom [111, 112], sposobnostjo imunomodulacije [113] in terapevtskim učinkom [114] MSC pri ljudeh in živalih. V naši raziskavi smo našli le manjše razlike med spoloma, ki nakazujejo na to, da spol živali ne vpliva bistveno na osnovne lastnosti AMSC, a vendar bi prisotne razlike lahko pomenile potencialno različno regeneracijsko sposobnost AMSC med spoloma. Več razlik med spoloma bi potencialno lahko našli na molekularni ravni, zaradi česar bi razlike med spoloma morali proučevati v prihodnjih študijah. Proučevanje razlik med spoloma v raziskavah, ki vključujejo celice, živali in ljudi, lahko pomembno vplivajo na razvoj novih strategij zdravljenja različnih bolezni [161].

Poleg proučevanja proliferacijskega in diferenciacijskega potenciala smo v nadaljevanju raziskave analizirali tudi izražanje površinskih označevalcev in živost celic obeh vrst živali. Pri obeh analizah smo našli medvrstne razlike, ki smo jih vključili med rezultate.

Minimalna merila za identifikacijo človeških MSC je leta 2006 oblikovalo Mednarodno združenje za celična zdravljenja z namenom poenotene karakterizacije MSC in primerljivosti rezultatov raziskav z njimi. Zahtevana merila vključujejo sposobnost adhezije MSC na plastiko v standardni celični kulturi, izražanje celičnih označevalcev CD105, CD73, CD90, odsotnost izražanja celičnih označevalcev CD45, CD34, CD14 (ali CD11b), CD79α (ali CD19) in HLA–DR ter sposobnost diferenciacije v osteoblaste, hondroblaste in adipocite [16]. MSC živali so, podobno kot MSC ljudi, sposobne adhezije na plastiko in trilinijske diferenciacije, vendar ne izražajo enakih celičnih označevalcev kot MSC ljudi. Večina MSC živali izraža CD29 in CD44, medtem ko je izražanje CD105, CD73 in CD90 med živalskimi vrstami različno [162]. Minimalni kriteriji za identifikacijo MSC živali zaenkrat zato še niso določeni. Predhodne raziskave so pokazale, da MSC psov [84, 163-165] in mačk [87, 157] stalno izražajo označevalce CD44 in CD90 in ne izražajo CD34, medtem ko je izražanje ostalih površinskih označevalcev, CD105 in CD73, spremenljivo in odvisno od tkiva izvora MSC [84] oziroma so izraženi samo pri psih [98, 166], ne pa tudi pri mačkah [157]. V naši raziskavi smo analizirali površinske označevalce, za katere je znano, da so na MSC psov in mačk stalno izraženi. Med pozitivnimi označevalci smo analizirali CD44 in CD90, med negativnimi pa CD34. V skladu z

rezultati predhodnih raziskav smo pokazali, da večina živih AMSC psov in mačk izraža CD44 in CD90 in ne izraža CD34. Odstotek celic, ki so izražale CD44 in CD90 oziroma niso izražale CD34, je bil statistično značilno večji pri celicah psov kot pri celicah mačk. Razlik med spoloma nismo našli. Glede na znano heterogenost MSC, ki se kaže v različnih populacijskih skupinah znotraj celične populacije MSC [156], bi kot enega izmed možnih vzrokov za medvrstne razlike v relativnem izražanju preiskovanih celičnih označevalcev lahko navedli večjo intrinzično heterogenost populacije celic mačk v primerjavi s populacijo celic psov. CD34 je transmembranski fosfoglikoprotein, ki je bil prvotno identificiran na hematopoetskih matičnih in hematopoetskih progenitornih celicah [167, 168], pri katerih na eni strani z zaviranjem adhezije spodbuja migracijo HSC [169], na drugi strani pa s spodbujanjem adhezije HSC omogoča interakcijo celic z okoljsko stromo [170]. V splošnem velja prevladujoče mnenje, da MSC ne izražajo CD34 [16, 171]. Vendar številne raziskave kažejo, da je CD34 izražen tudi na MSC in na številnih drugih nehematopoetskih celicah, kot so satelitske celice mišičnega tkiva [172], stromalne celice številnih organov [173, 174], progenitorne celice epitelija [175] in progenitorne celice žilnega endotela [176]. CD34 lahko izražajo tudi stromalne celice maščobnega tkiva, ki se nahajajo ob žilnem endotelu in poleg CD34 izražajo označevalce, značilne za pericite in MSC ter so sposobne adhezije na plastiko in imajo multidiferenciacijski potencial [29]. Variabilnost podatkov iz različnih raziskav, ki kažejo, da so stromalne celice maščobe ali $CD34^{-/-}$ ali $CD34^{+/+}$, so verjetno posledica adhezije celic na plastiko in zmanjševanja izražanja CD34 z večanjem števila pasaž [171, 174, 177]. Ali so v naši raziskavi celice psov in mačk, ki niso bile $CD34^{-/-}$, pripadale MSC ali ne, je težko predpostaviti, saj bi bila za bolj natančno identifikacijo celic poleg analize večjega števila celičnih označevalcev potrebna tudi analiza funkcionalnosti in diferenciacijskih sposobnosti posameznih celičnih populacij [174]. Na podlagi raznolikosti podatkov v literaturi o fenotipu MSC je v prihodnosti verjetno pričakovati spremembe v zvezi z identifikacijo MSC [171]. Z ozirom na že znane medvrstne razlike v izražanju površinskih označevalcev je pričakovati tudi nadaljne primerjalne raziskave fenotipa MSC med posameznimi živalskimi vrstami. V naši raziskavi smo pokazali, da je relativno izražanje tudi že znanih označevalcev MSC med vrstami različno. Ko bodo znani tkivno in vrstno specifični označevalci MSC in ko bo na voljo večji izbor vrstno specifičnih protiteles, bo olajšano tudi bolj podrobno proučevanje izražanja označevalcev MSC med različnimi vrstami veterinarskih pacientov.

V raziskavi smo med analizo proliferacijskega potenciala in površinskih označevalcev AMSC merili tudi celično živost ter našli razlike v živosti med AMSC psov in mačk. Živost celic smo merili med analizo proliferacijskega potenciala pri vsaki pasaži neposredno po tripsinizaciji celic z uporabo hemocitometra ter med analizo površinskih označevalcev pri tretji pasaži z uporabo pretočnega citometra, po predhodnem shranjevanju celic čez noč v PBS pri 4°C. Živost celic pri tretji pasaži, merjeno z obema metodama, smo primerjali med seboj, z namenom ugotoviti morebitne razlike v živosti med vrstama in med spoloma. Razlik med spoloma nismo našli, prav tako je bila živost, merjena s hemocitometrom, med živalskima vrstama podobna. Živost celic, merjena s pretočnim citometrom po predhodnem shranjevanju celic čez noč v PBS pri 4°C, pa je bila statistično značilno večja pri celicah psov kot pri celicah mačk. V večih raziskavah je bilo pokazano, da so podatki o živosti celic, merjene pod mikroskopom ali s pretočno citometrijo, med seboj primerljivi [178-180], zato razlaga za dobljene rezultate najverjetneje leži v različni občutljivosti AMSC psov in mačk na manipulacijo z njimi v manj optimalnih pogojih. Večja dovzetnost celic mačk na manj optimalne pogoje nakazuje na pomen upoštevanja živalske vrste pri pripravi celic za klinično uporabo. Za namen uporabe AMSC v kliniki je običajno potreben tudi transport celic, ki v odvisnosti od razdalje laboratorija do pacienta terja različen čas. Vpliv dodatne manipulacije in hrambe celic na celično živost pri različnih vrstah živali bi bilo zato potrebno podrobneje proučevati tudi v prihodnjih študijah.

Rezultati prvega dela naše raziskave so pokazali, da AMSC mačk izkazujejo slabši proliferacijski in diferenciacijski potencial kot AMSC psov in da pozne pasaže bolj negativno vplivajo na proliferacijski in diferenciacijski potencial AMSC mačk kot na AMSC psov. Z rezultati smo potrdili prvo in tretjo hipotezo, ter delno potrdili drugo hipotezo. Rezultati so tudi pokazali, da AMSC mačk v manjši meri izražajo preiskovane celične označevalce in v manj optimalnih pogojih izkazujejo slabšo živost.

Ena izmed možnih razlag za slabše karakteristike AMSC mačk v primerjavi z AMSC psov morda leži v večji dovzetnosti AMSC mačk na kisik v atmosferi gojenih celic. Značilna asimetrična delitev matičnih celic vodi v nastanek ene nove matične celice in druge progenitorne celice – delno diferencirane celice, usmerjene v razvoj določene celične linije [120]. Delitev MSC tako vodi v dve populaciji MSC gojenih *in vitro* – majhne, vretenaste, hitro deleče se celice in večje, ploščate celice nepravilnih oblik s počasno proliferacijo [181], ki z

večanjem števila celičnih delitev vztrajno nadomeščajo prve [182]. Življenjska doba MSC v pogojih *in vitro* je tako omejena zaradi replikativne senescence, procesa, ki vodi v prenehanje delitve celic, akumulacijo metabolnih produktov in celično smrt [183]. V naravnem okolju se MSC nahajajo v mirujočem stanju, za katerega so značilne visoka potentnost celic, počasna proliferacija in glikoliza kot vodilni proces energetskega metabolizma. V celični kulturi z visoko vsebnostjo hranilnih snovi je proliferacija pospešena, energetski metabolizem pa odvisen predvsem od oksidativne fosforilacije [184, 185], ki vodi v akumulacijo reaktivnih kisikovih spojin (ROS) [186]. Kljub temu, da se MSC v telesu nahajajo perivaskularno, je koncentracija kisika v niši MSC nizka – 2% – 9% [187]. Senescenca v pogojih *in vitro* je pospešena zaradi kisika, ki v atmosferi gojenih celic štiri– do desetkrat presega vsebnost kisika v telesu znotraj tkiv [186]. V kulturi z zmanjšano vsebnostjo kisika je senescenca celic zmanjšana kot posledica ohranjanja glikolize in zaviranja oksidativne fosforilacije [188, 189]. Večja vsebnost kisika v atmosferi gojenih celic pa vodi do poškodbe proteinov in telomer deoksiribounkleinske kisline (DNK) ter posledične aktivacije s senescenco povezanih signalnih poti [186], vključujoč MAPK in molekul p53 in p16 [190], ključnih za napredovanje in ustavitev celičnega cikla [191]. Senescenca celic poleg učinka na manjšanje proliferacijskega potenciala vpliva negativno tudi na migracijske sposobnosti MSC in njihov diferenciacijski potencial [192]. Z vplivom na imunomodulatorne lastnosti MSC, ključne pri njihovem terapevtskem delovanju, pa senescenca pomembno zmanjšuje terapevtski potencial MSC [185, 193]. V naši raziskavi bi predpostavljena morebitna večja dovzetnost AMSC mačk na kisik lahko bila posledica različnega metabolizma AMSC mačk v primerjavi z AMSC psov, kar bi bilo potrebno proučiti v prihodnjih raziskavah. Z znižanjem koncentracije kisika v atmosferi bi morda do neke mere lahko posnemali pogoje v naravnem okolju MSC in s tem morebiti dosegli boljše rezultate gojenja AMSC mačk, kot je bilo to pokazano v predhodnih raziskavah pri ljudeh in drugih vrstah živali [194-198].

Kljub temu, da bistvenih razlik med spoloma v preiskovanih parametrih AMSC psov in mačk nismo našli, so rezultati naše preiskave pokazali pomembne medvrstne razlike, kar kaže na bistven vpliv vrste živali na značilnosti AMSC, gojenih na običajni plastični podlagi v pogojih *in vitro.* Ker je za namen zdravljenja bolezni z MSC potrebna obsežna namnožitev celic in transport celic od laboratorija do pacienta, bi medvrstne razlike v značilnostih MSC lahko

potencialno vplivale tudi na njihov terapevtski učinek. Živalsko vrsto bi zato morali upoštevati kot spremenljivko pri pripravi celic za regenerativno zdravljenje.

Raziskave na področju matičnih celic se osredotočajo na iskanje in razumevanje mehanizmov, potrebnih za vodenje celične usode v želeno smer. Podobnosti med lastnostmi v laboratoriju gojenih AMSC z lastnostmi celic v naravnem okolju v telesu so vprašljive. Običajne plastične podlage za gojenje celic tako nadomeščajo 3D načini gojenja celic, saj omogočajo opazovanje celic v pogojih, ki bolje posnemajo naravno celično okolje in hkrati nudijo možnost uvajanja novih načinov vnosa celic v telo. V drugem delu naše raziskave smo AMSC psov gojili na celičnem nosilcu iz SF z namenom ugotoviti, ali biomaterial iz SF omogoča adhezijo, proliferacijo in diferenciacijo celic. Iz SF smo pripravili 2D filme in 3D porozne nosilce. V raziskavi smo potrdili, da SF omogoča adhezijo celic, a ne omogoča njihove proliferacije, temveč sproža hondrogeno diferenciacijo celic.

Hondrogeno diferenciacijo MSC na običajni plastični podlagi je moč doseči z uporabo vlažne atmosfere, veliko gostoto naseljenih celic in uporabo ustreznih rastnih dejavnikov, kot je transformirajoči rastni dejavnik beta (TGF–β) [199]. Ključni dejavniki, odgovorni za celično diferenciacijo, niso dobro znani. V predhodnih raziskavah so pokazali, da nekateri biomateriali kot so polimeri, kompozitni in nanofibrozni materiali lahko usmerjajo MSC proti adipogeni [200] ali osteogeni [201, 202] diferenciaciji celic. Rezultati predhodnih raziskav, v katerih so proučevali vpliv SF na proliferacijo in diferenciacijo MSC, so pokazali, da SF omogoča proliferacijo MSC. Rodriguez–Lozano F in sodelavci (2014) so pri proučevanju vpliva SF na MSC iz pozobnice pokazali, da je proliferacija MSC na filmih SF slabša kot na plastiki [147], medtem ko so Collado–Gonzalez in sodelavci (2018) pokazali, da je bila proliferacija MSC, izoliranih iz zobne pulpe človeških zob, gojenih na 3D poroznem nosilcu SF, podobna kot pri celicah, gojenih na plastiki [148]. V primerjavi z omenjenimi raziskavami smo v naši raziskavi pokazali, da SF ne omogoča proliferacije AMSC psov, ampak sproža hondrogeno diferenciacijo celic. Hondrogeno diferenciacijo smo dokazovali z analizo morfologije celic, z barvanjem celic z alcian modrim in analizo izražanja genov. Celice, gojene na fibroinskih filmih, so bile morfološko zelo podobne tistim iz pozitivne kontrole. Celice pozitivne kontrole smo z uporabo diferenciacijskega gojišča na plastiki diferencirali v hrustančne celice in so se združevale v tako imenovane hrustančne skupke s številnimi vezikli zunajceličnega matriksa,

zaznanih pod vrstičnim elektronskim mikroskopom. Številni vezikli zunajceličnega matriksa so bili prisotni tudi pri celicah, gojenih na filmih in poroznih nosilcih SF. Celice, gojene na poroznih nosilcih, so se večinoma združevale v amorfne plasti, združevanje v hrustančne skupke je bilo opazno le mestoma. Celice, gojene na filmih in poroznih nosilcih so se, tako kot celice pozitivne kontrole, barvale modro z alcian modrim, specifičnim barvilom, s katerim se modro obarvajo proteoglikani hrustanca, kot je hondroitin sulfat [203].

Za MSC, diferencirane na plastiki v hondrogenem diferenciacijskem gojišču v ustreznih atmosferskih pogojih velja, da v začetku združevanja v skupke izražajo kolagen tipa 1, po dveh do treh tednih pa začno izločati zunajcelični matriks z vsebnostjo kolagena tipa 2 [204]. Za diferenciacijo MSC je značilno zmanjševanje proliferacije in povečevanje izražanja za diferenciacijsko linijo značilnih genov [123]. TGF–β je znan dejavnik, ki z aktivacijo signalne poti SMAD in SOX9, prvega transkripcijskega dejavnika, ključnega za hondrogenezo, sproža hondrogeno diferenciacijo MSC [205]. Pomemben označevalec hondrogene diferenciacije je tudi agrekan, značilen za zrel hrustanec in katerega izražanje se povečuje med hondrogeno diferenciacijo MSC [206]. V naši raziskavi smo s kvantitativno reakcijo s polimerazo v realnem času analizirali izražanje genov kolagen tipa 1, kolagen tipa 2, SOX9 in agrekan v celicah, gojenih na filmih SF in celicah pozitivne in negativne kontrole. Izražanje genov kolagen tipa 1 in kolagen tipa 2 je bilo med skupinami podobno, medtem ko je bilo v primerjavi z negativno kontrolo izražanje genov SOX9 in agrekan statistično značilno večje v celicah pozitivne kontrole in celicah, gojenih na filmih SF. Rezultati kvantifikacije izražanja genov potrjujejo rezultate morfološke analize in barvanja z alcian modrim v naši raziskavi in kažejo na hondrogeno diferenciacijo celic, gojenih na SF. Kot enega izmed možnih razlogov za statistično neznačilno izražanje kolagena tipa 2 bi lahko navedli morebitno prekratko časovno obdobje, v katerem je potekala diferenciacija celic. Do večjega izražanja kolagena tipa 2 bi morda prišlo po daljšem času gojenja celic.

Zakaj je v naši raziskavi SF vplival na hondrogeno diferenciacijo AMSC psov, je težko predpostaviti. V nekaterih raziskavah je bilo pokazano, da vzdrževanje konfluentne kulture AMSC ljudi lahko izzove izražanje označevalcev, značilnih za hrustančno, kostno ali maščobno linijo diferenciranih celic [207]. Podobno so pokazali tudi Bosnakovski in sodelavci (2004), ki so zgolj s kulturo celic v obliki peleta MSC iz kostnega mozga goveda dosegli hondrogeno

diferenciacijo celic [208]. V naši raziskavi smo celice na filme SF naseljevali v majhni gostoti. Celice zaradi nezmožnosti proliferacije niso dosegle konfluence, zato smo gostoto celic izključili kot morebiten razlog za nastop hondrogene diferenciacije na filmih SF. Visoka gostota celic bi lahko igrala vlogo v hondrogenezi celic, naseljenih na porozne nosilce, kamor smo celice naseljevali v večji gostoti zaradi bistveno večje površine nosilca. Poleg visoke gostote naseljevanja celic na celične plošče je bil v predhodnih raziskavah kot možen vzrok za spontano hondrogeno diferenciacijo MSC konj navedeno celično gojišče z vsebnostjo 10% fetalnega govejega seruma (angl., Foetal Bovine Serum, FBS) [209]. V naši razsikavi se celice negativne kontrole, gojene na plastiki v gojišču z vsebnostjo 10% FBS, niso diferencirale, zato dodatek FBS verjetno ni bil vzrok za hondrogeno diferenciacijo celic na SF. Spontano hondrogeno diferenciacijo so zaznali tudi pri naseljevanju celic iz periosta ljudi v primeru uporabe celic iz zgodnjih pasaž [210]. V naši raziskavi so bile tudi uporabljene celice zgodnje (druge do četrte) pasaže, vendar to najverjetneje ni bil razlog za diferenciacijo na SF, saj so bile celice negativne kontrole, ki se niso diferencirale, tudi iz zgodnje pasaže. Poleg omenjenih je bilo kot eden izmed možnih vzrokov za spontano diferenciacijo omenjeno tudi tkivo izvora MSC. MSC podgan, izolirane iz fetalne periferne krvi in gojene na plastični podlagi v običajnem celičnem gojišču, so bile v primerjavi z MSC podgan, izoliranih iz kostnega mozga, sposobne spontane hondrogene in osteogene diferenciacije [211]. V naši raziskavi smo uporabili AMSC psov, za katere v literaturi nismo našli podatkov o pojavu spontane hondrogene diferenciacije. Potencialna predpostavka, da bi tkivo izvora MSC v naši raziskavi lahko imelo vpliv na dane rezultate, ni verjetna, saj do diferenciacije v celicah negativne kontrole, izvirajočih iz istega tkiva, ni prišlo. Glede na to, da sta do sprožitve hondrogene diferenciacije AMSC privedla oba uporabljena, strukturno različna nosilca iz SF (filmi in porozni nosilci), tudi mehanske lastnosti SF najverjetneje niso vzrok za hondrogeno diferenciacijo celic. Verjetnejša potencialna razlaga za hondrogeno diferenciacijo celic na SF v naši raziskavi je povezana z adhezijo celic na SF. Znano je, da adhezija celic na zunajcelični matriks preko mehanotransdukcije pomembno vpliva na morfologijo in usodo MSC [212]. Bai in sodelavci (2015) so npr. s spreminjanjem trdnosti in površinskih lastnosti nanovlaken SF vplivali na proliferacijski in diferenciacijski potencial MSC podgan iz kostnega mozga in ugotovili, da lahko biomaterial sam aktivno spodbuja celice k diferenciaciji v mišične in endotelne celice [154]. Adhezija celic pa je tako odločilnega pomena tudi za hondrogeno diferenciacijo MSC [213]. V večih raziskavah so pokazali, da RGD, vezavna domena fibronektina zunajceličnega matriksa, pomembno prispeva

k boljši adheziji in hondrogenezi MSC [214]. Vpliv RGD na adhezijo MSC je bil pokazan tudi pri gojenju MSC na SF [149], vendar pa dodatek RGD k nosilcem iz SF, zanimivo, ni prispeval k boljši hondrogenezi človeških MSC iz kostnega mozga, izražanje COL2A1 v celicah je bilo celo večje na samem SF v primerjavi z SF z dodanim RGD [215]. Jaipaew in sodelavci (2016) so ugotovili, da na različno stopnjo hondrogene diferenciacije človeških MSC iz popkovine lahko vpliva tudi različna vsebnost hialuronske kisline v poroznem nosilcu iz SF [150]. V naši raziskavi SF ni bil vezan z RGD ali hialuronsko kislino, vendar pa bi lahko bila hondrogeneza posledica kombinacije vpliva SF in katere izmed komponent uporabljenega celičnega gojišča. Hondrogeno diferenciacijo MSC gojenih na SF so v predhodnih raziskavah dosegli npr. z uporabo gojišča z vsebnostjo askorbinske kisline [151] ali PRP [151, 152], pri čemer se je PRP izkazal kot ustreznejši za sprožitev hondrogene diferenciacije, saj je omogočal večjo akumulacijo glikozaminoglikanov kot askorbinska kislina [151]. Zaradi individualne sestave celičnih gojišč, uporabljanih med posameznimi laboratoriji, ki onemogočajo neposredno primerjavo posameznih študij, se bodo nadaljne analize za ugotovitev vzroka za hondrogeno diferenciacijo AMSC na SF v naši raziskavi najprej osredotočale na preiskovanje povezave vpliva kombinacije SF in komponent celičnega gojišča. V naši raziskavi smo na SF gojili AMSC psov. Za ugotovitev morebitne vrstne specifičnosti hondrogene diferenciacije MSC na SF bodo potrebni nadaljni poskusi gojenja MSC različnih vrst živali na SF.

Rezultati drugega dela naše raziskave so pokazali, da SF omogoča adhezijo AMSC, vendar ne omogoča njihove proliferacije, temveč vodi razvoj AMSC psov v smeri hondrogene diferenciacije. Četrto hipotezo smo tako delno potrdili in delno ovrgli. Hondrogeno diferenciacijo smo potrdili z analizo celične morfologije pod svetlobnim in vrstičnim elektronskim mikroskopom, barvanjem hrustančnih proteoglikanov v zunajceličnem matriksu diferenciranih celic ter kvantifikacijo izražanja genov, značilnih za hrustančne celice. Celice so se na SF diferencirale brez posebnih pogojev naseljevanja, atmosferskih pogojev ali diferenciacijskih gojišč, ki so sicer potrebni za hondrogeno diferenciacijo na običajni plastični podlagi. Rezultati raziskave predstavljajo osnovo za nadaljne raziskave v smeri ugotavljanja in razumevanja ključnih dejavnikov in mehanizmov, odgovornih za sprožitev diferenciacije AMSC na SF. Še posebno smiselno bi bilo nadgraditi proučevanje vpliva SF na AMSC v smeri ugotavljanja izražanja drugih hrustančnih in tudi hipertrofičnih označevalcev ter možnosti prilagajanja dejavnikov vpliva SF, z vizijo prenosa znanja v klinično veterinarsko medicino.

4 SKLEPI

1. Vrsta živali bistveno vpliva na lastnosti AMSC psov in mačk. AMSC mačk izkazujejo slabši proliferacijski in diferenciacjiski potencial kot AMSC psov. Potrdili smo prvo hipotezo, da vrsta živali vpliva na proliferacijski in diferenciacijski potenical AMSC. AMSC mačk so tudi bolj podvržene vplivu poznih pasaž kot AMSC psov, v manjši meri izražajo izbrane površinske označevalce CD90, CD44 in CD34 in izkazujejo slabšo živost v manj optimalnih pogojih, kar dodatno nakazuje na vpliv vrste živali na lastnosti AMSC v pogojih *in vitro*.

2. Spol živali ne vpliva bistveno na lastnosti AMSC psov in mačk. Spol živali nima vpliva na proliferacijski potencial AMSC psov in mačk, a ima manjši vpliv na diferenciacijski potencial AMSC, pri čemer samice psov in mačk v zgodnji pasaži osteogene diferenciacije izkazujejo večji diferenciacijski potenical v primerjavi s samci obeh vrst živali. Drugo hipotezo, da spol živali vpliva na proliferacijski in diferenciacijski potenical AMSC psov in mačk, smo tako delno potrdili in delno ovrgli.

3. Pozne pasaže negativno vplivajo na proliferacijo in diferenicacijo AMSC psov in mačk, s čimer smo potrdili tretjo hipotezo. Vplivu poznih pasaž so bolj podvržene AMSC mačk.

4. Svilni fibroin omogoča adhezijo AMSC psov, vendar ne omogoča njihove proliferacije, temveč vodi razvoj AMSC v smeri hondrogene diferenciacije, ki nastopi brez posebnih pogojev gojenja celic, sicer potrebnih za hondrogenezo AMSC na običajni plastični podlagi. Četrto hipotezo, da svilni fibroin omogoča adhezijo, proliferacijo in diferenciacijo AMSC, smo tako delno potrdili in delno ovrgli.

5 POVZETEK

Matične celice so nediferencirane celice, sposobne samoobnove in razvoja v specializirane celice posameznega tkiva. Zaradi svojih edinstvenih lastnosti matične celic predstavljajo potencialni temelj medicine v prihodnosti. Gojenje celic *in vitro* omogoča preiskovanje celic in fizioloških ter bolezenskih procesov tkiva izven oganizma. Običajna podlaga za gojenje matičnih celic je 2D osnova iz polistirena. Ker različne lastnosti MSC v pogojih *in vitro* lahko vodijo v potencialno različen terapevtski učinek, je za lažjo presojo učinkovitosti zdravljenja z MSC pomembno odkrivanje vplivov različnih dejavnikov na osnovne značilnosti MSC *in vitro*. Med njimi sta vpliv vrste in vpliv spola v veterinarski regenerativni medicini redko proučevana. Namesto običajne plastične podlage za gojenje MSC se zaradi boljšega posnemanja naravnega celičnega okolja, stremenja po možnosti nadzora in vodenja usode MSC *in vitro* ter možnosti uvajanja novih načinov vnosa celic v telo za gojenje celic vedno bolj poudarja pomen uporabe celičnih nosilcev. Zaradi svojih mehanskih lastnosti, elastičnosti, biokompatibilnosti in nadzorovane razgradnje v ospredje številnih raziskav s področja biomaterialov stopa svilni fibroin.

Prvi namen našega dela je bil gojiti AMSC psov in mačk na običajni podlagi iz plastike ter ugotoviti, kako vrsta, spol živali in pozne pasaže vplivajo na proliferacijski in diferenciacijski potencial AMSC pri psih in mačkah. Drugi namen našega dela je bil gojiti celice tudi na celičnih nosilcih iz svilnega fibroina in ugotoviti, kako svilni fibroin vpliva na AMSC.

V prvem delu raziskave smo pokazali, da AMSC mačk izkazujejo slabši proliferacijski potencial, saj je bilo med merjenjem proliferacijskega potenciala od druge do osme pasaže število celičnih podvojitev bistveno manjše, čas, ki je potreben za podvojitev števila celic, pa bistveno daljši pri AMSC mačk kot pri AMSC psov. AMSC mačk so v primerjavi z AMSC psov izkazovale tudi slabši diferenciacijski potencial, saj so se lahko diferencirale pri pasaži 6, ne pa tudi pri poznejših pasažah, kot smo dokazali za AMSC psov. Osteogeni in hondrogeni diferenciacijski potencial je bil na podlagi kvantifikacije slik diferenciacije slabši pri AMSC mačk kot pri AMSC psov. Pozne pasaže so bolj negativno vplivale tako na proliferacijski kot na diferenciacijski potencial AMSC mačk kot na AMSC psov. Dodatno smo pokazali tudi, da AMSC mačk v manjši meri izražajo preiskovane celične označevalce. Odstotek celic, ki so izražale CD44 in CD90 oziroma niso izražale CD34, je bil večji pri celicah psov kot pri celicah

mačk. AMSC mačk so v primerjavi z AMSC psov v manj optimalnih pogojih izkazovale tudi slabšo živost, merjeno z metodo pretočne citometrije. V raziskavi smo našli le manjše razlike med spoloma, ki so bile prisotne v zgodnji pasaži osteogene diferenciacije, pri čemer so AMSC samic izkazovale večji diferenciacijski potencial kot AMSC samcev obeh vrst živali. Drugih razlik med spoloma nismo našli. Z rezultati prvega dela raziskave smo pokazali, da vrsta živali lahko bistveno vpliva na značilnosti AMSC v pogojih *in vitro* in da bi živalsko vrsto morali upoštevati kot spremenljivko pri pripravi celic za regenerativno zdravljenje.

Rezultati drugega dela naše raziskave so pokazali, da SF omogoča adhezijo AMSC in vodi usodo AMSC psov v smeri hondrogene diferenciacije. Hondrogeno diferenciacijo smo najprej potrdili z analizo celične morfologije, ki je bila med celicami pozitivne kontrole in celicami, gojenimi na SF, podobna, celice obeh skupin so se združevale v značilne hondrogene skupke. Hondrogeno diferenciacijo smo v nadaljevanju potrdili z barvanjem zunajceličnega matriksa diferenciranih celic z uporabo barvila alcian modro, ki modro obarva hrustančne proteoglikane. Celice, gojene na SF, so se, tako kot celice pozitivne kontrole, obarvale modro. Kot tretjo metodo za dokazovanje hondrogene diferenciacije smo uporabili kvantitativno verižno reakcijo s polimerazo v realnem času, s katero smo potrdili, da celice, gojene na SF, izražajo gene, značilne za hrustančne celice. Z rezultati drugega dela raziskave smo pokazali, da je hondrogeno diferenciacijo celic moč doseči brez posebnih pogojev gojenja celic, ki so sicer potrebni za hondrogenezo AMSC na običajni plastični podlagi.

Rezultati naše raziskave predstavljajo izvirni prispevek k znanosti na področju razumevanja osnovnih bioloških lastnosti MSC. Omogočajo poglobitev pomena poznavanja razlik matičnih celic med živalskimi vrstami in med spoloma ter posledično presojo zdravljenja živali z matičnimi celicami v veterinarski praksi. Hkrati predstavljajo tudi pomembno osnovo za prihodnje študije in potencial za uvajanje novih zdravljenj z matičnimi celicami v veterinarsko klinično medicino.

6 SUMMARY

Stem cells are undifferentiated cells capable of self–renewal and development into specialized cells of various tissues. Because of their unique properties, stem cells represent a potential basis of the medicine in the future. Cell cultures allow studies of cells, as well as physiological and pathological processes in various tissues outside the organism. The usual surface for stem cell culture is a two–dimensional polystyrene.

Different properties of MSC *in vitro* can potentially lead to different therapeutic effects. The influence of various factors on the basic properties of MSC *in vitro* is being studied with regard to the therapeutic potential of MSC. However, factors such as species and sex are rarely studied in regenerative veterinary medicine. Instead of the established plastic surface for MSC culturing, the use of cell carriers is increasingly studied in recent years as they provide a better imitation of the natural cellular environment, which might control the MSC fate *in vitro*. Furthermore, such carriers might represent potential novel ways of cell administration. Because of the suitable mechanical properties, good elasticity and biocompatibility, silk fibroin is at the forefront in the field of biomaterials research.

The first aim of our study was to culture AMSCs on a conventional plastic surface to determine the influence of animal species and sex, and late passages on the proliferation and differentiation potential of canine and feline AMSCs. The second aim of our work was to grow AMSCs on silk fibroin carriers to find out how silk fibroin affects AMSCs.

In the first part of the study, we established that feline AMSC showed poorer proliferative potential, as the number of cell doublings from the second to the eighth passage was significantly lower and the time required for cell number to double was significantly longer in feline AMSC than in canine AMSCs. AMSCs from cats also showed poorer differentiation potential compared to AMSCs from dogs, as feline cells differentiated at passage 6 but not at later passages, while canine cells successfully differentiated also in later passages. Moreover, osteogenic and chondrogenic differentiation potential of feline AMSCs was lower in comparison to canine AMSCs, based on the quantification of differentiation images. Late passages had a more negative effect on both the proliferation and differentiation potential of feline AMSC than on canine AMSCs. In addition, we also established that AMSCs from cats

showed lower expression of several cell surface markers. The percentage of cells expressing CD44 and CD90 and not expressing CD34 was higher in canine cells than in feline cells. Feline AMSCs also showed poorer viability under less optimal conditions, as measured by flow cytometry. However, in the present study, only minor differences between sexes of both species were found. Sex differences were present in the early passage in osteogenic differentiation, with AMSCs of females showing greater differentiation potential than AMSCs of males of both animal species. No other sex differences were found. The results of the first part of the study therefore showed that the animal species can significantly affect the basic characteristics of AMSCs *in vitro.* Animal species should therefore be considered as a variable in the preparation of cells for regenerative treatment.

The results of the second part of our study showed that SF enables canine AMSC adhesion and directs AMSC fate towards chondrogenic differentiation. Chondrogenic differentiation was confirmed by analysis of cell morphology, which was similar between positive control and cells grown on SF, with cells from both groups coalescing into characteristic chondrogenic clusters. Chondrogenic differentiation was further confirmed by staining the extracellular matrix of differentiated cells with the Alcian blue dye, known to stain cartilaginous proteoglycans blue. Cells grown on SF were stained blue, as did positive control cells. As a third method for confirming chondrogenic differentiation, real–time quantitative polymerase chain reaction was used and the expression of cartilage specific genes in cells grown on SF was confirmed. The results of the second part of the study showed that chondrogenic cell differentiation can be achieved without the specific conditions, otherwise required for chondrogenesis of AMSCs on a conventional plastic surface.

The results of this dissertation represent an original contribution to science in understanding the fundamental biological properties of animal MSCs. The results of our research allow us to deepen the knowledge about importance of the interspecies stem cells differences and, at the same time, provide a relevant basis for future studies and the potential for introducing new stem cell therapies into clinical veterinary medicine.

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