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Umbilical Cord Mesenchymal Stem Cells in Cats: biology and clinical application for embryo culture *in vitro*

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TABLE OF CONTENTS

LIST OF ARTICLES....7
SUMMARY IN ENGLISH....8
SUMMARY IN POLISH....10
LIST OF ABBREVIATIONS....12
CHAPTER 1: GENERAL INTRODUCTION....14
1. Introduction 15
2. Progress in the culture systems used for domestic cat oocytes and embryos....16
3. Oocyte, embryo communication in the absence of maternal tract....18
4. Co-culture system for oocytes and embryos utilising stem cells....19
5. Mesenchymal stem cells – an overview....21
6. Mesenchymal stem cells derived soluble factors 22
CHAPTER 2:AIMS OF THE THESIS....24
CHAPTER 3: MATERIALS AND METHODS 26
1.1.Obtaining umbilical cord tissue....27
1.2.Tissue harvest....27
1.3.Mesenchymal stem cell isolation and culture....27
1.4. Storage of cells....28
1.5. Thawing the cells....28
1.6. Cell doublings and doubling time....28
1.7. RNA extraction and real-time reverse transcription (qRT-PCR) 29
1.8. Immunophenotyping by flow cytometry....29
1.9. Tri-lineage differentiation assay....30
2.1. Preparation of mesenchymal stem cells for the co-culture....31
2.2. Source of ovaries and oocytes recovery....32
2.3. In vitro maturation of cat oocytes....32
2.4. Assessment of oocyte maturation....32
2.5. In vitro fertilisation....33
2.6. In vitro embryo culture and assessment of the embryo development....33
2.7. Study design....33

2.8. Statistical analysis.....34
CHAPTER 4:MESENCHYMAL STEM CELLS: GENERALITIES AND CLINICAL SIGNIFICANCE IN FELINE AND CANINE MEDICINE.....36
CHAPTER 5: MESENCHYMAL STEM CELLS: ISOLATION AND IN VITRO CHARACTERISATION FROM DISTINCT PARTS OF THE UMBILICAL CORD 54
CHAPTER 6: FELINE WHARTON'S JELLY-DERIVED MESENCHYMAL STEM CELLS AS A FEEDER LAYER FOR OOCYTES AND EMBRYOS CULTURE *IN VITRO*.....67
CHAPTER 7: GENERAL DISCUSSION AND CONCLUSION 79
REFERENCES.....86

Table of articles

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Feline umbilical cord mesenchymal stem cells: Isolation and in vitro characterisation from distinct parts of the umbilical cord	Published	Meriem Baouche, Agnieszka Krawczenko, Maria Paprocka, Aleksandra Klimczak, Pascal Mermillod, Yann Locatalli, Małgorzata Ochota, Wojciech Nizanski	2.923	140	doi.org/10.1016/j.theriogenology.2022.11.049	Theriogenology
Feline Wharton's Jelly-derived mesenchymal stem cells as a feeder layer for oocyte maturation and embryos culture in vitro	Accepted	Meriem Baouche, Małgorzata Ochota, Pascal Mermillod, Yann Locatalli, Wojciech Nizanski	3.471	70	doi:10.3389/fvets.2023.1252484	Frontiers in Veterinary Science

SUMMARY IN ENGLISH

Wild cats are facing a population decline, making it challenging to obtain germ cells for assisted reproduction techniques (ART). However, domestic cats can serve as a biomedical model for the ART of endangered species due to their biological similarities with other felids. While advancements have been made, success rates for ART in cats are lower than *in vivo* development. Stem cells have been used to improve germ cell development in vitro, and contact with MSCs can help obtain *in vitro*-derived embryos with levels of development similar to those derived *in vivo*.

Due to the above, the proposed research project aimed to isolate and characterise mesenchymal stem cells (MSCs) from the different anatomical regions of the feline umbilical cord and determine whether the *in vitro* co-culture of cat oocytes/embryos with feline Wharton's jelly-derived mesenchymal stem cells (fWJ- MSCs) will improve the results of the feline embryo *in vitro* culture.

During the initial research phase, the MSCs were derived and cultured from different segments of the feline umbilical cord, vessels, Wharton's jelly, and the whole cord. We evaluated the proliferative capacity of the MSCs by measuring the cumulative population doubling level and doubling time. Additionally, we validated the differentiation potential via chondrogenic, osteogenic, and adipogenic induction under each differentiation condition. The expression of surface markers was examined with flow cytometry, and the pluripotency gene expression was assessed using RT-PCR.

In the second research phase, feline Wharton's jelly-derived MSCs were used as a feeder layer for the oocytes during *in vitro* maturation and embryos during *in vitro* culture. In oocytes, the degree of cumulus expansion and the nuclear maturation were assessed, whereas for embryos, the developmental competence, measured as the cleavage, morula and blastocyst rate, was compared to the groups cultured without Wharton's jelly-derived MSCs addition.

The cells isolated possessed MSCs characteristics, including a typical spindle shape, proliferation capacity and the ability to differentiate into various lineages (chondrogenic, osteogenic, and adipogenic). These cells express mesenchymal (CD44+, CD90+) and pluripotency markers (NANOG, Oct4, SOX2) but not hematopoietic (CD34, MCH I) markers.

The Wharton's jelly-derived MSCs displayed the highest proliferation ability and tremendous differentiation potential compared to those isolated from the whole umbilical cord and from the umbilical cord vessels.

The use of feline Wharton's jelly-derived MSCs in co-culture with oocytes resulted in an increased proportion of cumulus cells and oocytes exhibiting cumulus expansion. Although there were no significant differences in the percentage of matured oocytes (metaphase II) among the groups, embryo development showed a significant improvement. Oocytes matured with MSC co-culture conditions had higher cleavage, morula, and blastocyst rates compared to commercial media alone ($P < 0.05$). Similarly, in the second part of the co-culture experiment, the embryos co-cultured with MSCs displayed higher morula and blastocyst rates ($P < 0.05$).

Based on the results obtained from our study, it has been found that the feline umbilical cord is a highly suitable source of MSCs. In addition, it was observed that co-culturing MSCs during oocyte maturation led to improved embryo development, while the co-culturing of MSCs during embryo culture resulted in a higher number of morula and blastocysts.

It is important to note that further research is needed to gain a complete understanding of how to best utilise MSCs for improving oocyte maturation and embryo *in vitro* conditions in domestic and wild feline species.

SUMMARY IN POLISH

Liczebność gatunków dzikich kotowatych zmniejsza się gwałtownie, co utrudnia pozyskiwanie ich komórek rozrodczych do badan mających na celu poprawę wyników obecnie stosowanych technik wspomaganego rozrodu prowadzonych w celu ochrony zasobów genetycznych zagrożonych gatunków. Z drugiej strony, stale rosnąca populacja kotów domowych, może służyć, jako model biomedyczny dla rozwoju i optymizacji biotechnik stosowanych u dzikich kotów. Pomimo badań prowadzonych już od wielu lat, wskaźniki sukcesu biotechnik u kotowatych są nadal znacząco niższe w porównaniu do wyników uzyskiwanych in vivo.

Dane literaturowe wskazują, że dodatek komórek macierzystych podczas hodowli gamet in vitro sprzyja pozyskiwaniu zarodków, których dynamika rozwoju i jakość są zbliżone do wyników obserwowanych in vivo. W związku z powyższym, celem przedstawionego projektu badawczego było wyizolowanie i scharakteryzowanie macierzystych komórek mezenchymalnych (MSC), pochodzących z różnych regionów anatomicznych sznura pępowinowego kota domowego. A następnie sprawdzenie czy dodatek MSCs pochodzących z części sznura pępowinowego zwanej galaretą Whartona (fWJ-MSC) podczas dojrzewania oocytów i hodowli zarodków, wpłynie korzystnie na proces dojrzewania i potencjał rozwojowy zarodków kota in vitro.

Niezróżnicowane MSCs pozyskiwano z różnych odcinków pępowiny kota: naczyń, galarety Whartona i całej pępowiny podczas początkowej fazy badawczej. W kolejnym etapie oceniano zdolność proliferacyjną pozyskanych MSCs w oparciu o czas podwojenia populacji. Dodatkowo weryfikowano potencjał pozyskanych komórek do wielokierunkowego różnicowania, poprzez indukowanie ich hodowli w kierunku chondrocytów, osteocytów i adipocytów. Ekspresję markerów powierzchniowych badano za pomocą cytometrii przepływowej, a ekspresję genu pluripotencji oceniano za pomocą RT-PCR.

W drugim etapie badań, mezenchymalne komórki macierzyste pochodzące z galarety Whartona sznura pępowinowego kota domowego, były dodawane do pożywek hodowlanych przeznaczonych dla oocytów podczas ich dojrzewania in vitro oraz dla zarodków podczas ich hodowli in vitro. W przypadku oocytów oceniano stopień rozszerzenia komórek wieńca promienistego i dojrzałość jądrową (metafaza II). Natomiast u zarodków oceniano kompetencję

rozwojową, mierzoną, jako wskaźnik bruzdkowania oraz odsetek morul i blastocyst. Uzyskane wyniki porównywano z grupami hodowanymi bez dodatku mezenchymalnych komórek macierzystych.

Uzyskane wyniki wskazują, że komórki mezenchymalne izolowane ze sznura pępowinowego, posiadały cechy komórek macierzystych, w tym typowy kształt, zdolność proliferacji i zdolność różnicowania się w różne linie komórkowe (chondrogenna, osteogenna i adipogenna). Komórki te posiadały również typowe markery mezenchymalne (CD 44+, CD90+) i markery pluripotencji (NANOG, Oct4, SOX2), ale nie posiadały typowych markerów hematopoetycznych (CD34, MCH I). W naszych badaniach, największą liczbę MSCs izolowano z galarety Whartona, ponadto te komórki wykazywały najwyższą zdolność proliferacyjną i najlepszy potencjał do wielokierunkowego różnicowania, w porównaniu z komórkami izolowanymi z całej pępowiny i naczyń pępowinowych.

W drugim etapie badań, MSCs pochodzące z galarety Whartona były dodawane do hodowli in vitro oocytów/zarodków kota domowego. W przypadku oocytów ich dodatek znacząco zwiększał stopień rozszerzenia komórek wieńca promienistego, ale nie wpływał na dojrzałość jądrową (metafaza II). Dodatkow zarodki pochodzące z oocytów dojrzewających z dodatkiem MSCs, wykazywały wyższy odsetek podziałów, morul i blastocyst ($P < 0,05$). Natomiast zarodki, pochodzące z oocytów dojrzewających in vitro w pożywce bez dodatku MSCs, a otrzymujące dodatek MSCs podczas rozwoju zarodkowego in vitro, również wykazywały wyższy odsetek morul i blastocyst ($P < 0,05$).

Na podstawie uzyskanych wyników stwierdzono, że pępowina kota domowego jest odpowiednim źródłem mezenchymalnych komórek macierzystych. Dodatkowo zaobserwowano, że dodatek MSCs podczas dojrzewania oocytów prowadził do poprawy rozwoju zarodkowego, podobnie jak dodatek MSCs podczas hodowli zarodków skutkował wyższą liczbą morul i blastocyst.

Należy zauważyć, że potrzebne są dalsze badania do pełnego zrozumienia tego, jak najlepiej wykorzystać MSCs podczas dojrzewania oocytów i hodowli zarodków kota domowego in vitro w celu optymalizacji technik wspomaganego rozrodu przeznaczonych dla tego gatunku, z nadzieją na możliwość ich przyszłego zastosowania u dzikich kotowatych.

LIST OF ABBREVIATIONS

ART	Assisted reproduction techniques
bFGF	Basic-Fibroblast Growth Factor
BoM	Bovine medium
BRL	Buffalo rat liver
BSA	Bovine serum albumin
CCL5	C-C Motif Chemokine Ligand 5
CM	Conditioned medium
CS	Calf serum
CSG	Cellular synthesis of Glutathione
COCs	Cumulus-oocytes complexes
DMEM- LG	Dulbecco's Modified Eagle's Medium-low glucose
EDTA	Ethylene diamine tetra-acetic acid
EPO	Erythropoietin
EqM	Equine medium
EVs	Extracellular vesicles
FBS	Foetal bovine serum
FCS	Foetal calf serum
FSH	Follicle-stimulating hormone
GSH	Glutathione
HGF	Hepatocyte Growth Factor
HLA	Human Leukocyte Antigen
HLA-G5	Human Leukocyte Antigen-G5
HO-1	Heme oxygenase 1
ICSI	Intracytoplasmic sperm injection
IDO	Indoleamine 2, 3-dioxygénase
IGF	Insulin-like growth factor
IGF1	Insulin-like growth factor 1
IL	Interleukin
IMS	Imaging mass spectrometry
IR	Implantation rate
IVC	*In vitro* culture
IVF	*In vitro* fertilisation
IVM	*In vitro* maturation
KGF	Keratinocyte Growth Factor
LH	Luteinising hormone
MALDI	Matrix-assisted laser desorption/ionisation

MDBK	Madin-Darby bovine kidney
MMP-2	Matrix metalloproteinases 2
MMP-9	Matrix metalloproteinases 9
MSCs	Mesenchymal stem cells
MZT	Maternal-to-zygotic transition
PBS	Phosphate buffer saline
PGE2	Prostaglandin E2
PPP	Pentose phosphate pathway
PR	Pregnancy rate
PS	Penicillin-streptomycin
ROS	Reactive oxygen species
SCNT	Somatic cell nuclear transfer
SDF1	Stimulated gene 6 protein
TGF	Stromal cell Derived Factor-1
TSG6	Transforming growth factor
UCV	Umbilical cord vessels
VEGF	Vascular Endothelial Growth Factor
WJ	Wharton's jelly
WUC	Whole umbilical cord

CHAPTER 1

GENERAL INTRODUCTION

1. Introduction

There are currently 40 cat species globally, among which only the domestic cat (Felis Catus) is not endangered or threatened with extinction because of habitat loss, degradation, and illegal hunting. The constantly decreasing wild cat population results in a growing demand for conservation strategies: translocation, protection, habitat management, captive breeding and improving assisted reproduction techniques to save their gene reserve [1].

Protecting endangered species through assisted reproduction techniques (ART) is crucial for maintaining biodiversity and preventing the extinction of valuable species, but the apparent constraints to obtain wild and endangered cats' germ cells cause the domestic cat to become the best available biomedical model for ART for these endangered species[2]. Since domestic cats are biologically very similar to other felids, it is strongly anticipated that methods developed for them in the future could be applied to wild cats as an innovative way of endangered species protection. Indeed, in Felidae, the *in vitro* embryo production system might become an essential tool for wildlife preservation [3].

The first successful *in vitro* fertilisation (IVF) for cats was reported in 1970[4]; since then, more advances have been made in ART in cats. It has been shown that oocytes matured *in vivo* can be fertilised and developed *in vitro*; the first blastocyst formation after IVF and embryo development using the oocytes matured *in vivo* was reported in 1977 [5]. Later, the first kitten birth of a domestic cat using IVF was reported in 1988[6]. Moreover, a series of studies have been made to improve the efficiency of IVF, and more advanced techniques, such as somatic cell nuclear transfer (SCNT) and intracytoplasmic sperm injection (ICSI), have also been applied successfully[7]. The first ICSI kittens derived from *in vivo* and *in vitro* matured oocytes were born in 1998 and 2000, respectively, and the first domestic cat generated by SCNT was born in 2002[8,9].

However, the success rate is lower than in most other species. The cause may lie in the differences in reproductive physiology between cats and other species. The maturation of reproductive cells *in vitro*, their fertilisation, and subsequent embryo development are still not fully understood[10]. Significant improvements were made to the culture systems for the evolution of embryo technology, and different protocols have been designed to optimise the development rate and quality of the embryos generated. Still, the quality of embryos in cats

obtained *in vitro* remains inferior to those produced in vivo, leading to increased embryo loss[11,12].

2. Progress in the culture system for domestic cat oocytes and embryos

It has been reported that IVM and IVC media contain different exogenous components that might affect *in vitro* oocyte maturation and division, blastocyst formation, and hatching after IVF. Some of these factors may have favourable and harmful effects on *in vitro* maintained cells. After years of investigations, the basic nutritional requirements for feline oocytes and embryos have been established, guaranteeing success *in vitro* development[13,14]. Different media supplements such as progesterone, oestrogen, gonadotropins or extra-pituitary gonadotropins (luteinising hormone - LH and follicle-stimulating hormone - FSH) were exploited and found that LH and FSH in the culture medium promote the preservation of the functional gap junctions (Transmembrane communication between oocytes and cumulus cells required for the diffusion of various compounds) enhancing the oocytes maturation *in vitro* [15]. Moreover, a recent study has shown that different sources (porcine vs. human) and concentrations (0.02 vs. 1.06 IU/ml) of FSH were used as a supplement for cat cumulus-oocytes complexes (COCs) and highlighted that an optimal hormone supplementation resulted in full maturation of oocytes and transcription ability of target gene[16].

The antioxidants and growth factors have been shown to have a major role in the regulation of apoptosis, cell proliferation and differentiation; cat oocytes matured in IVM with epidermal growth factor, resulting in late-stage embryo development (blastocyst) and higher rat (37.5%) and developmental competence (60.9%), further confirming the positive effect of this growth factor[17]. During the *in vitro* maturation, the oocytes are exposed to an adverse impact of reactive oxygen species (ROS) to stimulate the endogenous cell defence system. The supplementation of antioxidant is crucial for oocyte's functional integrity; adding a cysteine alone or in association with cysteamine or ß-mercaptoethanol into IVM maintains reduced cysteine available for the cellular synthesis of Glutathione (GSH) and avoid the depletion of GSH[15,18].

While it is difficult to determine what compounds are necessary for a good maturation of cat oocytes, some supplements like a calf (FCS) or foetal bovine serum (FBS) may cause an inhibitory effect on maturation, unlike bovine serum albumin (BSA), which is a good protein supplement[19,20].

As with all cells, cat oocytes metabolise glucose via glycolysis, the tricarboxylic acid cycle, and the pentose phosphate pathway (PPP)[21]. Resumption of meiosis is related to elevated activity of glycolysis and the PPP, as well as increased activity of hexokinase (glycolysis and PPP), phosphofructokinase (glycolysis), and glucose-6 phosphate dehydrogenase (PPP) within the cytoplasm of the oocyte[21,22].

In mice, gonadotropin-induced meiosis is dependent on the presence of glucose. Increased glucose metabolism through one or more metabolic pathways also co-occurs with the progression of meiosis to MII in oocytes from cats [23,24].

In primate oocytes, glucose is necessary for cytoplasmic maturation, although nuclear maturation can occur in the absence of carbohydrates. In addition, elevated glucose metabolism in mature oocytes is correlated with and predictive of improved embryonic development in cats and cattle. Similarly, oocytes matured *in vitro* from cats and pigs metabolise significantly less glucose and have lower developmental potential than oocytes matured *in vivo* [25,26]. These findings highlight the importance of glucose metabolism in oocyte maturation and the interactions between nuclear and cytoplasmic maturation.

To mimic the natural environment for fertilisation and embryo development, synthetic oviduct fluid based on biochemical and physiological research of the ovine oviduct, Earle's salt solution or Tyrode's salt solution, has been successfully used in cats during embryo production *in vitro*[27,28].

To identify the factors involved in the early stage of embryo development, the distribution of the proteins in cat oviduct was investigated using the matrix-assisted laser desorption/ionisation imaging mass spectrometry (MALDI-IMS), peptides involved in cellular damage response were identified in the oviduct, infundibulum, isthmus and ampulla among these peptides are thymosin, keratin and defensin[29,30].

In order to understand the specific cat embryo requirements during IVC and the effect of *in vitro* conditions, embryo gene expression was investigated, and it has been proved that gene expression depends on several parameters, *in vivo* and *in vitro* derived embryos, the culture medium, the embryo stage, and spermatozoa status. Moreover, the interruption of cellular division after a few cell cycles exists in cat species[31,32]. Studying the link between the consumption and the production of amino acids during IVC and the developmental capability of the embryos might also be exciting to understand cat embryo requirements better and, for

instance, to explain whether the developmental block *in vitro*, the interruption of cellular divisions after a few cell cycles, really exists in this species[33].

Current IVF results for cat species are considered unsatisfactory; approximately 60% of fertilised oocytes do not complete the pre-implantation phase. The cleavage of a one-cell embryo characterises this period until just beyond the blastocyst stage and represents a highly dynamic period of embryogenesis. At this point, the embryo must undergo several cell divisions, activation of the embryonic genome, epigenetic reprogramming, differentiation into two cell types, compaction, and development of the blastocoel cavity[34]. The embryos unable to accomplish their mission do not survive beyond the eight-cell stage; this phenomenon, known as developmental block, usually occurs at earlier embryo stages and is often associated with the maternal-to-zygotic transition (MZT), the embryonic genome activation[35]. In cats, a morula-to-blastocyst block coincident with the timing of *in vivo* embryo compaction, blastocoele formation and transition from the oviductal to the uterine environment was reported[36]. However, the exact timing of MZT is still unknown.

Few *in vivo* and *in vitro* embryogenesis studies in cat suggested that the developmental block may occur at the five- to eight-cell stage[37,38]. In contrast, a more recent study describes a "first wave" of embryonic gene activation at the 2-cell stage, followed by a greater wave during later development[39]. The final result of oocyte maturation, fertilisation and embryo development *in vitro* depends on the chemical factors in the medium, but it's important to consider the physical elements. Acting on biological factors may have a remarkable impact on chemical factors.

3. Oocytes, embryos communication in the absence of maternal tract

In the absence of the genital tract, when the gametes are being cultured *in vitro*, there is a lack of interaction between the gametes and several components present in the reproductive system during oocyte maturation, fertilisation and early stages of embryo development. In a semi-defined culture medium, the gametes lack the presence of paracrine or endocrine factors because all communication with the maternal tract is missing. Nevertheless, even without contact with the cells from the genital tract, oocytes and preimplantation embryos can promote their own development in vitro by the production of autocrine factors, and, in this way, they can

communicate with each other[40,41]. This accumulation of autocrine factors is typically achieved by culturing the oocytes or embryos in large groups, around 10–15 embryos in a droplet of medium covered by oil to avoid evaporation[42]. Recent advances point out that contact of oocytes and embryos with somatic oviduct cells, mesenchymal stem cells (MSCs), granulosa cells and extracellular vesicles (EVs) in the reproductive environment helps to obtain in vitro-derived embryos with developmental levels close to those embryos derived *in vivo*[41,43].

4. Co-culture system using stem cells

Over recent years and up to the present, we have seen a significant increase in ART. Most commercial *in vitro* production systems of mammalian embryos involve static co-culture systems to create physical contact between the gametes and somatic cells[44]. These systems use different helper cells/feeders such as primary epithelial cells from oviducts, oviductal ampullae, granulosa and cumulus cells, uterine endometrial cells, umbilical derived mesenchymal stem cells (MSCs), as well as established cell lines such as VERO cells have been used extensively for co-culture techniques to study their beneficial effect on the maturation of oocytes and the development of greater quality embryo, implantation rate (IR) and pregnancy rate (PR) (Table1). It has been demonstrated that these feeder cells have more than one action. It may be that such cells can release embryo-trophic factors into the co-culture medium and can remove inhibitory or toxic factors present in the medium or derived from the embryo [45]. There is also evidence that these cells may be beneficial by lowering the concentrations of ions and metabolising the glucose in the medium, allowing the embryos to be exposed to bearable levels of glucose[46]. The co-culture system also appears to overcome the developmental block[47]. This may be mediated by the release of growth factors such as vascular endothelial growth factor, insulin-like growth factors (IGF) I and IGF-II, platelet-activating factor and epidermal growth factor, transforming growth factor (TGF)-α and TGF-β, essential for the activation of the embryonic genome and for normal embryonic development [48]. The feeder cells may also protect the embryos from oxygen toxicity. Co-culture of embryos provides favouring growth up to the blastocyst; it also increases the availability of more cells for biopsy from the blastomere for genotypic evaluations and by better synchrony between the embryonic stage and site of placement of transfer (uterus) [49]. Recently, mesenchymal stem cells have been used most in a co-culture system with embryos and /or oocytes in mammals, including pigs, cows, rabbits, mice, and horses. These studies have

been based on the fact that MSCs have different tissue origins, are easy to culture and are capable of secreting a variety of substances that are collectively competent in modulating the surrounding microenvironment and enhancing germ cell development *in vitro*[44].

Feeder cell type	**co-culture stage**	**References**
Granulosa cells	IVC	[50]
Cumulus cells	IVM	[51]
Ampullary cells	IVM/IVC	[52]
Tubal/oviductal epithelial cells	IVC	[53]
Endometrial epithelial cells	IVC	[54]
Endometrial stromal cells	IVC	[55]
Epithelial cancer cells	IVC	[56]
Vesicles: ciliated oviduct cells, trophoblast	IVC	[57,58]
Vero cells	IVC	[59]
Fibroblasts: skin, placental, embryonic, oviduct cells	IVM/IVC	[60–62]
Buffalo rat liver (BRL) cells	IVC	[63]
Decidual stromal cells	IVC	[64]
Granulosa-lutein cells	IVC	[65]
Mesenchymal stem cells(placenta, umbilical cord, adipose tissue, amniotic fluid)	IVM	[66–68]

Table 1: Cells used as a feeder layer in the culture systems for oocytes and early embryos in vitro Adapted from Nicolas M et al.[69]

5. Mesenchymal stem cells - an overview

The most common sources of MSCs are of adult origins, such as bone marrow or adipose tissue, but their removal requires an invasive procedure. Perinatal sources like the umbilical cord and Wharton's Jelly offer more accessibility and significant MSCs with a higher proliferation rate and more potent immunomodulatory action. They were recently isolated from different adult tissues and represent a promising candidate for cell-based therapies. Many studies have shown that MSCs acquire morphology and change phenotype to express many specific markers depending on the environmental conditions in which they are cultured. Differentiations in cardiomyocytes, neurons, pancreatic cells, hepatocytes, renal tubular cells, and skeletal or smooth muscle cells have been observed. The most essential clinically applicable characteristics of mesenchymal stem cells depend on their self-renewing and the ease of proliferation in culture.

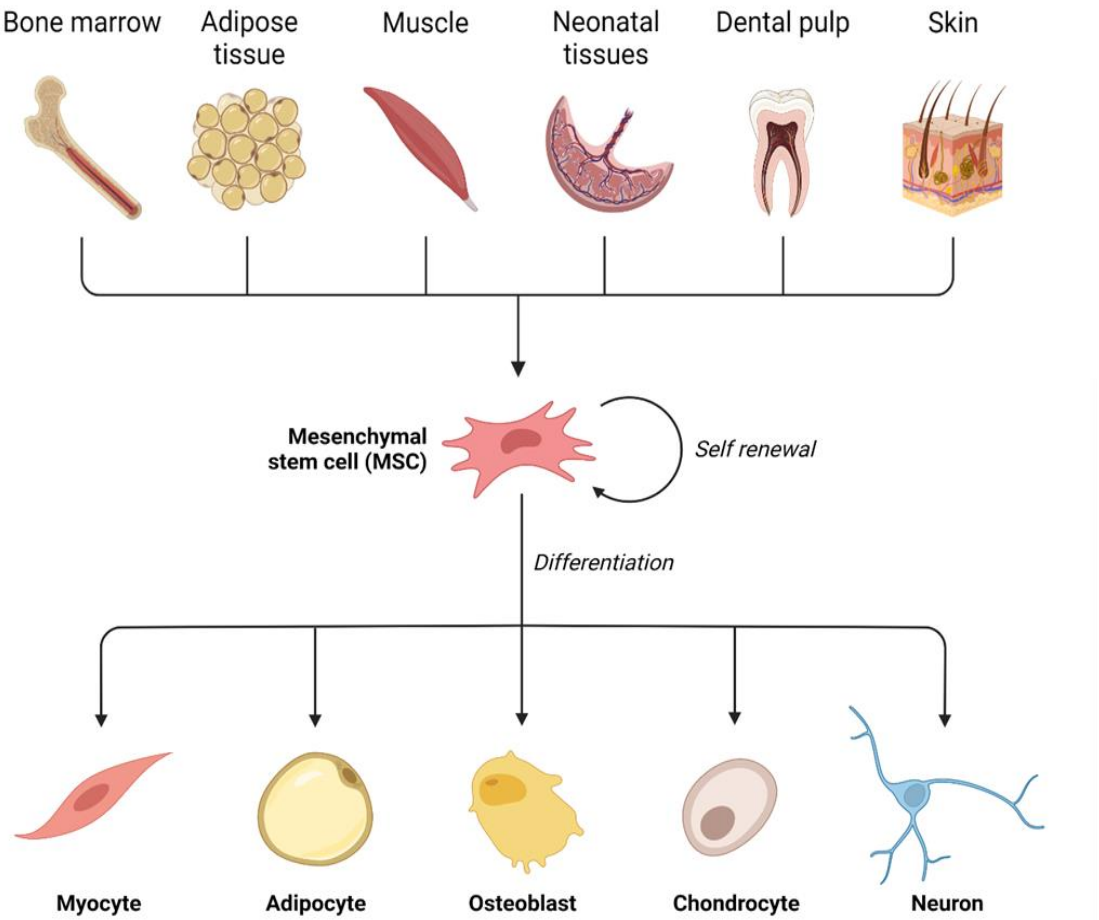

Figure 1: The picture summarises the variety of origins and differentiation potential of mesenchymal stem cells (MSCs)

6. Mesenchymal stem cells derived soluble factors

MSCs have already been applied in preclinical and clinical studies to successfully treat diseases such as type I diabetes, multiple sclerosis, cirrhosis of the liver, and Crohn's disease[70]. These studies demonstrate the crucial clinical potential of mesenchymal stem cells. MSCs can be very immunosuppressive and have been found to suppress T-cell proliferation and cytokine production. The HLA-G protein (Human Leukocyte Antigen), a non-classical HLA class I molecule, has also been considered to have an immunosuppressive effect on MSCs. The ability of MSCs to regenerate injured tissues is closely linked to their anti-inflammatory properties[71]. MSC**s** act locally through cell-cell interactions based on receptor-ligand bonds or through nanotubes that transfer molecules and organelles. However, they intervene mainly at the systemic level by secreting trophic factors that can be transported by extracellular vesicles (EVs)**.** They can thus promote cell viability and angiogenesis by producing growth factors (VEGF, PDGF, bFGF). They also stimulate the recruitment of endogenous stem cells by secreting chemokines, such as SDF1 or CCL5, and reduce fibrosis by producing KGFs, MMP-9, bFGF, MMP-2, and HGF; they intervene in the regulation of apoptosis, via the production of HGF, b Fgf, IGF1, and oxidative stress, by the release of HO-1 or EPO. Finally, they possess anti-inflammatory activity by releasing IDO, HLA-G5, PGE2, TSG6, IL-6, IL-1, etc. The secretion of all these factors thus gives these cells an unusual trophic activity[70,71].

It has also been shown that injection of MSCs conditioned medium, not the cells themselves, can induce the same effects as MSCs. This is due to the composition of MSCs secretions containing soluble factors and EVs. EVs are involved in tissue repair, immunomodulation, and proliferation. A diverse population of EVs can be found in biological fluids (blood, saliva, semen, vaginal fluids, mucus and urine). Scientists mainly describe three types of EVs according to their size and biogenesis. The biggest vesicles are secreted after cell apoptosis (large EVs of 1 to 5 µm) called apoptotic bodies. EVs of 0.1 to 1 µm are called microparticles, ectosomes, or macrovesicles. They are usually produced by cells during stress or metabolic changes and result from the appearance of the plasma membrane. According to the literature, the latest EVs are

called exosomes, with a size varying from 30 to 150 nm according to the literature, and they are secreted continuously, regardless of the cell state. MSCs have been shown to generate more EVs than other cell types, making them an attractive source of vesicles for therapeutic applications[72].

MSCs possess multi-potentiality and properties of immunological and inflammatory regulation. Cell therapy based on their transplant appears to be a promising approach at present, as these cells can lead to adipocytes, osteoblasts, chondrocytes, smooth muscle cells, and endothelial cells[73]. The use of MSCs in cell therapy appears to be due to different soluble factors summarised in Figure 2.

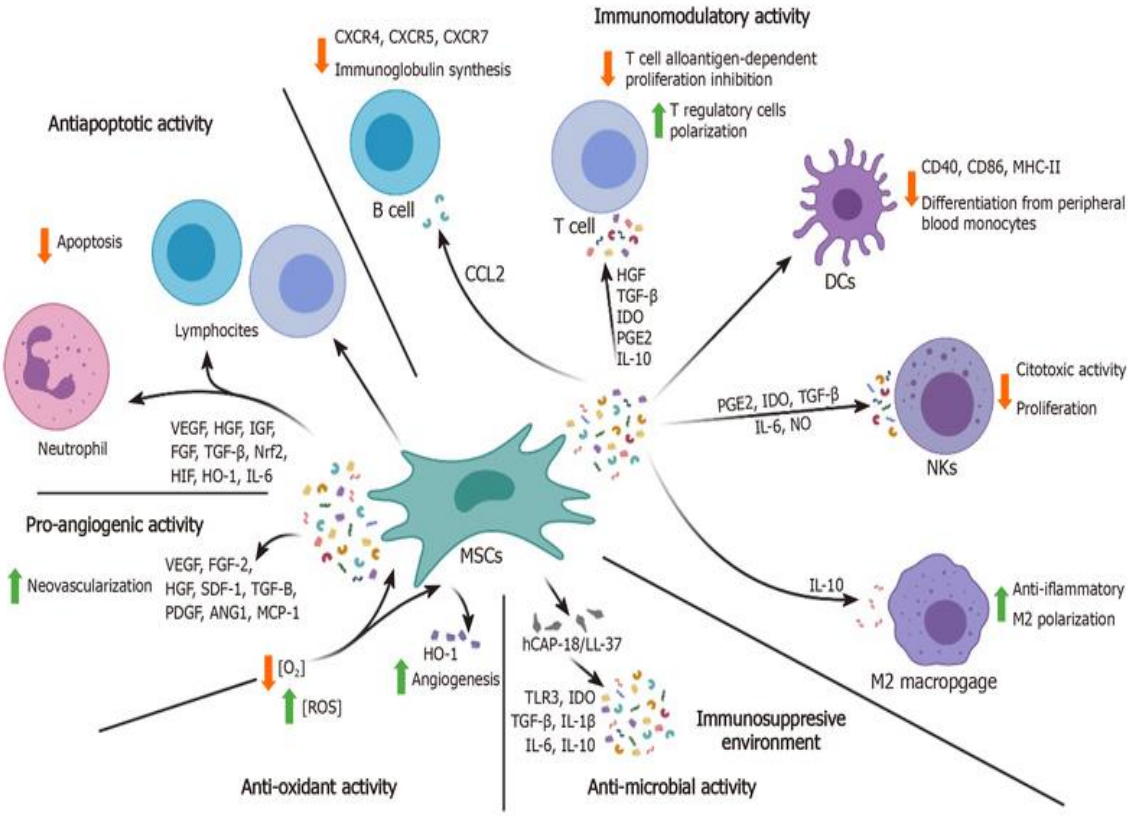

Figure 2: Summary of the soluble factors released by mesenchymal stem cells and their biological function (Adapted from Alberto et al)[73]

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CHAPTER 2

AIMS OF THE THESIS

Understanding the nutrient requirements and physiology of oocytes and embryos would led to the development of more suitable culture media. The success of such media can be attributed to catering to the oocytes and embryo-changing nutrient requirements while minimising the culture-induced stress. Co-culture systems are more effective than chemically defined mediums in producing mammalian embryos. Recently, Mesenchymal Stem Cells (MSCs) have emerged as a promising source of stem cells in co-culture systems. Furthermore, it has been shown that MSCs are able to secrete a variety of autocrine and paracrine factors, including chemokines, cytokines, growth factors, extracellular matrix (ECM) and proteases, enabling the possibility of using them as feeder layers in a co-culture to improve the quality of the oocytes maturation and the embryo development *in vitro* mammalian species.

The specific scientific aims of the presented thesis can be defined as follows:

1. Review of the current knowledge on mesenchymal stem cells in feline and canine medicine: their origin, characteristics, *in vitro* therapeutic applications, mechanisms of biological action and challenges in their therapeutic application (Chapter 4).

2. Isolation and characterization of feline mesenchymal stem cells from the umbilical cord and two different anatomic regions: vessels, and Wharton's jelly (Chapter 5).

3. Evaluation of the addition of mesenchymal stem cells as a feeder layer during feline oocyte maturation and embryo development *in vitro* (Chapter 6).

CHAPTER 3
MATERIAL AND METHODS

In order to isolate and characterise Mesenchymal stem cells from feline umbilical cords, we followed the methodology below. The chemicals and reagents were purchased from Sigma Aldrich or Thermo Fisher Poland, unless stated otherwise. Ethical approval was not sought, as it is not required for studies carried out on cells obtained from tissues that were surgical waste (Decision No. 004/2021).

1.1.Obtaining umbilical cord tissue:

The full available length of umbilical cords were collected from queens of different breeds aged from 1,5 to 5 years old. The queens were patients of the Department of Reproduction and Clinic of Large Animals in Wroclaw. Directly after natural delivery or during caesarean sections, the umbilical cords were dissected with a scalpel blade from the placenta on one side, and on the other side about 1 cm far from the kitten's abdomen. The umbilical cords were stored in a PBS with addition of 1% penicillin-streptomycin solution at 4°C before the transfer to the laboratory. The general patient data: the age of the queen and the number of newborns were registered.

1.2. Tissue harvest:

Within 2 hours of collection, umbilical cords were cleansed with alternating washes of cold PBS +1% penicillin-streptomycin. Using sterile surgical forceps and a #10 scalpel blade, the anatomical regions of each cord were separated: the amniotic epithelium, the umbilical arteries and veins (UCV) were resected, and the Wharton's jelly (WJ) were separated.

1.3. Mesenchymal stem cell isolation and culture:

Mesenchymal stem cells were isolated and cultured as described below, the collected WJ, UCV and the entire cord were placed in an individual sterile culture dish. The tissue was minced into a 2 mm square using a bistoury blade and then transferred to 15 ml centrifuge tubes containing 0.02% of collagenase type-I in Dulbecco's Modified Eagle's Medium-low glucose (LG-DMEM). Samples were incubated at 37 °C for 20 minutes for tissue digestion. Afterwards, the samples were centrifuged at 300 ×g for 5 minutes and washed in PBS. Next, the stromal vascular fraction was resuspended in a stromal medium: LG-DMEM with 10% fetal bovine serum (FBS) and 1% antibiotic solution and cultured in T-25 flasks (Techno Plastic Products AG, Switzerland). Half of the medium was changed after 24 hours, and then the entire medium was changed twice per week; after one week, the stromal vascular fractions were removed, and the flasks were washed with 3 mL of PBS. The cells were cultured to reach 80% confluence.

1.4. Storage of cells:

When the culture wells are about 80% confluence, the cells are washed three times with 3 mL of PBS and then detached using trypsin-EDTA (3 mL of trypsin-EDTA solution per flask). After 3 min of incubation at 37°C, the action of trypsin is inhibited with 3 mL of stromal culture medium. The cell suspension is then centrifuged (300 ×g, 5 min) and resuspended in a culture medium, and then the cells are counted in the Thoma counting chamber. After further centrifugation (300 ×g, 5 min), the cell pellet was suspended in 10% of the final DMSO cryopreservation solution and cooled for 1 hour at a temperature of -20, then at -80°C within the next 24 hours. The cells were then stored in liquid nitrogen (-196°C) before further use.

1.5. Thawing the cells:

The cells were rapidly thawed in a water bath at 37°C, then re-suspended in 10 mL of culture medium. They are then centrifuged (300 ×g, 5 min), and the cell pellet is diluted in 1 ml of culture medium. Cells were counted using Trypan blue and Thoma chamber; finally, cells were seeded at 1000 cells/cm² in T25 with 3 mL of a stromal medium per culture flask or T75 with 9 mL of medium. These culture flasks were placed in a humidified incubator (5% CO2, 5% O2) at 37°C until reaching (80% to 90%) confluence. The medium was changed twice per week.

1.6. Cell doublings and doubling time:

Cells isolated from WJ, UCV, and WUC were quantified in 12 well plates(Techno Plastic Products AG, Switzerland), as previously described by Zhang et al. Cells at passage 1 were seeded in triplicate at a density of 5×10^3 cells/cm. Cell numbers were assessed after 2, 4 and 6 days of culture using a trypan blue and hemocytometer. Cells doubling numbers (CD) and doubling time (DT) were calculated according to the equation below:

$$CD = \ln(Nf /Ni)/\ln(2)$$

$$DT = CT/CD$$

Where culture time CT = culture time, Nf = final cell number, and Ni = initial cell number.

The mean and the standard error were calculated for all data obtained.

1.7. RNA extraction and real-time reverse transcription (qRT-PCR)

Feline-specific primers were designed for SOX2, NANOG and OCT-4 (Table); cells were seeded in 6-well plates at a density of 1×10^6 cells per well in a stromal medium until reaching around 90% confluence. Cells were rinsed twice with PBS then total RNA was isolated using TRI Reagent according to the manufacturer's instructions. RNA concentration and purity were measured using a nano spectrophotometer (denovix ds-11). Complementary DNA (cDNA) was prepared using isolated RNA and a Tetro cDNA Synthesis Kit (Bioline, London, UK). To determine the expression levels of MSCs pluripotency markers, target gene mRNA levels were quantified with real-time reverse transcription-polymerase chain reaction (qRT-PCR) using the SensiFAST SYBR Green Kit (Bioline, London, UK) in a CFX Connect™ Real-Time PCR Detection System (Bio-Rad). For the 10 µl reaction volume, the following cycling conditions were applied: 95 °C for 2 min, followed by 40 cycles for 15 at 95 °C, annealing for 15 s, and elongation at 72 °C for 15 s. The results were determined relative to the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The relative level of the expression was calculated using the $2^{-\Delta CQ}$ method.

Table 2: Primer sequence information for feline of WJ-MSCs, UCV-MSCs and WUC-MSCs.

Gene	Primer	Sequence 5'–3.'	Annealing temperature (°C)
SOX2	F : R :	CCGAGTGGAAACTTTTGTC AAAATCTGCAGGAGATATGC	65.4
OCT-4	F : R :	AAAATCTGCAGGAGATATGC ACTCGGTTCTCGATACTTG	54.60
NANOG	F : R :	GTGACAACTTCACAAAATCG TCCAGTTTCTCTTCTAGTTCC	54.45
GAPDH	F : R :	GATGCCCCAATGTTTGTGA AAGCAGGGATGATGTTCTGG	55.60

1.8. Immunophenotyping by Flow cytometry

Antibodies against CD90, CD44, CHMII, and CD 34 (Table 2) specific for feline antigens or validated for feline cross-reactivity were used to label the cells for flow cytometry. With the exception of CD44 and CHMII, antibodies were conjugated with PE or FITC. Cells at the 2nd passage were detached using trypsin EDTA, then centrifuged, and thereafter the cells pellet was resuspended in PBS with 1% FBS and counted. Cells aliquots containing 10^5 cells from each

population were resuspended in PBS of labelled antibodies, and the mixtures were incubated in the dark at room temperature for 30 min. Following the incubation with antibodies, cells were rinsed with PBS. For CHMII and CD44 detection, cells were additionally incubated with a secondary antibody. An appropriate isotype-matched control antibody was used. Cell fluorescence was quantified using FACSCalibur, equipped with a 488-nanometer laser. Data were recorded for at least 5,000 events using CellQuest version 3.3 software. Unlabelled cells and isotype controls were included in all assays.

Table 3: Antibodies used for feline umbilical cord mesenchymal stem cells

Antibody	Isotype	Label	Cross / reactivity	Host	Manufacturer	Catalogue #	Antibody dilution
MHC Class II	IgG2b	Purified	Cat	Mouse	Bio-Rad	MCA2723	4:100
CD90	IgG1,k	PE	Human	Mouse	BD Biosciences	555596	1:10
CD34	IgG1,k	FITC	Human	Mouse	Bio-Rad	555821	1:10
CD44	IgG1	Purified	Human	Mouse	Bio-Rad	MCA1719GA	4:100
Control	IgG1	PE	NA	Mouse	Antibodies	ABIN376413	1:10
Control	IgG1	FITC	NA	Mouse	Invitrogen	GM4992	4:100
Secondary Antibody	IgG (H+L)	FITC	Mouse	Goat	Invitrogen	A16079	1:1000

1.9. Tri-lineage differentiation assay

Osteogenic, chondrogenic, and adipogenic differentiation ability was confirmed in cell isolates (WJ-MSCs, UCV-MSCs, and WUC-MSCs) cultured in 6 well plates at passage number 3. The experiments were conducted in two groups- a treated group cultured in a stromal medium to reach about 80% confluence, then maintained in a specific induction medium, and a control group cultured in a stromal medium only. The results were observed and analysed under an Olympus IX73 inverted microscope.

To determine the adipogenic differentiation, cells were cultured at a density of $1x10^5$ per well in Adipogenic Differentiation Medium (Mesenchymal Stem Cell Adipogenic Differentiation

Medium. Sigma-Aldrich. Poland) for 3 weeks. The culture medium was changed every 3 days.to detect lipid droplet accumulation, the cells were washed two times with PBS, fixed with 4% formaldehyde (PFA) (Sigma-Aldrich. Poland) and incubated at room temperature for 45 min, followed by three rinses with PBS. Finally, the cells were incubated for 5 min in 60% 2-propanol and then stained with Oil Red O (Sigma-Aldrich. Poland) for 5 min at room temperature.

To access the osteogenesis, the cells were cultured in the osteogenic induction medium (Mesenchymal Stem Cell Growth Medium. Sigma-Aldrich. Poland) for 3 weeks; the medium was changed twice a week, and the accumulation of calcium in colonies was confirmed using 2% Alizarin Red S(Sigma-Aldrich. Poland). First, cells were washed two times with PBS, fixed with 4% PFA for 30 min, and then incubated with 2% Alizarin Red S in the dark for 15 min at room temperature.

The cells were also cultured in a chondrogenic induction medium (Mesenchymal Stem Cell Chondrogenic Differentiation Medium. Sigma-Aldrich. Poland) for 21 days. After the differentiation steps were completed, the cells were rinsed gently with PBS. Then, the cells were incubated in 4% PFA at room temperature to fix the cartilage for 40 min. Followed by two washes with distilled water to confirm the cartilage formation; the cells were then stained with Alcian Blue staining solution (Merck KGaA, Darmstadt, Germany) and incubated at room temperature in the dark for 50 minutes.

The results of this study were published in the article titled: **'Feline umbilical cord mesenchymal stem cells: Isolation and in vitro characterisation from distinct parts of the umbilical cord'** Theriogenology (2023) 201:116–25. doi:10.1016/j.theriogenology.2022.11.049

2.1. Preparation of mesenchymal stem cells for the co-culture

To prepare the fWJ-MSCs for co-cultivation with feline oocytes or embryos, the cells at a density of 5×10^3 cells/cm were cultured in a stromal medium until they reached about 90% confluence. MSCs were then treated with10 μg/ mL mitomycin C for 2 hours to inactivate them. After 5 washes with PBS, the cells were maintained in LG-DMEM for 24 hours before co-cultivation.

2.2. Ovaries and oocytes recovery

Ovaries from sexually matured domestic queens subjected to a routine ovariohysterectomy or ovariectomy were obtained at the University clinic or from local veterinarians in Wroclaw. After surgical removal, ovaries were stored at 4 °C in PBS with 1% of Antibiotic Antimycotic solution over a period ranging from 2 to 24 h before the recovery of cumulus-oocyte complexes (COCs). To collect COCs, ovaries were sliced with a #10 scalpel blade in an OPU medium (IVF Bioscience, Bickland Industrial Park, Falmouth, UK). Isolated COCs were selected under a dissecting microscope. Only oocytes with dark ooplasm and several layers of cumulus cells were selected for further procedures.

2.3. *In vitro* maturation of cat oocytes

In order to evaluate the effect of different culture systems on the in vitro oocytes maturation, groups of 15-20 COCs were matured in a four-well plate in 400 µl of plain equine maturation medium (EqM) (IVF Bioscience, Bickland Industrial Park, Falmouth, UK) and plain bovine maturation medium (BoM) (IVF Bioscience, Bickland Industrial Park, Falmouth, UK) and/or in the same medium with MSCs co-culture: EqM+MSCs or BoM+MSCs, covered with sterile mineral oil and cultured for 24 h at 38.5°C in 5% CO2 in the air with maximum humidity.

2.4. Assessment of oocytes maturation

After 24 hours of the oocytes maturation period, the oocytes were denuded from the remaining cumulus cells using a glass pipette that was overheated and pulled to achieve diameters of approximately 165 µm, slightly larger than the oocyte. Oocytes were aspirated and blown out repeatedly until removing the most of cumulus cells. The oocytes were then washed twice and fixed with 4% PFA for 15 minutes flowed by washing in PBS and then incubated in 4, 6-diamino-2-phenylindole (DAPI) stain solution for 10 min in the dark at room temperature and mounted on glass slides in drops of Vectashield (Vector Laboratories, LTD UK). The oocytes were classified as degenerated oocytes (irregular border and fragmentation of cytoplasm) and immature (without the first polar body extrusion), or metaphase II (MII) stages of the maturation process with distinct polar bodies or two separate and bright chromatin spots under a fluorescence microscope (Olympus IX73) at 360 excitations and 450 nm emission.

2.5. *In vitro* fertilisation:

For *in vitro* fertilisation, the matured and denuded oocytes were fertilised using frozen-thawed semen cryopreserved and thawed according to the protocol described by Partyka et al. 2012.[74] In brief: frozen semen straw was thawed in a water bath at 37°C for 30 seconds, then washed in IVF medium (IVF Bioscience, Bickland Industrial Park, Falmouth, UK) followed by centrifugation at 35000 rpm for 5min. The oocytes were incubated with 1×10^6 motile spermatozoa/ml in 400 µl of IVF medium for 18 hours under mineral oil at 38.5°C in the maximum humidified air atmosphere with 5% CO2.

2.6. Embryo culture and assessment of the embryo development:

After fertilisation, the presumptive zygotes were transferred to a new plate in a droplet of 50 µl of either pure BoM or EqM (IVF Bioscience, Bickland Industrial Park, Falmouth, UK) medium or to the co-culture BoM+MSCs, EqM+MSCs medium (depending on the part of the experiment), covered with mineral oil and incubated at 38.5°C in 5% CO2 in the air with maximum humidity for up to 8 days. To assess embryo development, morphological changes were evaluated and noted every 8 to 12 hours. The subsequent developmental stages were noted and compared for each group, and the blastocyst formation was recorded.

2.7.Study design:

Experiment 1: The effect of the MSCs co-culture on cumulus cell expansion and oocyte maturation.

This study evaluated the nuclear maturation and cumulus cell expansion of oocytes after 24 hours of incubation in different maturation media. 180 oocytes were matured in four experimental groups compared, including 1) Maturation in BoM, 2) Maturation in EqM, 3) maturation in BoM+MSCs, and 4) Maturation in EqM+MSCs. In each group, 45 oocytes were matured; in each group, 3 independent replicates of 15 oocytes were performed.

a. Assessment of cumulus cells expansion:

We evaluated the degree of cumulus cell expansion after 24 hours of oocyte maturation in EqM, BoM EqM+MSCs and BoM+MSC. The scoring system was as follows: degenerated (oocytes with no cumulus cells attached), expended (more than three layers of cumulus cells expanded), and limited expansion (less than three layers of cumulus cells expended) and no expansion.

b. Assessment of nuclear maturation

An extrusion of the first polar body (Metaphase II) from each denuded oocyte was assessed using DAPI staining under the fluorescence microscope (Olympus IX73) at excitation 360 and 450 nm emission. Oocytes with distinct polar bodies or two separate and bright chromatin spots were classified as entering the MII stage.

Experiment 2: The effect of the co-culture with MSCs during oocyte maturation on embryo development.

In this part of the study, we assessed the effect of the co-culture system with MSCs during maturation in BoM and EqM on the subsequent embryo development after in vitro fertilisation. In total, 565 oocytes were matured and cultured in four experimental groups and 10 replicates per group: 1) Maturation in BoM / embryos culture in BoM (n = 103), 2) Maturation in EqM/ embryos culture in EqM (n = 109), 3) Maturation BoM+MSCs / embryos culture in BoM (n = 124), 4) Maturation in EqM+MSCs / embryos development in EqM, (n = 109).

Experiment 3: The effect of co-culture with MSC during embryo development.

In this experiment, the maturation of oocytes was performed in pure EqM or in BoM, while the embryo development was carried out in pure BoIVC or EqIVC medium or with MSCs in addition to evaluate their effect on the morula and blastocyst formation. In total, 486 oocytes were matured and cultured in four experimental groups, and 10 replicates per group: 1) Maturation in BoM/ embryos culture in BoM (n =103), 2) Maturation in EqM/ embryos culture in EqM (n =109), 3) Maturation BoM/ embryos culture in BoM+MSCs (n =132), 4) Maturation in EqM / embryos development in EqM+MSCs (n =142).

2.8.Statistical analysis:

In this study, the data were analysed using one-way ANOVA followed by Tukey's multiple comparison test using Statistica software (TIBCO, USA). Values are shown as means ± SEM. The significance level was $P < 0.05$, and at least three independent replicates were performed in all experiments. Nonparametric data, such as differences in the percentage values between groups, were assessed using the chi-square test. The result of this second study was published in the article with the title **Feline Wharton's Jelly-derived Mesenchymal Stem Cells as a Feeder**

Layer for Oocytes and Embryos Culture *in vitro*. Frontiers in veterinary sciences journal (2023). 10:1252484. doi: 10.3389/fvets.2023.1252484.

CHAPTER 4
MESENCHYMAL STEM CELLS: GENERALITIES AND ITS CLINICAL SIGNIFICANCE

Mesenchymal stem cell therapy is an exciting and rapidly advancing area of veterinary medicine that could have significant implications for the future. Before commencing our laboratory experiments, we extensively reviewed previously published research on this topic. The comprehensive survey was titled "Mesenchymal Stem Cells: Generalities and Clinical Significance in Feline and Canine Medicine" and was published in the Animals Journal (2023) 13:1903. doi: 10.3390/ani13121903.

Our primary goal was to gain a comprehensive understanding of mesenchymal stem cells (MSCs), including their various sources, characteristics, properties, and clinical applications in both cats and dogs. Therefore, the first publication provided an overview of the different tissues that have been investigated for isolating MSCs in cats and dogs, as well as their morphology. We also described the criteria that have been identified by other research teams, which served as a foundation and guidance during our study.

We mainly focused on the MSCs' properties, such as anti-inflammatory, immunosuppressive and immunomodulatory that make them an excellent option for treating inflammatory diseases in cats. Stem cell therapy has the potential to improve the health of felines suffering from various illnesses. Therefore, we provided a comprehensive overview of the current proposed mechanism of action of MSCs. Additionally, we summarized the numerous studies that used MSCs in clinical trials for the treatment of various diseases such as gingivostomatitis, chronic enteropathy, asthma, and kidney disease in feline; osteoarthritis, osteochondritis, tendonitis and ligament rupture in dogs. We also highlighted the importance of animal safety in veterinary clinical trials by checking the Quality-controlled cells (including: cells' origin, storage, composition, freedom from contamination, the long-term safety evaluation identifies additional risks i.e. toxicity and tumorigenicity).

Information gathered in this review article served as guidance during our study, helped to decide on the type of tissue for isolating MSCs, and provided essential data for the laboratory protocols we used for culturing and characterizing the MSCs from the feline umbilical cord.

animals

MDPI

Review

Mesenchymal Stem Cells: Generalities and Clinical Significance in Feline and Canine Medicine

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Simple Summary: Veterinary regenerative medicine is an area of active research in which mesenchymal stem cells are applied. Mesenchymal stem cells (MSCs)are cells that can be obtained from various adult tissues; these cells have an extraordinary quality of being able to self-renew and develop into other cells. MSCs can be used to treat orthopaedic conditions in dogs, asthma, kidney disease, chronic gingivostomatitis, and inflammatory bowel disease in cats. Most studies have used adipose tissue-derived MSCs because they are easily obtainable and easy to work with. However, other stem cells from different tissues may be more suitable for treating certain diseases. In this manuscript, we report the generalities and the use of mesenchymal stem cells in cats and dogs, and we believe that the ongoing research in this field will eventually bring us to a point where stem cell treatments for currently untreatable diseases will become a reality. Finally, veterinary medicine now has access to new treatments, giving hope for a cure to illnesses in our furry friends.

Abstract: Mesenchymal stem cells (MSCs) are multipotent cells: they can proliferate like undifferentiated cells and have the ability to differentiate into different types of cells. A considerable amount of research focuses on the potential therapeutic benefits of MSCs, such as cell therapy or tissue regeneration, and MSCs are considered powerful tools in veterinary regenerative medicine. They are the leading type of adult stem cells in clinical trials owing to their immunosuppressive, immunomodulatory, and anti-inflammatory properties, as well as their low teratogenic risk compared with pluripotent stem cells. The present review details the current understanding of the fundamental biology of MSCs. We focus on MSCs' properties and their characteristics with the goal of providing an overview of therapeutic innovations based on MSCs in canines and felines.

Keywords: mesenchymal stem cells; properties; characteristics; canine; feline

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1. Introduction

Current cell therapies use multipotent stromal cells isolated from adult tissue, representing an emerging branch of regenerative medicine that aims to restore tissues and organs damaged by trauma, pathology, or ageing processes. Research on the therapeutic properties of stem cells in humans over many years has shown the benefits that can be obtained in inflammatory and degenerative diseases through the use of adult stem cells, particularly multipotent stromal cells or mesenchymal stem cells (MSCs) obtained from the bone marrow. Although MSC administration is performed with the support of immunosuppressive treatment, autologous MSC, which allows personalised immunomodulation, seems an interesting approach, limiting the risk of immunisation or faster apoptosis of MSC. However, this approach requires either isolation from the patients without systemic diseases or keeping the MSCs for an extended period before transplantation, causing high additional costs [1]. Several teams have turned to the use of allogeneic MSCs, allowing

the creation of therapeutic batches [2]. This approach is reinforced by the fact that the injection of allogeneic MSCs seems to have the same immunoregulatory properties in vitro and in vivo. There is significant interindividual variability of the MSCs, making selecting a batch with a high immunoregulatory capacity preferable [3]. Stem cell therapy is not limited to humans. It is also of great interest in veterinary medicine and has already been used to treat animals affected by degenerative disorders, inadequate diet, and genetic disorders. It has also been used in animals with various musculoskeletal tissue injuries, primarily cartilage wear in joints and spinal discs, tendonitis, fractures, and bone degeneration [4]. Veterinarians mainly use mesenchymal stem cells (MSCs), and in recent years, treatments have been used in companion animals. MSCs provide innovative therapeutic options for diseases that previously lacked indicated treatments. Thus, protocols for regenerating damaged structures in joints, ligaments, menisci, and cartilage, similar to those observed in horses, have emerged in dogs, cats, and rabbits [5–8]. MSCs have vast potential in the treatment of many animal and human diseases. Randomised and controlled clinical studies are still necessary to apply such therapies in humans, but the success of many animal models attests to stem cells' efficacy and therapeutic potential [9]. This review summarises the general characteristics and properties of MSCs with a particular focus on feline and canine MSCs. It also provides an overview of the use of MSCs in cell therapy and regenerative medicine.

2. Mesenchymal Stem Cells

MSCs are immature cells derived from mesenchyme or embryonic connective tissue, part of the mesoderm. In adults, they occur in connective tissue. MSCs are present in varying quantities and with different potentials throughout postnatal life, depending on the individual source tissue, age, and health [10,11]. The cells can be isolated significantly from different connective tissues, particularly bone marrow, umbilical cord, and adipose tissue [12]. Adult MSCs can self-renew and generate multiple types of mature and functional differentiated cells, with differentiation into specific cells of mesodermal origin (adipocytes, myoblasts, osteoblasts, and chondroblasts), depending on the environment [13,14]. However, studies have shown that MSCs can also be oriented in vitro towards endodermal phenotypes (hepatocytes, pancreatic cells) and/or ectodermal (astrocytes and epithelial cells) phenotypes [15,16]. MSCs have immunomodulation potential and positive effects on tissue tropism, and these characteristics make them ideal candidates for cell therapy and immunomodulatory strategies, particularly in systemic or local inflammatory diseases [17].

MSCs were initially isolated from the stromal compartment of bone marrow [18]. They were subsequently found in almost all postnatal connective tissues [19], umbilical cord and umbilical cord blood [20], adipose tissue [21], placental tissue [22], and cutaneous connective tissue [23]. Regardless of their course, all cells have the same basal biological characteristics, although they may differ in their potential for expansion and differentiation [24]. MSCs are a heterogeneous population of multipotent cells characterised by clonogenic abilities and differentiation potential. The International Society for Cellular Therapy's definition of MSCs is based on three criteria: (i) their ability to adhere to plastic; a phenotype of CD73+/CD90+/CD105+ and CD45−/CD34−/CD14−, CD11b−/CD19−, or CD79a−/HLA-DR−; and their potential for differentiation into osteoblasts, chondrocytes, and adipocytes [25]. Some authors [26,27] have also suggested using other markers to select multipotent subpopulations, such as the STRO-1 marker. This marker expressed precociously on the cell surface is used to isolate mesenchymal progenitors within a cell population; its expression decreases gradually in culture. Embryonic stem cell markers, such as Oct-4, Nanog, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81, have also been described on the surface of MSCs derived from dental pulp. Some properties of MSCs are particularly promising in therapeutics, but they are only identifiable in vivo and are mainly related to the immune system.

MSCs have immunosuppressive and anti-inflammatory capabilities. The cells can modulate the immune response through their synthesis of anti-inflammatory molecules and

mediators, such as interleukin (IL)-6 and macrophage colony-stimulating factor, secretion of interferon (IFN)γ, tumor necrosis factor (TNF)-α, and control of monocyte maturation [28]. MSCs have a significant role in tissue regeneration. Both transplanted and resident MSCs can contribute to tissue repair by secreting molecules involved in homeostasis, including soluble glycoproteins of the extracellular matrix, cytokines, and growth factors, which are responsible for reducing inflammation and stimulating tissue regeneration [29]. MSCs also have a significant angiogenic ability. This ability is critical for repairing and restoring organ function because oxygen supply to the tissue depends on restoring blood vessels. Some factors produced and secreted by MSCs appear to be primarily responsible for this effect. Molecules that have been identified in their secretome play a significant role in angiogenesis, including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)-2, angiopoietin-1, chemokine (C-C motif) ligand (CCL)2, IL-6, placenta growth factor, and cysteine-rich protein and angiogenic inducer 61 [30,31]. More factors are released by MSCs, including soluble factors rich in immunomodulatory molecules, chemokines, growth factors, and cytokines. The vesicular fraction contains extracellular vesicles (EVs), which are classified primarily by their size [32,33]. Exosomes originate from the endocytic pathway and range in size from 30 to 200 nm on average and are composed of secondary metabolite, nucleic acids, proteins, and lipids. The macrovesicles originate from the cell plasma membrane and, in size from 200 to 1000 nm, contain lipids, proteins, secondary metabolites, and nucleic acids. Apoptotic bodies released by dying cells with an average between 50 and 100 μm in diameter contain nucleic acids, organelles, and proteins. All EVs participate in intercellular communication except for apoptotic bodies, which typically function in phagocytosis [32–34]. The properties of MSCs are maintained due to the interactions between these cells and factors in their environment, including stromal cells, signalling molecules, the extracellular matrix, and adhesion molecules. Once the cells leave these environments, they begin differentiation; however, the molecular and environmental mechanisms that control differentiation are not fully elucidated. Therefore, many studies within the veterinary field are focused on expanding the understanding of these cells [35].

3. The Therapeutic Role of MSCs In Vitro

The ability of MSCs to regenerate injured tissues is closely linked to their anti-inflammatory properties. MSCs act locally through cell–cell interactions based on receptor–ligand bonds or nanotubes that transfer molecules and organelles. However, they intervene mainly at the systemic level through trophic factors secreted directly in the microenvironment or transported by extracellular vesicles [36]. The MSCs can thus promote cell viability, proliferation, and angiogenesis by producing growth factors (VEGF, platelet-derived growth factor, basic fibroblast growth factor [bFGF]) [37]. They also stimulate the recruitment of endogenous stem cells by secreting chemokines, such as CXCL12 or CCL5, and reduce fibrosis by producing keratinocyte growth factor, matrix metalloproteinase (MMP)-9, bFGF, MMP-2, and hepatocyte growth factor (HGF) [38]. In addition, they intervene in the regulation of apoptosis through the production of HGF, bFGF, and insulin-like growth factor 1 (IGF1) and through the regulation of oxidative stress by releasing heme oxygenase-1 or erythropoietin. Finally, they exhibit anti-inflammatory activity by releasing indoleamine 2,3-dioxygenase (IDO), HLAG5, prostaglandin E2 (PGE2), TNFα-stimulated gene-6, IL-6, and IL-1 receptor antagonist, among other molecules. The secretion of all these factors gives these cells an unusual trophic activity [39].

4. Therapeutic Application of MSCs

Stem cell-based regenerative therapy is recognised as a future therapeutic option for treating many diseases in humans and animals. MSCs are good candidates for cell therapies because they are easily isolated from various tissues and have extensive and rapid proliferation [40]. The use of MSCs in regenerative medicine allows considering new therapies to treat different pathologies in cardiology, immunology, neurology, and many other diseases [41]. The development and application of cell therapy may eventually be

used to treat common diseases in the population, such as diabetes and liver cirrhosis [42,43]. Cardiology could also take advantage of the advances possible through stem cell therapy. In ischemic cardiomyopathies, such as angina, acute coronary syndrome, and infarction, MSCs have shown a natural capacity to repair the heart muscle [44,45]. The transplantation of MSCs to the myocardium reduces the lesions caused by ischemia, improves wound healing, restores tissue contractile function, and increases myocardial flow by optimising left ventricular function [46,47].

Cell therapy developments are also expected to occur in neurology, with spinal cord injuries and strokes seeming to benefit from treatment with MSCs [48,49]. The injection of MSCs enhanced endogenous neuroprotection and brain plasticity through paracrine neurotrophic effects: immunomodulation, angiogenesis, synaptogenesis, oligodendrogenesis, and neurogenesis. Moreover, the apoptosis of neural cells decreased. Indeed, due to the antiapoptotic effect of certain factors, such as brain-derived neurotrophic factors, ischemic tissue was repaired, and neural function was restored [50,51]. In other investigations, clinical trials of treatments for Parkinson disease and macular degeneration have been successful owing to the ability of MSCs to increase the level of tyrosine hydroxylase and to promote the production of dopamine [52,53].

Treating neurological diseases using MSCs relies on the cells' neuroprotective capacity after they migrate into damaged brain tissue. Although MSCs benefit rain lesions and tissue through various trophic factors, such as nerve growth factor (NGF), brain-derived neurotrophic glial-derived neurotrophic factor (GDNF), vascular endothelial growth factor (VEGF), and insulin-like growth factor (IGF), they also have immunomodulatory, angiogenic, and antiapoptotic effects [54,55]. In addition, they stimulate endogenous regeneration by activating neural progenitor cells quiescent in brain tissue [56,57]. Other medical fields use MSCs to influence wound healing, angiogenesis, and reepithelialisation [58]. They also seem to regenerate the function of specific specialised tissues, such as sweat glands [59]. In addition, clinical trials on ischemic tissue regeneration in diabetic patients have shown revascularisation and healing of damaged tissue after stem cell treatment [60].

The immunomodulatory properties of MSCs arouse great clinical interest. Their ability to produce trophic, immunomodulatory, and immunosuppressive factors enables their use to treat graft-versus-host and certain autoimmune diseases [61,62]. In addition, the application of MSCs in treating type 1 diabetes results in the arrested destruction of β cells, increasing the differentiation of stem cells into insulin-producing cells and tissue repair by stabilising the inflammatory response [63]. However, using MSCs in cartilage regeneration is still challenging because large amounts of cells need to be injected [64]. Recently, MSCs were used to treat premature ovarian insufficiency. The results were not precise, but the procedure offers a promising treatment option to improve lipid metabolism and restore ovarian function by activating the phosphoinositide 3-kinase pathway, promoting the level of free amino acids, and reducing the concentration of monosaccharides [65]. MSCs-derived secretomes have been used in different clinical trials and shown to produce the same or even enhanced therapeutic effect compared with MSCs [66]. Moreover, MSC-derived secretomes have been shown to display a dual function in tumor promotion and tumor suppression [67].

5. Veterinary Use of Mscs in Companion Animals

5.1. Canine MSCs

5.1.1. Sources and Characteristics

Canine MSCs (cMSCs) were initially obtained from adipose tissue [68]. They have since been isolated from allogenic and autologous sources, including bone marrow muscle and periosteum [69], umbilical cord blood [70], Wharton's jelly [71], umbilical cord tissue [72] amniotic membrane [73], amniotic fluid [74], the limbal epithelium [75], endometrium [76], and the dental pulp [77]. In addition, cMSCs have also been harvested from olfactory epithelium [78], periodontal ligament [79], synovium [80], placenta [81], peripheral blood [82], and ovary [83]. cMSCs obtained from different sources are plastic

adherent with a spindle-shaped morphology. Some studies have shown that the morphology of cMSC varies from more cuboid to very thin with cytoplasmic extension [84]. The cells are positive for CD90, CD105, CD44, and CD73 markers, but they lack the expression of the hematopoietic cell surface markers CD34, CD45, CD146, and CD11b. In addition, some studies have shown the absence of other markers, such as CD14, D11b, CD19, CD29α, CD45, CD34, and HLA-DR [85]. The different sources and genetic differences between various breeds may cause variation in their biological characteristics; dissimilarities in multi-lineage differentiation and proliferation level can define their clinical uses [86].

5.1.2. Canine MSC Therapy

Dogs have been extensively studied in cartilage repair work because, like humans, dogs lack an intrinsic repairability of cartilage and can experience the same cartilage diseases as humans, including osteoarthritis and osteochondritis dissecans [87,88].

Osteoarthritis (OA)

Canine osteoarthritis (OA) is a degenerative disease of joint tissues that leads to the loss of joint cartilage and the release of inflammatory and regulating cytokines, causing pain. The cartilage's ability to heal is inadequate because of its avascular nature. After a lesion, the fibrous tissue is formed with various functional properties of the native hyaline cartilage, promoting joint degeneration [89,90]. The OA pathophysiology is multifactorial, with a robust inflammatory component, and it is frequently secondary to anatomical anomalies or injuries, causing joint instability. It is widespread in large animals but can also affect dogs from all breeds; there is no cure for OA, and the treatment routine focuses on pain reduction and symptom management [89,91]. Conventional treatment is based on diet, long-term nonsteroidal anti-inflammatory drugs, weight management, and dietary supplements. More therapies have been used and studied, such as acupuncture and shockwave therapy. More recently, MSCs have been used as a promising tool for treating different OA cases [91,92]. Studies evaluated the therapy improvement of hip joint OA using the subjective method, including a range of motion scores, pain, and lameness [93,94]. Black et al. [94] conducted a study on 21 dogs with chronic hip joint OA: the dogs were treated with 4.2–5 $\times$ 10^6 intra-articular autologous ASCs for 6 months, and the study results showed a significantly improved score for pain, lameness, and range of motion compared with the control group. Marx et al. [93] evaluated the effect of allogeneic ASCs, and autologous stromal vascular fraction injected into acupuncture in 6 dogs; after 60 days, all 5 dogs showed an improvement in lameness, range of motion, and pain manipulation. More studies carried out by Viral et al. [95,96] used the objective method to analyse the approach using a force platform to demonstrate the effectiveness of a single AI injection of ASCs. The first research revealed how the effect of the combination of ASCs and PRGF was extended over 6 months [95]. In the second study, the same team showed that using ASCs alone improves the dogs' conditions after the first month of the treatment with a reduction in lameness and pain; however, this effect gradually eased between the first and the third month [96]. In the third study, Vilar and his team [97] used the force platform to compare the pain scales for the same animal treated with ASCs six months after therapy. The results showed that using pain assessment scales to measure lameness associated with OA did not reveal high accuracy compared with the quantitative force platform gait approach [97].

Numerous studies have shown notable results using the intra-articular administration of ASCs for canine elbow OA therapy, with improved pain, lameness, amplitude of motion, and functional capacity [93,98,99]. Éva Kriston-Pál et al. [98] used MSCs resuspended in 0.5% hyaluronic acid to treat dogs suffering from elbow dysplasia and OA; the results reported a significant improvement demonstrated by the degree of lameness during the follow-up period of one year. Controlled arthroscopy also showed that cartilage had completely regenerated in one dog. In a more recent study, Olsen et al. [99] used IV injections of allogeneic ASCs (1–2 $\times$ 10^6 cells/kg body weight) to treat 13 dogs with elbow OA 2 weeks apart. No acute adverse effects were observed, and a significant improvement in clinical

signs and the owner's perception was noted. However, synovial fluid OA biomarkers did not change after MSCs administration. Despite subjective outcomes showing good enhancements, such as the dog's clinical signs, objective outcome measures did not confirm similar results, such as reducing the OA biomarkers measurement in synovial fluid. Larger sample sizes and CGs are needed to interpret these findings [99]. According to previous studies, treating OA using MSCs in combination or alone improves the clinical signs, reducing lameness and providing remarkable recovery after the last limited sports activity [100–103].

Osteochondritis

Dissecting osteochondritis is common in large dogs of predisposed breeds before the age of one year. The joints mainly affected are the shoulders, elbows, and ankles (tarsus). The treatment uses arthroscopically guided excision of free cartilage fragments in the joint combined with stem cells and plasma enriched with growth factors [101]. Robert Harman et al. (2016) carried out a study of a treatment for osteoarthritis using MSCs obtained from adipose tissue. In this trial, 43 dogs in the treatment group received a dose of 12×10^6 cryopreserved allogeneic MSCs intra-articularly. The study measured the effects of MSCs on pain during handling and assessed the dogs' abilities to perform daily activities for two months [90]. No severe side effects were associated with the treatment in this study, and there was a notable reduction in pain and improved functional abilities. Intra-articular injection of MSCs has also proved to be a promising technique [102].

Tendonitis and Ligaments Rupture

In dogs, tendonitis is another frequently diagnosed disorder that can cause significant locomotor disorders. In addition to tendonitis, ruptures and lacerations are other common tendon disorders in dogs [103]. These disorders rarely resolve spontaneously and invariably require treatment followed by physiotherapy; therefore, an effective treatment that heals the scar tissue as closely as possible to resemble the healthy tendon properties is needed. Autologous adipose MSCs used in the tendon treatment modulate the tendon's post-repair inflammatory response by increasing prostaglandin reductase1, M2 macrophage, and proteins involved in tendon formation. Moreover, the anti-inflammatory effect of MSCs is thought to cause a decrease in collagen fibre alteration [95,104]. Currently, MSC therapy is an exciting prospect. Studies have demonstrated a histologically significant improvement in tendon healing following treatment with adipose-derived progenitor cells (ADPC) or bone marrow aspirate concentrate (BMAC) and platelet-rich plasma (PRP) combination on partial cranial cruciate ligament rupture CCL. This investigation reviewed 36 medical records of client-owned dogs diagnosed with an early partial tear of the craniomedial band of the CCL treated with BMAC–PRP or ADPC–PRP from 2010 to 2015. The data collected are mainly the results of the diagnostic arthroscopy on days 0 and 90, the physical and orthopaedic examination, the medical history, the x-rays, and the objective analysis of the temporospatial gait [105]. In another study, dogs with unilateral cranial cruciate ligament rupture confirmed by arthroscopy were treated as follows: The first group received an intra-articular injection of allogeneic neonatal MSCs after tibial plateau levelling osteotomy, followed by a placebo for one month. The second group received the same concentration of MSCs after tibial plateau levelling osteotomy, followed by nonsteroidal anti-inflammatory drugs (NSAIDs). After one month, the results showed tendon healing in the group treated with MSCs. The same result was recorded in the other group treated with nonsteroidal anti-inflammatory drugs, and insignificant differences between the two groups in gait evaluation after three months were reported [106].

Other studies have explored the use of MSCs to treat systemic or local inflammatory pathologies and autoimmune diseases in dogs [107]. In addition, the canine model offers certain advantages, such as the possibility of conducting long studies involving physiotherapy or exercise protocols. Finally, dogs are considered model animals for human research [108].

5.2. Feline MSCs

5.2.1. Sources and Characteristics

MSCs have been isolated from different tissues in cats. The initial isolation of MSCs from bone marrow and characterisation of the cells were reported in 2002, followed by isolation from fetal fluid, fat, peripheral blood, amniotic membrane, umbilical cord blood [7], and from different parts of the umbilical cord tissue [109]. As with all MSCs, feline MSCs (fMSCs) have the capacity for self-renewal. They also display a typical fibroblast-like appearance and plastic adherence, express numerous surface markers (CD90, CD44, CD105), and are negative for leukocyte markers (CD4, CD18) and histocompatibility complex (MHC) II [110]. These characteristics can be altered after extended culture. Lee et al. [111] showed that proliferation and the expression of surface markers of adipose-derived fMSCs decreased after multiple passages; for this reason, their use in cell therapy will be more effective during the early passages. fMSCs also have the potential to differentiate into adipogenic, osteogenic, and chondrogenic cells [112].

MSCs can modulate both adaptive and innate immune systems: T lymphocytes are the primary mediators of the adaptive immune response, and fMSCs have the same immunomodulatory gene expression and response to inflammatory cytokines as human MSCs. The secretion of IFN-γ and TNF-α stimulate MSCs, which attracts T lymphocytes by chemotaxis for cell contact; however, MSCs are poor immunogenic cells because they do not express HLA class II molecules HLA-DR or the costimulatory molecules CD40, CD80, and CD86 [113–115]. As a result, they escape recognition by CD4+ T lymphocytes and cause them to become energy sources [115]. As a result, they escape recognition by CD4+ T lymphocytes and cause them to become energy sources [116]. These molecules inhibit the proliferation of T lymphocytes and the activation of T lymphocytes by antigen-presenting cells, and they induce the differentiation and survival of regulatory T lymphocytes. In addition, the expression of IDO enzyme by fMSCs inhibits the proliferation of T lymphocytes by reducing the amount of tryptophan in the surrounding environment, an amino acid essential for cell multiplication [117].

In later passages, FMSCs develop giant foamy multinucleated cells, causing proliferation arrest and syncytial cell formation. These cytopathic effects are caused by infection with the feline foamy virus (FeFV), a very common, asymptomatic retrovirus in cats. The impacts of FeFV infection on fMSC function make their use in therapy impossible [118–124]. However, a recent study conducted by Boaz et al. shows that treating fMSCs infected by FeFV using an antiretroviral drug, tenofovir, in early passages effectively prevents the harmful effects of the infection and supports in vitro expansion [119].

Current and potential clinical applications of mesenchymal stem cell therapy in cats are explored in several diseases. Based on the immunomodulatory properties of feline stem cells, clinical trials show interest in this new therapeutic strategy for treating illnesses such as gingivostomatitis, chronic inflammatory bowel diseases, asthma, and even kidney failure [120].

5.2.2. Feline MSCs and Their Clinical Use

Current and potential clinical applications of MSC therapy in cats have been investigated in several diseases. Based on the immunomodulatory properties of feline stem cells, clinical trials have been conducted for the treatment of disorders such as gingivostomatitis, chronic inflammatory bowel diseases, asthma, and even kidney failure [121].

Feline Asthma

Cats with asthma have a progressive decline in respiratory function linked to structural remodelling of the airways, characterised by subepithelial fibrosis and bronchial smooth muscle hypertrophy. These structural changes result from communication between cells of different bronchial structures, including fibroblasts, epithelial cells, smooth muscle cells, and immune cells present within the bronchial mucosa [122]. Current therapies for asthma are mainly based on steroidal anti-inflammatory drugs, but many side effects appear over

time. MSCs have been used to treat cats with chronic, acute, and allergic asthma. Cats have been treated in different ways; in one study, adipose-derived MSCs intravenous injections at a dose (0.36–2.5 $\times$ 10^7 MSCs/infusion) were administered every two months over one year. Results show that MSCs positively affect airway remodelling at eight months, diminished airway hyperresponsiveness, and decreased airway eosinophilia compared with placebo, but no effect on airway inflammation [123]. In the second study, serial intravenous infusions of allogeneic, adipose-derived MSCs were administered at different doses (2×10^6, 4×10^6, 4.7×10^6, 1×10^7, and 1×10^7); after 133 days, treatment of allergic asthma experimentally induced by allogeneic MSCs resulted in significant improvement in airway hyperresponsiveness, airway inflammation, and airway remodelling [124]. These two studies showed that MSCs positively affected airway remodelling, diminished airway hyperresponsiveness, and decreased airway eosinophilia compared with placebo but had no effect on airway inflammation and improvement [123,124].

Feline Kidney Disease

The effectiveness and safety of MSC administration were investigated in the treatment of cats with chronic kidney disease (CKD), using a single unilateral intrarenal injection of autologous adipose tissue-derived or bone marrow-derived MSC (bmBM MSC or aMSC) via ultrasound guidance. A total of 6 cats were used for this study, including 2 healthy 1.5-year-old cats and 4 cats with CKD whose ages varied between 6 and 13 years. Intrarenal injection resulted in a mild decrease in serum creatinine concentration and a modest improvement in glomerular filtration rate without inducing adverse effects [125–131]. In another investigation, cats with naturally occurring CKD were treated with feline amniotic membrane-derived allogenic MSCs via internal and intravenous injection [127]. Unfortunately, the internal injection of aMSCs was unsuccessful because of stress, sedation, bleeding, and anaesthesia complications. In contrast, data obtained from intravenous administration revealed significant improvement in proteinuria, decreased serum creatinine, and mild improvements in urine-specific gravity [128].

In another research study, the treatment of acute kidney injury (AKI) in an ischemic kidney model in adult research, cats underwent unilateral renal ischemia for 60 min with fibroblasts (five cats), aMSCs (five cats), or bm-MSCs (five cats). Three cats that had undergone ischemia previously were used as a control. The results of the study revealed no AKI influence or smooth muscle actin staining [129]. Thus, despite decreased serum creatinine concentrations, using MSCs in treating CKD did not lead to a clinically meaningful improvement in renal function. Furthermore, none of the tests in cats with CKD have reproduced the positive results obtained in rodents [129].

Feline Chronic Gingivostomatitis

Feline chronic gingivostomatitis (FCGS) results from an inadequate immune response of the cat to different antigenic stimulations. The disorder affects the gums and other parts of the oral cavity, and its treatment is long and complex [130]. Nevertheless, the ability of MSCs to downregulate the activation of T lymphocytes makes their use in the treatment of chronic stomatitis in feline medicine remarkable [131] Boaz Arzi et al. applied allogenic aMSCs in clinical cases of FCGS: FCGS cats refractory to full-mouth tooth extraction were enrolled [132,133]. In each trial, 7 FCGS cats received 2 intravenous injections of 2 $\times$ 10^7 aMSCs 3–4 weeks apart. In the first experiment, the seven cats received autologous aMSCs; in the second experiment, the seven cats received unmatched, allogeneic aMSCs from SPF donor cats. The results demonstrated that cats treated with allogenic and autologous aMSCs recovered from their clinical condition, with the clinical cure being shown by the histopathology resolution of B- and T-cell inflammation. Moreover, neutrophil counts, normalisation of the CD4/CD8 ratio, and numbers of circulating CD8+ T cells were decreased, while serum IL-6 and TNF-α concentrations were temporarily increased [132–138].

In a different experiment, allogenic aMSCs were used to treat cats with chronic clinical enteropathy, and the results showed no side effects, with a significant improvement in clinical signs [132].

Inflammatory Bowel Disease

Feline inflammatory bowel disease (IBD) is a condition in which a cat's gastrointestinal (GI) tract becomes chronically irritated and inflamed; the possible causes can include bacterial or parasitic infection, intolerance, or allergy to a specific protein in the diet [7,134]. To date, there is no single best treatment for IBD, so veterinarians may need to try several combinations of medications or diet to determine the best therapy [135]. For this reason, alternative approaches became necessary; applying MSCs as an alternative treatment for IBD is still a very recent concept in veterinary medicine [120]. Tracy et al. [136] conducted a clinical trial involving seven cats with diarrhea for at least three months. They received two IV injections of 2×10^6 cells/kg from cryopreserved feline ASCs, while four cats with a similar clinical condition received a saline placebo. Clinical signs improved in five out of seven cats treated with stem cells after one to two months, unlike the placebo group, which did not show any progress. With this trial, it is possible to conclude that MSC therapy was well tolerated and potentially effective in treating feline chronic enteropathy. However, these preliminary results require a significant follow-up study.

In recent research performed by Tracy et al. [137] fMSCs were used as a treatment for IBD after the failed diet trial and compared with prednisolone treatment. The endoscopic biopsies confirmed the histopathologic diagnosis of IBD, and the cats were randomly assigned to either the prednisolone or fMSC groups.

In total, 12 cats were treated, 6 cats in each group. The cats that received fMSCs were between 4.5 to 13 years old and included 3 neutered males and 3 spayed females with weights varying between 4 and 5.9 kg; cats received IV injections of 2×10^6 cells/kg of freshly allogenic adipose-derived MSCs separated by 2 weeks. The prednisolone group included spayed females with a mean age of 8.3 years and a mean weight of 3.6 kg. They received a 1–2 mg/kg PO q24h. In each group, one cat failed the treatment at the second-month recheck, and five completed the six months study with no changes in diet and medications [137]. The results showed that freshly allogenic adipose-derived MSCs were safe and easily administrated in the cat with IBD without any side effects; the response to therapy was similar between the group that received MSC infusions and the group that received standard prednisolone therapy. However, a more extensive study is needed to confirm the efficacy and duration of the effect [137].

The studies were carried out on a small number of treated animals, making the published results interesting and promising. More studies would certainly be required to confirm its beneficial influence. Other diseases that could benefit from this new therapeutic strategy in veterinary medicine remain to be investigated.

6. Conclusions

Along with a scientific interest in regenerative medicine, interest in MSCs has grown over time. Many studies have made it possible to characterise these cells and demonstrate their regenerative potential, and it appears that their use in new therapeutic approaches is inevitable. Indeed, two essential properties of MSCs make them critical in regenerative medicine: their ability to proliferate without losing their undifferentiated character and ability to differentiate into specialised cells. Other properties found more recently, such as their abilities to modulate the immune system and to secrete molecules influencing their environment, make them even more attractive. The evolution of our understanding of MSCs and their use will enable the development of new therapeutic strategies, particularly in veterinary regenerative medicine. In addition, MSC therapy is a promising option for treating several diseases.

Nevertheless, many factors remain to be investigated regarding the protocols of use, the most suitable source of stem cells, the optimal route of administration, and the impact

of the donor's status on stem cell function. For that reason, when selecting a donor for cell-based products in veterinary clinical trials, screening them for infectious diseases and other risk factors is crucial to prevent the transmission of disease agents and ensure the safety of the animal subjects involved in the trial. Therefore, besides the quality-controlled cells, it is essential to clearly understand their origin, storage conditions, and product composition. In addition, it is also necessary to demonstrate that cellular function and integrity have been preserved throughout the process and prove that the cells are free of contamination from viruses, bacteria, fungi, mycoplasma, and endotoxins.

Conducting long-term safety evaluations to ensure no adverse effects is highly recommended. If any adverse events occur after stem cell intervention, reporting them and, more importantly, considering the potential risk factors, such as toxicity, tumorigenicity, and immune reactions, is essential. Moreover, there are regulations and guidelines for using stem cell-based products in veterinary practice made by the European Medicine Agency (EMA), the United States Food and Drug Administration (FDA), and the Animal and Plant Quarantine Agency (APQA) of Korea [138–141] to ensure the safety assessment of cell-based products for animal use.

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CHAPTER 5

MESENCHYMAL STEM CELLS: ISOLATION AND IN VITRO CHARACTERISATION FROM DISTINCT PARTS OF THE UMBILICAL CORD.

In this study, we decided to obtain MSCs from feline umbilical cords that are considered surgical waste products if obtained during Caesarean section, or waste biological material when obtained during natural delivery. Thus its use as the source of MSCs would not raise specific ethical questions. That was also supported by the local legislation and institutional requirements Decision No. 004/2021. Moreover, umbilical cords are easy to obtain in non-invasive methods both during surgery and parturition. The feline umbilical cord (UC) is a complex organ. Therefore, we decided to evaluate for the first time in cats whether it is possible to obtain MSCs from the whole cord, as well as from its parts (separately from Wharton's jelly and vessels). Furthermore, we evaluated whether the cells collected from the feline umbilical cord tissues would show MSCs characteristics.

This study aimed to isolate, characterise and compare the mesenchymal stem cells from different anatomical regions of the feline umbilical cord, including vessels, Wharton's jelly and the whole umbilical cord. The aim was to be able to indicate the best part of umbilical cords for MSCs isolation in cats. The detailed results were published in the Theriogenology journal under the title of **Feline umbilical cord mesenchymal stem cells: Isolation and in vitro characterisation from distinct parts of the umbilical cord.** Theriogenology (2023) 201:116–25. doi:10.1016/j.theriogenology.2022.11.049.

As it was mentioned in the Materials and Methods chapter 4, 36 umbilical cords were collected during natural delivery or caesarean section from a healthy queen aged from 1.5 to 5 years old. The two different parts of the umbilical cord were separated (Wharton's jelly and vessels). Each part separately was minced into small pieces and then subjected to digestion using collagenase type 1. The cells were cultured in a stromal medium and characterised using the minimal criteria for defining MSCs, their morphology, self-renewing, differentiation potential, immunophenotyping and gene expression to obtain an alternative source of MSCs.

In this study, mesenchymal stem cells were isolated and successfully cultured from all parts of the feline umbilical cord; the proliferative potential was measured by cumulative population doubling level and doubling time test; we performed chondrogenic, osteogenic, and adipogenic induction under each differentiation condition to confirm the differentiation potential. The

surface markers expression was performed using flow cytometry, then pluripotency gene expression by RT-PCR.

MSCs isolated have been shown to exhibit the MSCs characteristics: a typical spindle shape consistent with MSCs morphology and the ability to differentiate into multiple lineages, including chondrogenic, osteogenic, and adipogenic differentiation and high proliferation capacity. Mesenchymal markers of MSCs (CD44+, CD90+) and pluripotency markers (NANOG, Oct4, SOX2) were expressed in the cells isolated, but no hematopoietic markers (CD34, MCH I). The comparison between the MSCs isolated from the WUC, WJ and UCV revealed that WJ-derived MSCs showed more significant pluripotency gene expressions, the highest proliferation ability, and more tremendous differentiation potential than the mesenchymal cells isolated from WUC and UCV.

In conclusion, it can be stated that in cats, the MSCs can be obtained from all regions of the umbilical cord, but the most efficient tissue is the Wharton jelly, which serves as the foundation for further research concerning the non-invasive collection of mesenchymal stem cells and its further utilisation in feline clinical and regenerative medicine. However, it is crucial to emphasise that cells isolated from the WJ tissue exhibit the best MSCs characteristics and can offer the best clinical utility.

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Feline umbilical cord mesenchymal stem cells: Isolation and in vitro characterization from distinct parts of the umbilical cord

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ABSTRACT

Mesenchymal stromal/stem cells (MSCs) are a particular population of cells that play an essential role in the regeneration potential of the body. As a source of MSCs, the umbilical cord (UC) has significant advantages, such as a no-risk procedure of tissue retrieval after birth and the easiness of MSCs isolation. In the presented study, the cells derived from the feline whole umbilical cord (WUC) and two separate parts of the UC tissue, including Wharton's jelly (WJ) and umbilical cord vessels (UCV), were investigated to check whether they exhibit MSCs characteristics. The cells were isolated and characterized based on their morphology, pluripotency, differentiation potential, and phenotype. In our study MSCs were successfully isolated and cultured from all UC parts; after one week of culture, the cells had a typical spindle shape consistent with MSCs morphology. Cells showed the ability to differentiate into chondrocytes, osteoblasts and adipocytes cells. Two markers typical of MSCs (CD44, CD90) and three pluripotency markers (Oct4, SOX2 and Nanog) were expressed in all cells cultures; but no expression of (CD34, MCH II) was evidenced by flow cytometry and RT-PCR. In addition, WJ-MSCs showed the highest ability of proliferation, more significant pluripotency gene expressions, and greater differentiation potential than the cells isolated from WUC and UCV. Finally, we conclude in this study that cat MSCs derived from all the parts are valuable cells that can be efficiently used in various fields of feline regenerative medicine, but cells from WJ can offer the best clinical utility.

1. Introduction

In recent years, the possibility of creating tissues and organs by tissue engineering for research and therapy has generated increased interest in cells suitable for such use. However, working with young cells that have undergone a relatively small number of divisions and can renew themselves in the long term is challenging [1]. Therefore, the umbilical cord (UC) has become a popular target of scientific research [2,3].

UC is yellowish-white in color tissue, has a gelatinous appearance, and is slightly twisted; in mammals, it forms a connection between the fetus and the placenta [4]. This structure is responsible for the exchange of nutrients and oxygen during gestation. The umbilical cord's macroscopic structure contains the vein and arteries surrounding the connective tissue [5].UC is of interest to researchers because it can be easily non-invasive obtained during parturition, and because it is considered a clinical waste, it has no ethical controversy [6]. In addition, it is a source of several types of cells. At least five cell types have already been described in the UC tissue and blood, including epithelial cells, mesenchymal stem/stromal cells (MSCs) [7], smooth muscle cells, endothelial cells [8] and progenitor blood cells [9]. UC-MSCs were isolated from veins,

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extra gelatinous tissue around the vessels, and Wharton's jelly [10–12]; The umbilical cord-originated cell lines present many advantages compared to MSCs from other adult tissues, as they commonly exhibit higher proliferation abilities. In addition, their genetic and phenotypic stability is maintained even after a long-term in vitro culture, suggesting they are primitive compared to adult tissue-derived MSCs [13].

The UC-MSCs morphology, proliferation, immunophenotyping, and multi-directional differentiation abilities are similar to bone marrow (BM) derived MSCs [14]. Therefore, they are an ideal alternative source for BM-MSCs. Furthermore, UC-MSCs are proposed as an essential option in immunotherapy and regenerative medicine due to their anticancer properties and capacity to secrete cytokines and growth factors [15].

In feline species, MSCs were isolated from amniotic membrane, adipose tissue, bone marrow, amniotic fluid, and fat; up to date, the adipose-derived MSCs were the most commonly used in therapy for some feline diseases [16]. Mainly kidney diseases [17,18], asthma [19], gingivostomatitis [20,21] and chronic enteropathy [22].

Numerous published works documented the UC-MSCs isolation, characterization, and differentiation potential, including humans and animals like horses, dogs, rats, pigs, and sheep [23]. On the other hand, there is a lack of studies carried out in feline species about the MSCs isolation from distinct parts of the umbilical cord. Therefore, the present study aimed to isolate and culture MSCs derived from cats, separately from two parts of the feline umbilical cord (fUC): Wharton's jelly (WJ), umbilical cord vessels (UCV), and the whole umbilical cord (WUC). Moreover, we aimed to characterize the MSCs derived from different compartments of fUC for the first time by their morphology, plastic adherence, expansion capacity, differentiation ability and phenotype to find out which part of the cord is the most suitable for MSCs isolation in this species basis on their basic characteristics.

2. Material and methods

2.1. Obtaining umbilical cord tissue

The umbilical cords (n = 36) were collected from healthy queens, aged from 1,5 to 5 years, of different breeds during caesarean sections or natural delivery. The queens were patients of the Department of Reproduction and Clinic of Large Animals in Wroclaw. The umbilical cords were collected in an aseptic manner immediately after dissection, rinsed with cold buffered saline phosphate (PBS) (Sigma-Aldrich. Poland), and placed in falcons tubes containing PBS and 1% penicillin-streptomycin solution (Thermo Fisher Scientific), then stored at 4 °C until further procedures.

2.2. Mesenchymal stem cells isolation and culture

To isolate the MSCs, the umbilical cords were washed twice with cold PBS to remove blood clots in a sterile Petri dish (NunclonTM Delta Surface, Thermo Fisher Scientific, Denmark). Then, using sterile surgical forceps and a #10 scalpel blade, the two anatomical regions of each cord were identified and separated: Wharton's jelly and vessels (vein and arteries). Next, cell isolation was performed separately for the two parts of the umbilical cord: Wharton's jelly (WJ), umbilical cord vessels (UCV), and the whole umbilical cord (WUC). Using the protocol described by Qingqiu et al. [24]with some modifications: as described below, the collected WJ, UCV and the entire cord were placed in an individual sterile culture dish. The tissue was minced into a 2 mm square using a bistoury blade and then transferred to 15 ml centrifuge tubes containing 0.02% of collagenase type 1 (Merck KGaA, Darmstadt, Germany) in Dulbecco's Modified Eagle's Medium-low glucose (LG-DMEM) (Merck KGaA, Darmstadt, Germany). Samples were incubated at 37 °C for 20 min for tissue digestion. Afterwards, the samples were centrifuged at 300×g for 5 min and washed in PBS. Next, the stromal vascular fraction was resuspended in a stromal medium: LG-DMEM with 10% fetal bovine serum (FBS) (Merck KGaA, Darmstadt, Germany) and 1% antibiotic solution, and cultured in T-25 flasks (Techno Plastic Products AG, Switzerland). Half of the medium was refreshed after 24 h, changed every 3 days and passed when the cells reached 80% confluence.

2.3. Cell doublings and doubling time

Primary cells from WJ, UCV, and WUC at passage 1 were seeded in triplicate at a density of 5×10^3 cells/cm2 in 12 well plates (Techno Plastic Products AG, Switzerland) as previously described by Zhang et al. [25] using the above described stromal culture medium. Cell numbers were assessed after 2, 4 and 6 days of culture using a trypan blue and hemocytometer. Cells doubling numbers (CD) and doubling time (DT) were calculated according to the following formulas:

$$CD = \ln(Nf/Ni)/\ln \quad (2)$$

$$D\,T = CT/CD$$

Where CT is culture time, Nf is the final cell number, and Ni is the initial cell number.

Data from all days for each sample were combined within passages to calculate the mean and standard error.

RNA extraction and real-time reverse transcription PCR (qRT-PCR).

MSCs from WUC, WJ, and UCV were seeded in 6-well plates at a density of 1×10^6 cells per well in a stromal medium until reaching (80–90%) confluence. Total RNA was extracted from cells using TRI Reagent according to the manufacturers instructions. RNA purity and concentration were measured using a nano spectrophotometer (denovix ds-11). cDNA was prepared from total isolated RNA using a Tetro cDNA Synthesis Kit (Bioline, London, UK).To determine the expression levels of MSCs pluripotency markers (OCT-4, SOX2, NANOG), real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed using the SensiFAST SYBR Green Kit (Bioline, London, UK) in a CFX Connect™ Real-Time PCR Detection System (Bio-Rad). For the 10 μl reaction volume, the following cycling

Table 1
Sequences of primers used in the gene expression profiling of WJ-MSCs, UCV-MSCs and WUC-MSCs by using RT-PCR.

Gene	Primer	Sequence 5′–3.'	Annealing temperature (°C)
SOX2	F:	CCGAGTGGAAACTTTTGTC	65.4
	R:	AAAATCTGCAGGAGATATGC	
OCT-4	F:	AAAATCTGCAGGAGATATGC	54.60
	R:	ACTCGGTTCTCGATACTTG	
NANOG	F:	GTGACAACTTCACAAAATCG	54.45
	R:	TCCAGTTTCTCTTCTAGTTCC	
GAPDH	F:	GATGCCCCAATGTTTGTGA	55.60
	R:	AAGCAGGGATGATGTTCTGG	

conditions were applied: 95 °C for 2 min, followed by 40 cycles for 15s at 95 °C, annealing for 15 s, and elongation at 72 °C for 15 s. Sequences for all used primers are listed in Table 1. All results were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. The relative level of the expression was calculated using the $2^{-\Delta CQ}$ method.

2.4. Immunophenotyping

Flow cytometry was used to assess the MSC immunophenotyping of UC cells, using the standard minimal criteria for MSC described by the position paper of the International Society for Cellular Therapy (ISCT) [26]. Cells at Passage 2 were detached using trypsin EDTA and then centrifuged. The pellet was resuspended in PBS with 1% FBS and counted. One million cells of each population were used for flow cytometry. Cells were conjugated with antibodies against CD90, CD44, CHMII, and CD 34 in dark at 4 °C for 30 min. Following the incubation with antibodies, cells were rinsed with PBS. For CD44, CHMII detection cells were additionally incubated with a secondary antibody. An appropriate isotype-matched control antibody was used. Samples were analyzed using FACSCalibur, equipped with a 488-nm laser. Data were recorded for at least 5000 events using CellQuest version 3.3 software. The percentage of positive cells in each sample and the expression level of selected antigens for each antigen were evaluated. Data were presented as histograms using WINMDI 2.8 software. Antibodies information are shown in Table 2.

2.5. Tri-lineage differentiation assay

Osteogenic, adipogenic, and chondrogenic differentiation ability was confirmed in cells isolated from UCV, WJ, and WUC. All experiments were performed on cell samples at passage number 3. Every experiment consisted of a control group cultured in a stromal medium and a treated group cultured in a stromal medium to reach 80% confluence and then maintained in a specific induction medium. The results were visualized under an Olympus IX73 inverted microscope (Olympus Polska sp.z.o.).

2.6. Osteogenic differentiation

To determine the osteogenic differentiation capacity of WJ-

Table 2
Antibody information.

Antibody	Isotype	Label	Crosse/reactivity	Host	Manufacturer	Catalog #	Antibody dilution
MHC Class II	IgG2b	Purified	Cat	Mouse	Bio-Rad	MCA2723	4:100
CD90	IgG1,k	PE	Human	Mouse	BD Biosciences	555596	1:10
CD34	IgG1,k	FITC	Human	Mouse	Bio-Rad	555821	1:10
CD44	IgG1	Purified	Human	Mouse	Bio-Rad	MCA1719GA	4:100
Control	IgG1	PE	/	Mouse	Antibodies	ABIN376413	1:10
Control	IgG1	FITC	/	Mouse	Invitrogen	GM4992	4:100
Secondary Antibody	IgG (H + L)	FITC	Mouse	Goat	Invitrogen	A16079	1:1000

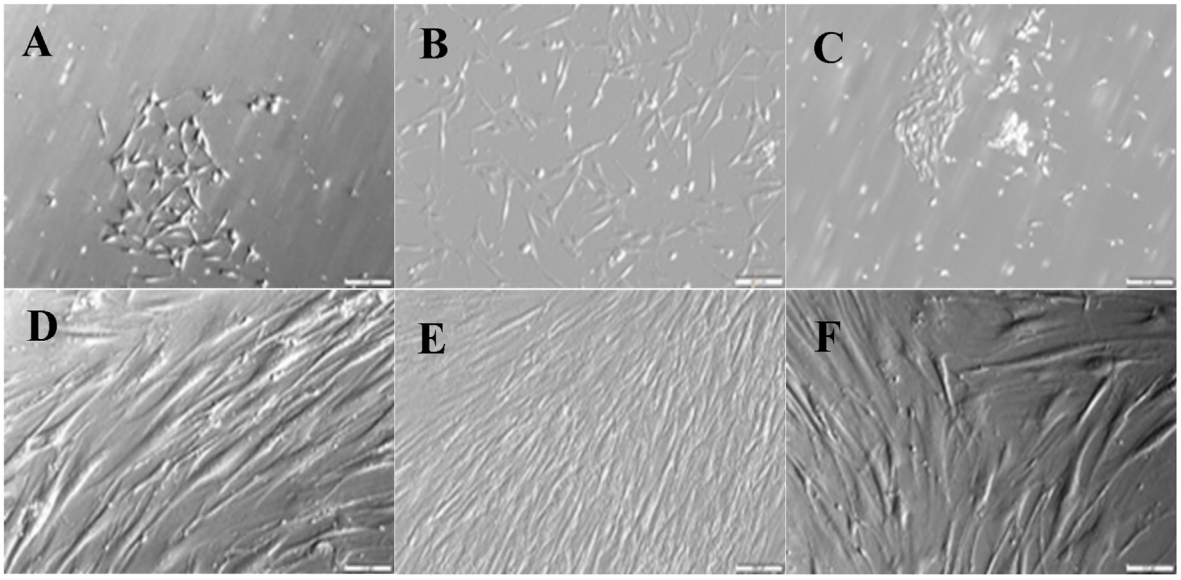

Fig. 1. Representative morphology of feline umbilical cord mesenchymal stem cells from Wharton's jelly (WJ) (A, D), umbilical cord vessels (UCV) (B, E), and the whole umbilical cord (WUC) (C, F) after 3 (A, B, C) and 10 (D, E, F) days of culture Bar = 100 um.

MSCs, UCV-MSCs, and WUC-MSCs cells at density 1×10^5 per well were cultured in the osteogenic induction medium (Mesenchymal Stem Cell Growth Medium. Sigma-Aldrich. Poland) for about 3 weeks; the medium was changed every 3 days. To confirm the calcium accumulation, the cells were washed two times with PBS and then fixed with 4% formaldehyde (PFA) (Sigma-Aldrich. Poland) for 30 min and next, stained with 2% Alizarin Red S (Sigma-Aldrich. Poland), incubated in the dark at room temperature for 15 min.

2.7. Adipogenic differentiation

For the adipogenesis, the WJ-MSCs, UCV-MSCs, and WUC-MSCs were cultured in an induction medium (Mesenchymal Stem Cell Adipogenic Differentiation Medium. Sigma-Aldrich. Poland) and incubated for 3 weeks; the medium was changed every 3 days. The cells were examined by Oil Red O staining (Sigma-Aldrich. Poland) to detect lipid droplets accumulation. First, cells were washed twice with PBS, then fixed with 4% PFA and incubated for 45 min at room temperature, followed by three rinses with PBS. Finally, the cells were incubated for 5 min in 60% 2-propanol (Firma Chempur. Poland) before staining in Oil Red O for 5 min at room temperature.

2.8. Chondrogenic differentiation

WJ-MSCs, UCV-MSCs, and WUC-MSCs were also cultured in an induction chondrogenic medium (Mesenchymal Stem Cell Chondrogenic Differentiation Medium. Sigma-Aldrich. Poland) for 21 days. After the differentiation steps were completed, cartilage formation was confirmed using Alcian blue staining. First, cells were washed gently with PBS to fix the cartilage. Then, the cells were incubated in 4% PFA for 40 min at room temperature, followed by two rinses with distilled water. The cells were then stained with Alcian Blue staining solution (Merck KGaA, Darmstadt, Germany) and incubated in the dark for 50 min at room temperature.

3. Results

3.1. Umbilical cord tissue isolation

A total of 36 UC were collected in this study. The average umbilical cords taken per birth were 3, with a length of 2.5 cm per umbilical cord; using the enzymatic method, we successfully isolated MSCs from WUC, WJ, and UCV and evaluated the morphology of available MSCs.

3.2. Cell isolation and culture

Primary cells isolated from the WJ, UCV, and WUC exhibited a rhomboid shape after 3 days of culture (Fig. 1 A, B, C). After 10 days of culture, cells from distinct parts formed a monolayer and

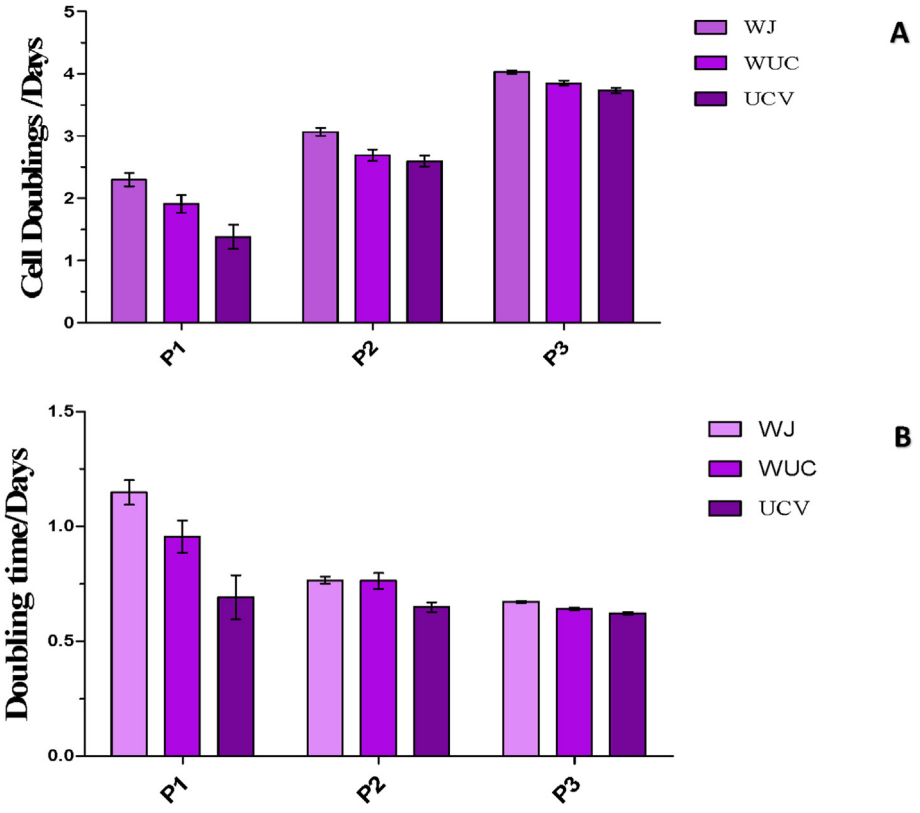

Fig. 2. Cell doubling (A) and doubling times (B) (mean ± SEM) for P1–P3 mesenchymal stem cells from (WJ) Wharton's jelly,(WUC) whole umbilical cord, and (UCV) umbilical cord vessels.

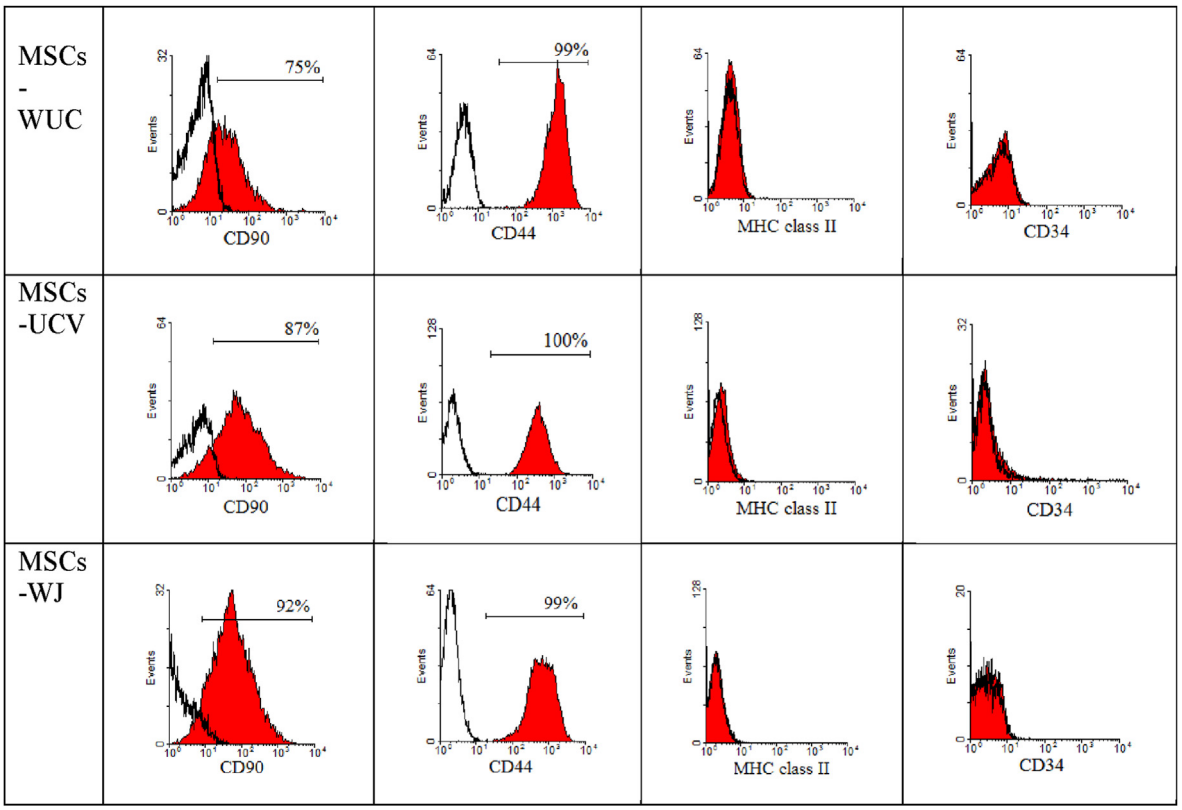

Fig. 3. Representative flow cytometry results showing the expression of CD90 and CD44 as well as the lack of expression of CD34, MHC class II on the MSC derived from the whole umbilical cord (WUC), Wharton's jelly (WJ) and umbilical cord vessels (UCV).

had a typical spindle shape compatible with the mesenchymal stem cell morphology (Fig. 1 D, E, F). However, the cells isolated from the WJ and UCV are larger than those isolated from the WUC.

3.3. Cell doublings and doubling time (passages P1–P3)

The expansion rate of cells isolated in vitro tended to increase with passage (Fig. 2-A). Tissue from the deferent part of the umbilical cord and the whole cord digest contained a high number of adherent MSC-like cells that proliferated rapidly. Cells isolated from WJ tissue proliferated faster than cells isolated from WUC and UCV (Fig. 2-B). Cell doubling values (CD) for P1, P2, and P3 for cells from WJ were 2.29 ± 0.10, 3.06 ± 0.06, 4.03 ± 0.03, from the WUC were 1.91 ± 0.14, 2.69 ± 0.09, 3.85 ± 0.04 and from UCV were 1.38 ± 0.19, 2.59 ± 0.08, 3.73 ± 0.03 (Fig. 2-A). Doubling time for P1,P2 and P3 cells from WJ were 1.14 ± 0.05, 0.76 ± 0.01, 0.67 ± 0.00 days/CD, from the WUC were 0.95 ± 0.07, 0.76 ± 0.04, 0.46 ± 0.01days/CD and from UCV were 0.69 ± 0.09, 0.64 ± 0.02, 0.62 ± 0.01 days/CD (Fig. 2-B).

3.4. Immunophenotyping (P3)

The MSCs derived from two parts of the umbilical cord (WJ, UCV) and the whole umbilical cord (WUC) were strongly positive for CD90 and CD44 and were negative for MHC class II and CD 34 (Fig. 3).

3.5. Pluripotency markers expression P(2)

Cells from the three experimental groups were expressed the pluripotency markers SOX2, OCT-4 and Nanog (Fig. 4). Cells from WJ, WUC and UCV showed a comparable level of mRNA expression. The highest expression level of all the genes tested was shown by WJ-MSCs at passage 2.

Isolated cells showed the capacity to differentiate toward more than one type of cell line (Figs. 5, 6 and 7); at Passage 3, MSC from WJ, WUC, and UCV displayed adipocytic (Fig. 5B–D-F), osteoblastic (Fig. 6B–D-F), and chondrogenic (Fig. 7B–D-F) differentiation ability, based on histochemical staining following culture in induction medium. Cells cultured in the basal stromal medium for the same period showed no differentiation (control group).

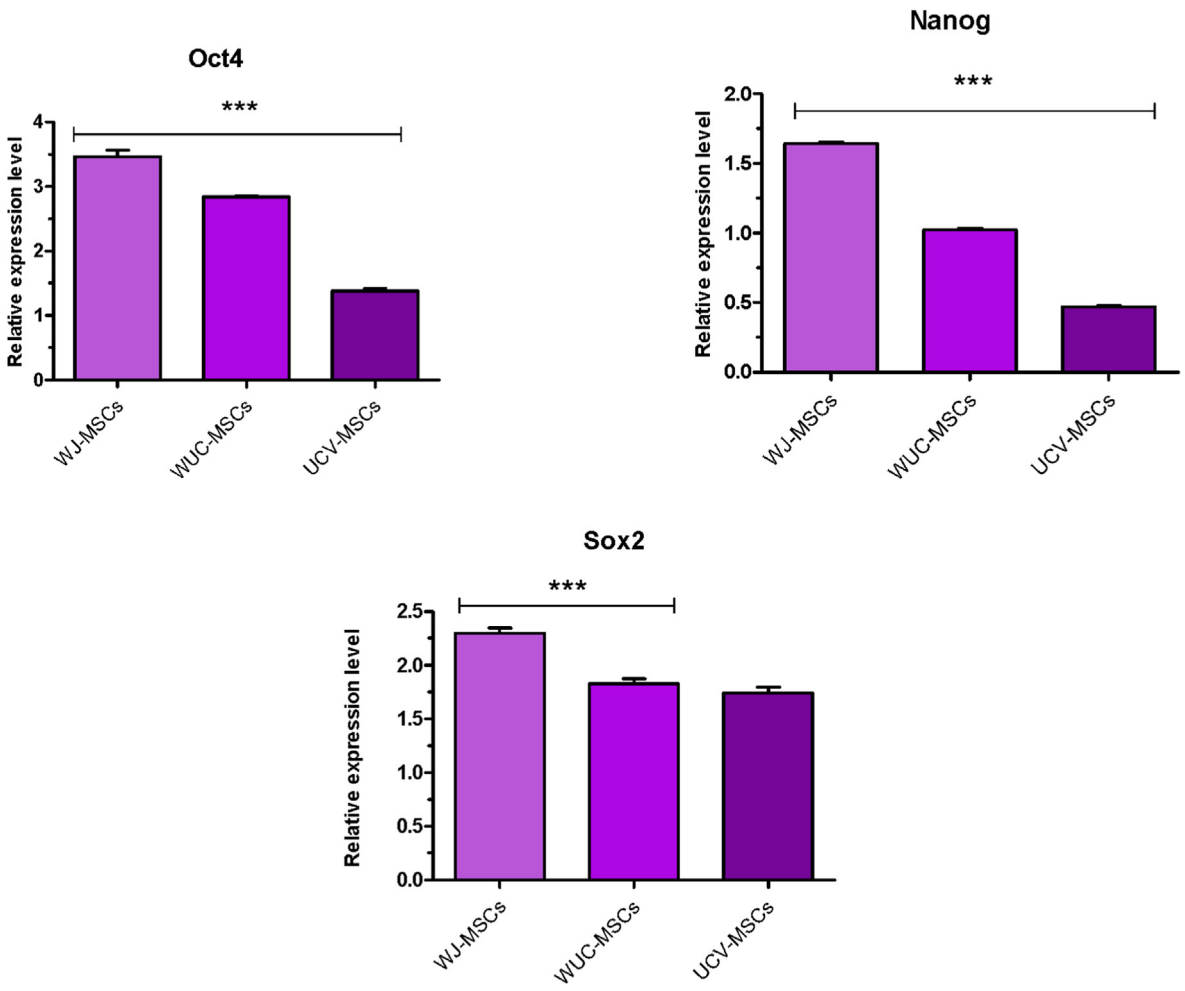

Fig. 4. Gene expression by a reverse transcription-polymerase chain reaction of MSCs-WUC, MSCs-WJ and MSCs-UCV. All the cells show the expression of the embryonic stem cell transcriptional factors such as OCT-4, SOX2 and NANOG. GAPDH was used as a reference gene. The results are expressed as the mean of 3 different experiments ± SD. Asterisk (*) refers to a comparison between the three groups of cells.***$p < 0.001$.

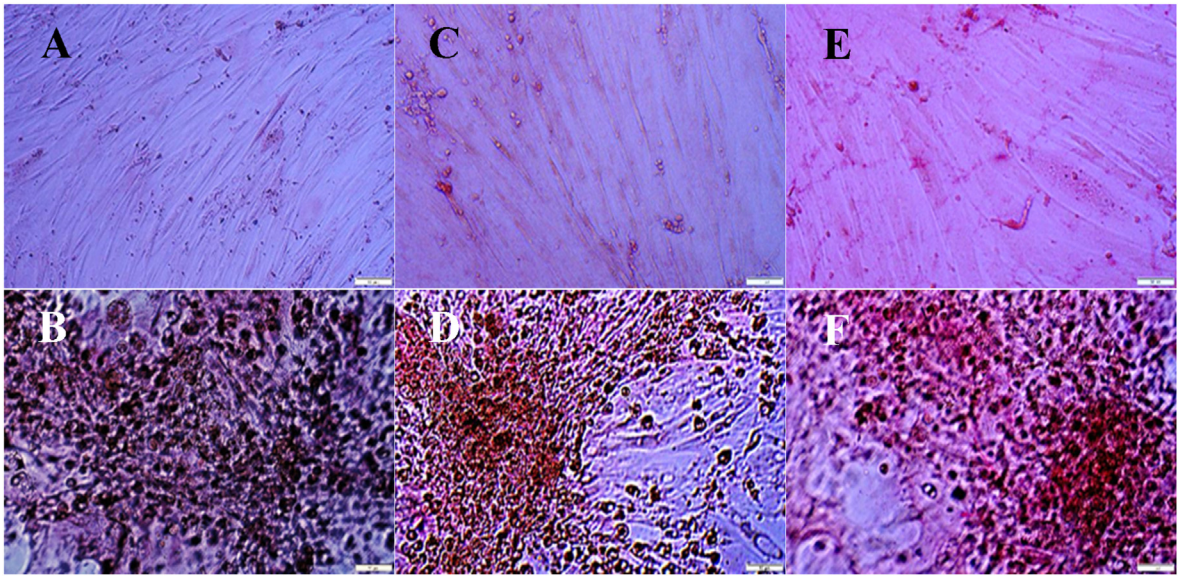

Fig. 5. Photographs of adipogenic differentiation and Oil Red O staining of WJ-derived MSCs (A, B), UCV-derived MSCs (C, D) and WUC-derived MSCs (E, F) at Passage 3. The MSCs were maintained in a basal stromal medium (A, C, E controls) and in an adipogenic induction medium (A, C, E) for 21 days. Staining neutral lipids by Oil Red O revealed no accumulation of lipid drops in control cultures (A, C, E), whereas differentiated cells (B, D, F) demonstrated lipid vacuole formation. Magnification 10 × , Bar = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

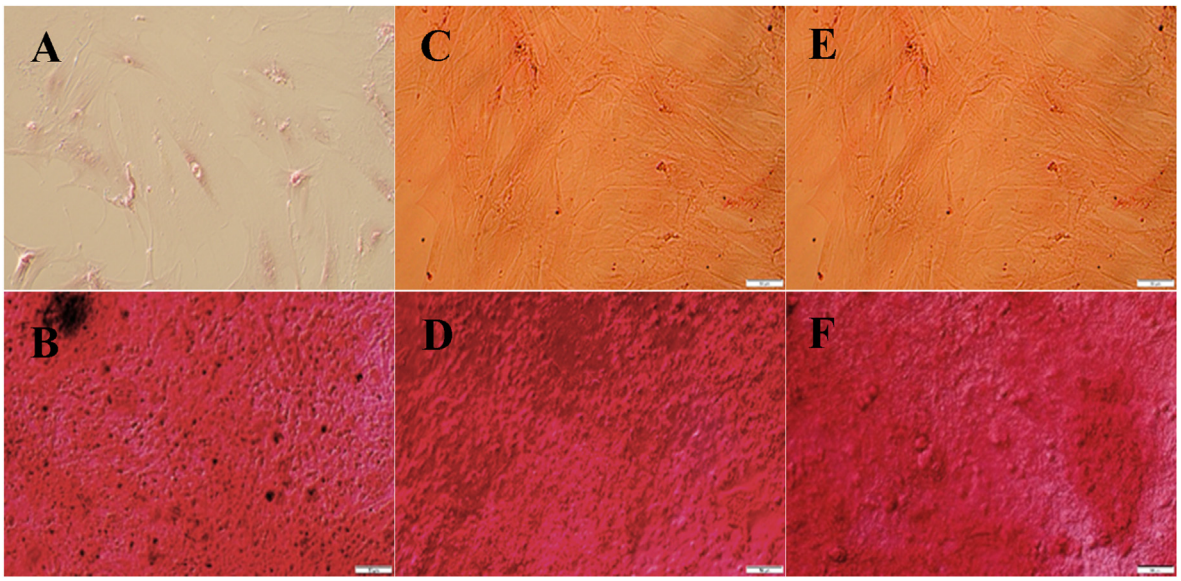

Fig. 6. Photographs of osteogenic differentiation and Alizarin Red staining of WJ-derived MSCs (A, B), UCB-derived MSCs (C, D) and WUC-derived MSCs (E, F). MSCs were maintained in a basal stromal medium (A, C, E controls) and in an osteogenic induction medium (B, D, F) for 21 days. The control culture (A, C, E) showed a lack of significant accumulation of calcium after Alizarin Red S staining. In contrast, the differentiated culture (B, D, F) stained deep red in areas of mineral deposition. Magnification 10 ×, Bar = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

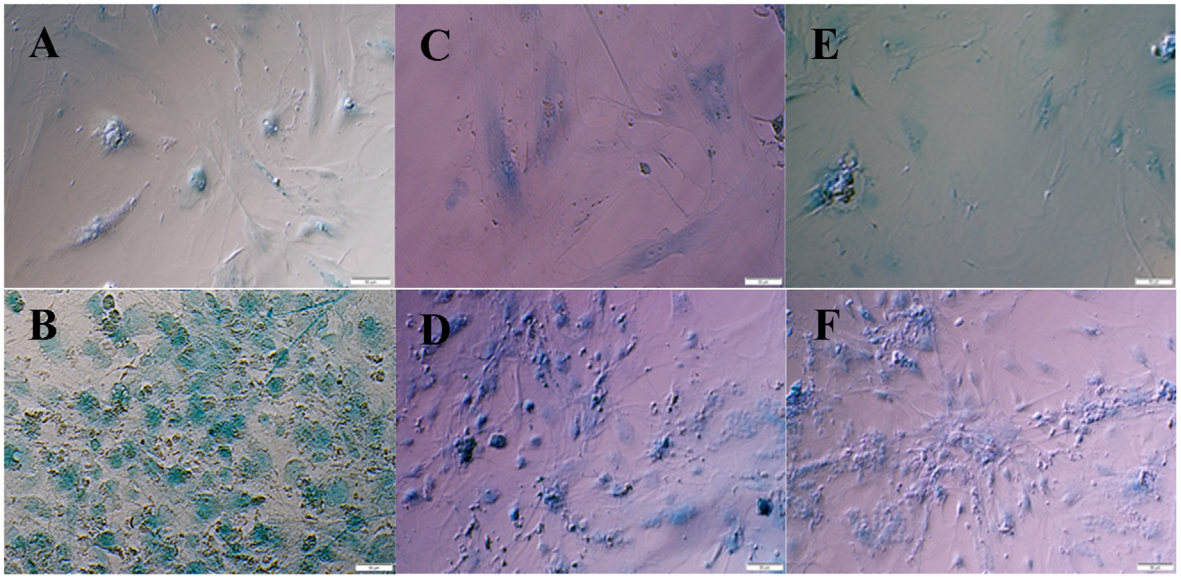

Fig. 7. Photographs of chondrogenic differentiation and Alcian Blue staining of WJ-derived MSCs (A, B), UCV-derived MSCs (C, D) and WUC-derived MSCs (E, F). MSCs were maintained in a basal stromal medium (A, C, E controls) and in an osteogenic induction medium (B, D, F) for 21 days. The control culture (A, C, E) showed a lack of significant accumulation of cartilage. The intense blue color indicates the cartilage formation in the differentiated culture (B, D, F) stained with Alcian Blue. Magnification 10 ×, Bar = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

Clinical trials using cellular therapies more and more often use small animals with naturally occurring diseases as a model for research to support new investigational human and veterinary drug applications [27,28] Cats are a valuable translational model of diseases similar to humans, including asthma [19], chronic enteropathy [22], chronic renal failure [29], and chronic oral mucosa inflammation [30].

Being able to proliferate and differentiate into several cell lines, MSCs represent a promising option for treating some of the diseases cited before in cats. Therefore, searching for new and reliable sources of MSCs is very important in the development of novel therapies. In the presented study, we aimed to evaluate for the first time the suitability of the feline umbilical cord compartments to isolate and culture MSCs. In addition, MSCs from the whole umbilical cord, Wharton's Jelly, and umbilical cord vessels were characterized using morphological characteristics, self-renewal, tri-lineage differentiation abilities, specific surface markers, and gene expression.

In this study, we isolated and cultured cells from the feline whole umbilical cord and separately from Wharton's jelly and

umbilical cord vessels. All the cells isolated were adherent to the plastic and showed fibroblast and spindle-like morphology as reported in studies performed on the feline MSCs, isolated from bone marrow-derived MSCs [31], adipose tissue-derived MSCs [32], fetal fluid-derived MSCs [33] and fetal membrane-derived MSCs [34].

Several parameters can affect cell yield during the isolation of the cells, including the temperature, time of incubation and type of the enzyme [35]. Umbilical cord tissue is considered as a source of young adult cells [36]. To minimize the negative impact of the isolation on the quality and the quantity of our MSCs, it was decided to process the isolation of the cells in our study using a shorter time of incubation and a lower concentration of the enzyme (collagenase type I at 0.02% for 20 min at 37 °C) comparing to other reports. Whereas the enzymatic digestion methods used by the other teams, utilized longer incubation time (30 min incubation) and higher concentration of collagenase (0,1%) [37], or the same 0.1% of the collagenase type I but much more prolonged incubation (3 h) [38]. The cell yield is very heterogeneous from one published protocol to another. Therefore, we decided to adjust our procedure, as our preliminary studies proved better results with shorter times and concentrations. In our study, the in vitro expansion rates of the Wharton's jelly-derived MSCs were slightly higher than those observed in the umbilical cord vessels-derived MSCs and the whole umbilical cord-derived MSCs, which is comparable to the results reported by Ekaterina et al. [39] who described the human MSCs derived from distinct parts of the umbilical cord: perivascular space, Wharton's Jelly and the umbilical membrane, and showed that only the MSCs isolated from Wharton's jelly were characterized by a high potential for proliferation and differentiation, and the populations obtained had higher purity than in the other groups.

Researchers have already described the presence of human MSCs in the umbilical cord compartments, including cord lining, Wharton's jelly, veins, and the perivascular region [40,41]. For example, in the study conducted by Subramanian et al., human MSCs were isolated from the whole umbilical cord, subamnion, amnion, perivascular and Wharton's jelly [24]. The authors reported that MSCs were cultured from all cord regions, demonstrated by their plastic adherence and ability to differentiate along chondrogenic, adipogenic, and osteogenic lineages. Moreover, MSCs derived from Wharton's jelly showed better differentiation and higher proliferation than MSCs isolated from individual cord regions. These data agree with our findings that morphology and adherence properties were not different within all derived MSCs populations, isolated from different compartments of cats' umbilical cord.

Furthermore, in our study, the trilineage differentiation: adipocytes, osteoblasts and chondrocytes was expressed in all MSCs isolated from different parts of the umbilical cord by showing positive staining for cells induced. Wharton's jelly MSCs showed the best differentiation to all lineage compared to MSCs derived from the whole umbilical cord and the umbilical cord vessels. Several teams have reported that Wharton's jelly MSCs could also differentiate very efficiently toward endothelial cells [42], pancreatic-like cells [43], hepatocytes [44] and skeletal muscle [45].

In animals, there are no minimal criteria for defining MSCs based on the surface antigens, as it is reported in human MSCs [46]. To date studies concerning MSCs in cats, mainly described adipose-derived MSCs, and noted that cells were positive for CD90, CD44 and negative for CD34. While the expression of CD73 and 105 depended on the origin of the tissue or were not expressed [31,32,47] Considering the above, in our experiments, we decided to test the following markers: CD44, CD90, CD105, CD34 and MHC II. It was decided not to include the CD73 as it was shown before that it is not expressed in feline cells [33]. Furthermore, we chose not to include the CD105 in the final results as the anti-feline CD105 antisera was not commercially available. On the other hand, we tested antibodies against CD105 that were reported to be used in cat MSCs [48] but we could not obtain positive results. For that reason, in this study, MSCs isolated from the whole umbilical cord, Wharton's jelly and umbilical cord vessels were investigated using flow cytometry; all the examined samples showed a positive expression for CD90 and CD44 and lacked the expression for major histocompatibility class II (MHC II) and hematopoietic marker CD34. Our results showed the accordance with other reports on feline MSCs isolated from different tissue [32,49].

In addition, the results obtained in our study demonstrated the presence of embryonic stem cells *SOX2*, Nanog and OCT-4 in feline WUC-MSCs, WJ-MSCs and V-MSCs at the mRNA level with significant differences among the investigated groups. Comparing the pluripotency expression from the different compartments of the UC, the genes tested were highly expressed in Wharton's jelly-derived MSCs compared to other parts of the umbilical cord. Unfortunately, previously published studies showed inconsistent results in the stem cell markers expression in MSCs. For example, Greco et al. and other teams have confirmed NANOG, OCT-4 and SOX2 stem cell markers expression in MSCs derived from human bone marrow, heart, adipose tissue, liver, dermis and Wharton's jelly [50–52]. Whereas, Pierantozzi et al. [53] have not detected SOX2 and OCT4 in the human heart, adipose tissue and bone marrow MSCs. *OCT-4* gene knockdown promotes differentiation, thereby that transcriptional factors play an essential role in stem cell self-renewal, while SOX2 is a transcription factor co-expressed with OCT-4. Park et al. [54]. and Han et al. [55] showed that Sox2 and OCT-4 are necessary for the enhanced proliferation of human MSCs. Therefore, the high expression of SOX2 and OCT-4 in Wharton's jelly-derived MSCs confirmed in our cells could be one of the reasons for the high proliferation activity in Wharton's jelly-derived MSCs.

In conclusion, MSCs were successfully isolated from the whole feline umbilical cord and the two separate anatomical regions of the umbilical cord: Wharton's Jelly and vessels. All the isolated cells displayed the MSCs characteristics: the ability to self-renew, stem cell markers expression, the capacity of cell doubling and trilineage differentiation potential. Wharton's jelly-derived MSCs showed higher expansion, more remarkable differentiation ability, and best pluripotency markers expression. Taken together, our data suggest that Wharton's jelly provides the best source for MSCs in cats umbilical cords. However, if necessary, the whole feline umbilical cord and umbilical cord vessels may also be considered a good source of MSCs.

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Conflicts of interest

The authors declare that there no conflict of interest.

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CHAPTER 6

FELINE WHARTON'S JELLY-DERIVED MESENCHYMAL STEM CELLS AS A FEEDER LAYER FOR OOCYTES AND EMBRYOS CULTURE *IN VITRO*

In the second part of the study, we used the mesenchymal stem cells obtained from feline umbilical cords using the materials and methods developed in the first stage of the experiments. In this part, we focused on utilising mesenchymal stem cells from feline Wharton's jelly, as they demonstrated highly favourable characteristics. In this part of the study, we would like to check its biological competence to support (and possibly enhance) the feline oocyte maturation and embryo development in vitro. The whole manuscript summarises that part of the research was published in Frontiers in Veterinary Sciences journal, with the title **Feline Wharton's Jelly-derived mesenchymal stem cells as a feeder layer for oocytes and embryo culture *in vitro.*** Frontiers in Veterinary Science Journal (2023), 10:1252484. doi: 10.3389/fvets.2023.1252484**.**

The study involved collecting feline oocytes, which were utilised in three distinct experiments described in the Materials and Methods chapter 4. That part of the study was divided into 3 experiments, as follows:

Experiment 1: *In vitro,* oocytes maturation were co-cultured in the presence of two different maturation mediums, bovine (BoM) and equine medium (EqM), with or without MSCs coculture in the maturation medium, then the nuclear maturation and the cumulus cell expansion were assessed.

The experiment 1 results showed that co-culturing oocytes with MSCs in the presence of a bovine or equine medium did not affect the maturation rate. However, the co-culture system did lead to a higher cumulus cell expansion rate than the group without MSCs coculture.

Experiment 2: *in vitro,* oocytes maturation were co-cultured in the presence of two different maturation mediums, bovine (BoM) and equine medium (EqM), with or without MSCs coculture. After fertilisation, the embryos' development was performed in BoM and EqM without MSCs.

The results from experiment 2 showed there was a noticeable increase in cleavage, morula, and blastocyst development percentages when using MSC co-culture conditions compared to commercial media used alone. The statistical significance difference was reported to be $p < 0.05$.

Experiment 3: The oocytes were matured in BoM and EqM medium, and then the embryo culture was performed in the bovine or equine medium and with or without MSCs coculture.

In the results obtained, we observed a notable rise in morula and blastocyst rate in the embryos that were co-cultured with MSCs, with a P value ($P < 0.05$). Furthermore, the pure equine media supported better the embryonic development and blastocyst rate than the pure bovine medium.

To conclude, our findings have shown that the presence of MSCs during oocyte maturation did not affect the nuclear maturation of the oocytes, but can significantly enhance further embryo development. Additionally, adding MSCs during embryo culture increased the number of morula and blastocysts, indicating their potential to support embryo growth. Further research is necessary to fully understand the benefits of MSCs in oocyte maturation and embryo development in cats and optimise their use in this important study area.

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Feline Wharton's jelly-derived mesenchymal stem cells as a feeder layer for oocytes maturation and embryos culture *in vitro*

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Introduction: Due to their capacity to release growth factors and cytokines, co-culture using mesenchymal stem cells has been considered a good alternative to promoting the maturation of the oocytes and the embryo's development quality *in vitro* in different mammalian species. In this regard, we investigated the effect of feline Wharton's jelly MSCs as feeders layer in oocyte maturation—consequently, the development of resulting embryos in co-culture.

Methods: Oocytes with dark cytoplasm and a few layers of cumulus cells were collected and subjected to *in vitro* maturation and embryo culture using commercial media with and without MSCs addition. The oocytes' nuclear maturation and the degree of cumulus expansion in different groups were assessed after 24 h; the development of the embryo was evaluated every 12 h until day eight.

Results: Although MSCs increased the proportion of cumulus cells oocytes exhibiting cumulus expansion, there were no significant differences in the percentage of matured oocytes (metaphase II) among the groups ($p > 0.05$). However, the embryo development differs significantly, with a higher cleavage, morula, and blastocyst percentage in oocytes matured with MSC co-culture conditions than in commercial media alone ($p < 0.05$). Also, we observed higher morula and blastocyst rates in the embryos co-cultured with MSCs during the *in vitro* culture ($p > 0.05$).

Conclusion: Based on our results, the co-culture with MSCs during the oocyte maturation resulted in better embryo development, as well as the MSCs addition during embryo culture returned an increased number of morula and blastocysts. Further research is needed to fully understand and optimize the use of MSCs in oocyte maturation and embryo development.

KEYWORDS

cat, oocytes, embryos, co-culture, mesenchymal stem cells, Wharton's jelly

Introduction

Due to the decreasing number of wild felids, domestic cats (*Felis catus*) as a model for studying reproduction physiology and developing new assisted reproductive technologies (ART) are gaining more and more importance (1).

ART has been used for several years to preserve genetic material, circumvent problems of subfertility, improve male reproduction, and increase the reproductive results and number of offspring that a single female can obtain. The post-fertilization period is an essential step in culturing embryos *in vitro*. Therefore, it is crucial to carefully plan the culture conditions during this period to ensure proper embryonic development. Inadequate culture conditions can significantly impact embryonic homeostasis, leading to short-term changes in morphology, cell proliferation, and metabolism and resulting in apoptosis. Such changes can ultimately lead to a reduction in both the number and quality of the formed blastocysts.

After years of investigations, the basic nutritional requirements for oocytes and embryos have been established, guaranteeing successful *in vitro* development in horses (2), cattle (3), pigs (4), mice and humans (5). However, ART in a feline species is still not as efficient as in other animals. Unfortunately, the culture conditions dedicated for cat oocytes and embryos have not yet been adequately investigated, and on average, *in vitro*, only around 60% of cats' oocytes reach the MII phase, and less than a half of the cleaved embryos become blastocyst (6). Furthermore, the low rate of embryo production from feline oocytes reflects the need for a better understanding of the developmental competence of feline oocytes and their specific requirements during *in vitro* maturation, fertilization and embryo development. Current ART procedures lack knowledge of the interaction of gametes with several components present in the reproductive system during the maturation of oocytes and early stages of embryo development. To mimic the *in vivo* complex microenvironment *in vitro*, recent advances used a co-culture of oocytes and embryos with oviduct epithelial cells, mesenchymal stem cells (MSCs), cumulus cells and extracellular vesicles (EVs) in the reproductive environment with the aim to obtain *in vitro* embryos with developmental levels similar to embryos derived *in vivo*.

MSCs possess multi-potentiality and properties of immunological and inflammatory regulation. Cell therapy based on their transplant is a promising approach, as these cells can develop into adipocytes, osteoblasts, chondrocytes, smooth muscle cells, and endothelial cells and can express many specific markers depending on the environmental conditions in which they are found (7). The most common sources of MSCs are of adult origins, such as bone marrow or adipose tissue, but their removal requires an invasive clinical procedure. Perinatal sources like an umbilical cord, mainly Wharton's jelly, offer higher practical accessibility and good quality MSCs with a higher proliferation rate and more potent immunomodulatory properties (8). *In vitro*, MSCs can thus promote cell viability and angiogenesis by producing growth factors. They also stimulate the recruitment of endogenous stem cells by secreting chemokines and acting locally through cell–cell interactions based on receptor-ligand bonds or through nanotubes that transfer molecules and organelles (9).

MSCs' properties make them a suitable candidate for improving the performance of *in vitro* production systems in mammalian species. In fact, many studies have used MSCs or their derived biomaterials in a co-culture system with oocytes and/or embryos, with most studies indicating improved embryo development (10). Furthermore, it has been shown that coculture with MSCs could rescue poor-quality embryos and enhance early embryonic development (11, 12). Additionally, coculture with MSCs has been observed to enhance the cytoplasmic and nuclear oocyte maturation *in vitro* (13, 14). Based on these findings, we hypothesise that feline Wharton's Jelly-derived MSCs could improve oocyte maturation and embryo culture *in vitro*. In this regard, we aimed to evaluate the *in vitro* effect of fWJ-MSCs added as a feeder layer in the co-culture system during cats' oocyte maturation and embryo development, in comparison to non-conditioned, commercial maturation and culture media.

Materials and methods

All chemicals and reagents were purchased from Sigma Aldrich Poland unless stated otherwise. Ethical approval was not sought, as it is not required for studies carried out on cells obtained from tissues that were surgical waste (Decision No. 004/2021). Commercial media were used for the oocyte manipulation and maturation: IVF Bioscience, Bickland Industrial Park, Falmouth, United Kingdom.

Mesenchymal stem cells isolation and characterization

MSCs were isolated and characterized, as mentioned in our previous study (8). Umbilical cords were collected from healthy queens (1.5–5 years old) after a normal birth and caesarean sections; the cells were obtained from Wharton's jelly parts of the umbilical cord (fWJ-MSC—feline Wharton's jelly mesenchymal stem cells) using collagenase type I at 0.02% in DMEM-LG. The cells were cultured in DMEM-LG containing 10% FBS and 1% PS at 37°C in humidity. Adherents' cells were grown until reaching 80 to 90% confluence before each passage, and the medium was changed three times a week. Before fWJ-MSCs were used, the cells were identified and characterized based on their expansion rate, tri-lineage differentiation (adipocytes, chondrocytes, and osteoblasts), cell surface markers (CD44, CD90, CD34, and MHC II) and pluripotency genes expression (OCT4, SOX2, NANOG).

Preparation of Wharton's jelly mesenchymal stem cells

To use fWJ-MSCs as a feeder layer, the cells at passage 2 to 3 were seeded in four well plates at a density of 1×10^4 cells/mL in DMEM-LG containing 10% FBS and 1% PS at 37°C in humidity until reaching 80% to 90% confluence, nonadherent cells were removed by washing twice with PBS. The adherent cells were inactivated with 10 µg/mL mitomycin C for 2 h to avoid nutrients competition. After a series of washes with PBS, the culture was maintained in DMEM-LG for 24 h before the oocytes or embryos were co-cultivated.

Ovaries and oocytes collection

Ovaries were obtained from sexually matured domestic queens subjected to a routine ovariohysterectomy or ovariectomy at the

University clinic and local veterinarians in Wroclaw. After surgical removal, ovaries were stored in PBS with 1% of Antibiotic Antimycotic Solution at 4°C for up to 24 h before the recovery of cumulus-oocyte complexes (COCs). COCs were collected by slicing ovaries with a #10 scalpel blade in an OPU medium. Isolated COCs were classified under a dissecting microscope. Only oocytes with evenly pigmented dark ooplasm and some layers of cumulus cells were selected for further procedures.

In vitro maturation of cat oocytes

The selected COCs were placed in a four-well plate in 400 µL of plain bovine maturation medium (BoM) and plain equine maturation medium (EqM) or in the same medium with MSCs co-culture: BoM + MSCs or EqM + MSCs, under mineral oil and matured for 24 h at 38.5°C in 5% CO_2 in the air with maximum humidity.

In vitro fertilization

For *in vitro* fertilization, the oocytes were fertilized with frozen–thawed semen and cryopreserved according to the protocol described by Partyka et al. (2012).(15) Semen straw was thawed in awater bath at 37°C then washed in IVF medium followed bycentrifugation at 35,000 rpm for 5 min. After 24 h of maturation,cumulus oocytes complex were washed in IVF medium; thenincubated with 1×10^6 motile spermatozoa/ml for 18 h in 400 µL ofIVF medium under mineral oil at 38.5°C in 5% CO_2 in the air withmaximum humidity.

Assessment of oocytes maturation

In order to establish the assessment of oocyte maturation after 24 h of IVM, all the cumulus cells were mechanically removed using a glass pipette overheated and pulled to achieve the diameters of approximately 165 µm, slightly larger than the oocyte. Oocytes were aspirated and blown out repeatedly until most cumulus cells were removed. After most of the cumulus cells were removed, the oocytes were washed twice and fixed with 4% formaldehyde for 15 min flowed by washing in PBS and then incubated in DAPI stain solution for 10 min in the dark and mounted on glass slides in drops of Vectashield (Vector Laboratories, Ltd. United Kingdom). The nuclear state of the stained oocytes was assessed under a fluorescence microscope (Olympus IX73) at 360 excitations and 450 nm emission. Oocytes with distinct polar body or two separate and bright chromatin spots were classified as entering the MII stage.

Embryo culture and assessment of the embryo development

After fertilization, presumptive zygotes were washed and transferred to a new plate in a droplet of 50 µL of either BoM or EqM medium or co-culture BoM + MSCs, EqM + MSCs medium (depending on the part of the experiment) covered with mineral oil and incubated at 38.5°C in 5% CO_2 in the air with maximum humidity for up to 8 days. To assess embryo development, morphological changes were evaluated and noted every 8 to 12 h. The subsequent developmental stages were noted for each group, and the blastocyst formation was recorded.

Study design

Experiment 1: the effect of the co-culture with MSCs on the oocyte maturation and cumulus cell expansion

This experiment evaluated nuclear maturation and cumulus cell expansion. Oocytes were matured in 400 µL of IVF medium under mineral oil at 38.5°C in 5% CO_2 in the air with maximum humidity. In total, 180 oocytes were used in this part of the study, and three independent replicates of 15 oocytes per experimental group were carried out. Study groups were as follows:

- Maturation in BoM ($n = 45$ oocytes).
- Maturation in EqM ($n = 45$ oocytes).
- Maturation in BoM + MSCs ($n = 45$ oocytes).
- Maturation in EqM + MSCs ($n = 45$ oocytes).

The degree of nuclear maturation was analyzed after 24 h.

Assessment of cumulus cells expansion

The degree of cumulus cells expansion after 24 h of oocyte maturation using two different commercial media and with or without MSC addition was assessed as described by Lee et al. (16). The evaluation system was as follows: no expansion, limited expansion (less than three layers of cumulus cells expended), expended (more than three layers of cumulus cells expanded) and oocytes with no cumulus cells attached were classified as degenerated) (Figure 1).

Assessment of nuclear maturation

The nuclear state of the stained oocytes was assessed under the fluorescence microscope (Olympus IX73) at excitation 360 and 450 nm emission. Oocytes with distinct polar bodies or two separate and bright chromatin spots were classified as entering the MII stage (Figure 2).

Experiment 2: the effect of the co-culture with MSCs during oocyte maturation on embryo development

This part of the study was done to assess the effect of the co-culture system with MSCs during maturation in two commercial media on the subsequent embryo development after *in vitro* fertilization. In total, 565 oocytes were matured and cultured in four groups,10 replicates per group, as illustrated in Figure 1. Embryonic development (cleavage, morula and blastocysts rate) was compared among all groups.

Experiment 3: the effect of co-culture with MSC during embryo development

At this stage, the oocytes were matured in EqM or in BoM then the MSCs were added during the embryo development to evaluate their effect on the morula and blastocyst formation. In total, 486 oocytes were matured and cultured in four groups, 10 replicates per group, as presented in Figure 1.

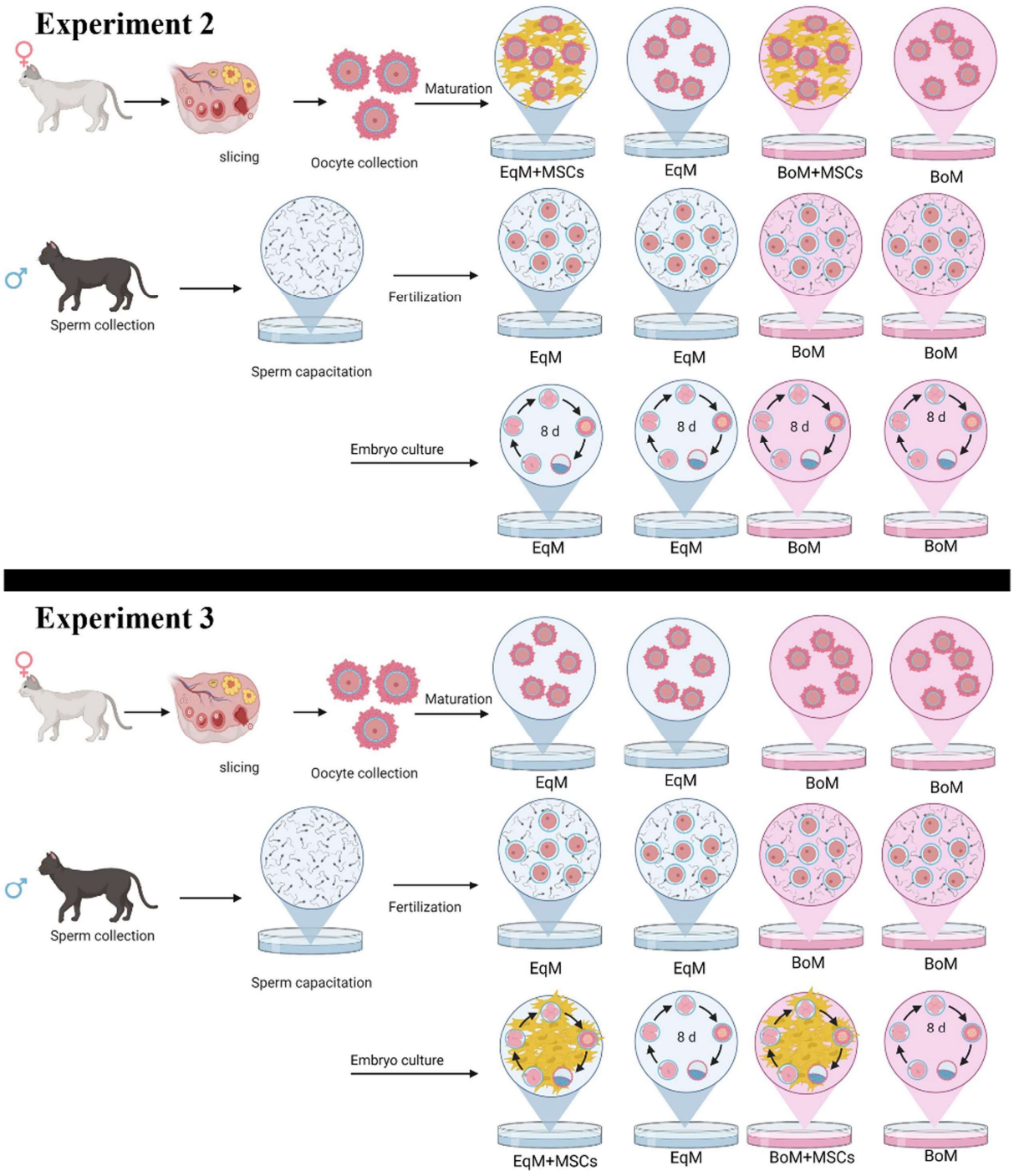

FIGURE 1
Study design of the experiment 2: the effect of the co-culture with MSC during oocyte maturation on the subsequent embryo development. Oocytes were matured and embryos cultured in four groups: EqM + MSCs/EqM: maturation in EqM + MSCs/embryo culture in EqM ($n = 109$). EqM/EqM: maturation in EqM/embryo culture in EqM ($n = 109$). BoM + MSCs/BoM: maturation in BoM + MSCs/embryo culture in BoM ($n = 124$). BoM/BoM: maturation in BoM/embryo culture in BoM ($n = 103$) and experiment 3: The effect of MSC addition during embryo development. Oocytes were matured, and embryo culture was carried out in four groups: EqM/EqM + MSCs: maturation in EqM/embryo culture in EqM + MSCs ($n = 142$). EqM/EqM: maturation in EqM/embryo culture in EqM ($n = 109$). BoM/BoM + MSCs: maturation in BoM/embryo culture in BOM + MSCs ($n = 132$). BoM/BoM: maturation in BoM/embryo culture in BOM ($n = 103$). The figure was prepared with BioRender.

Statistical analysis

Data were analysed using one-way ANOVA followed by Tukey's multiple comparison test using Statistical software (TIBCO, United States). Values are shown as mean ± S.E.M.. The significance level was $p < 0.05$, and at least three independent replicates were performed in all experiments. Nonparametric data, such as differences in the percentage values between groups, were assessed using the chi-square test.

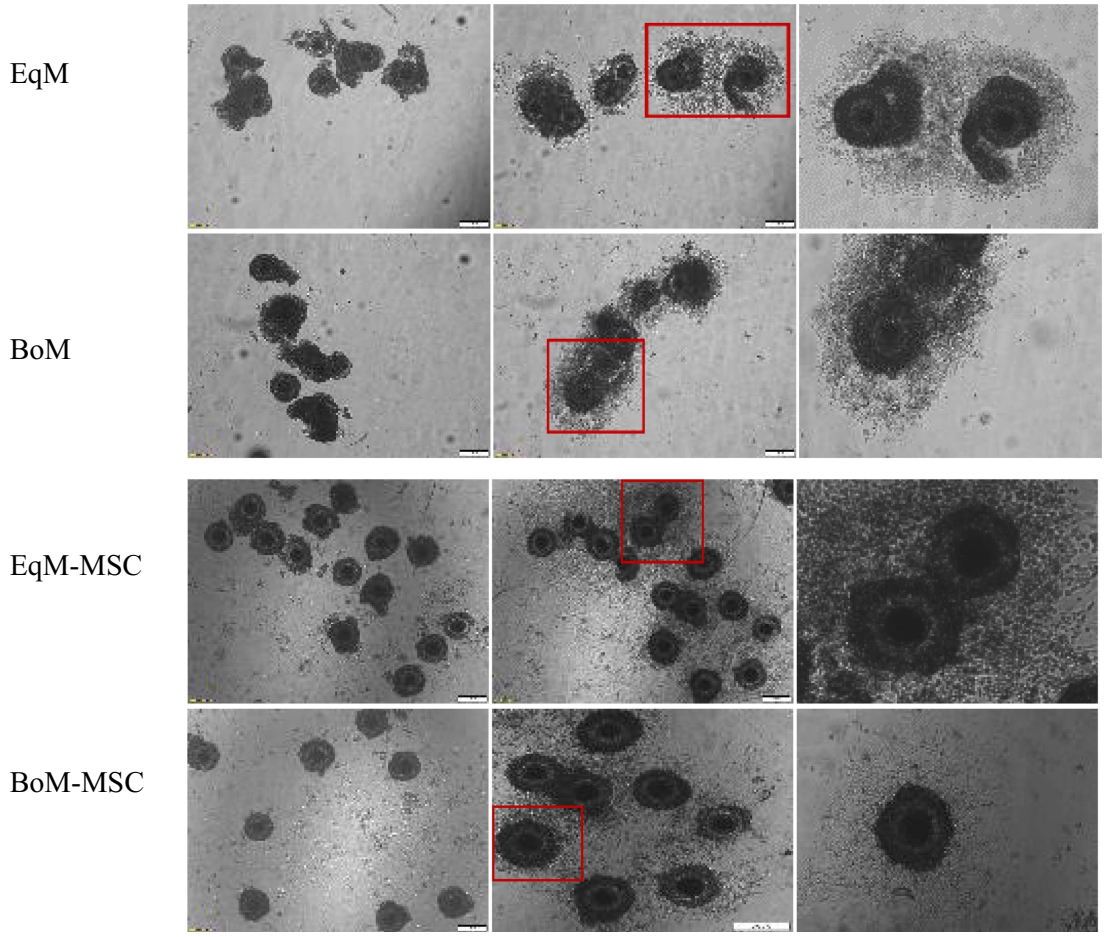

FIGURE 2
Effect of feline MSCs cells on cumulus expansion of oocytes after IVM. The degree of cumulus expansion at T0 and after 24 h of IVM in four groups EqM + MSC, BoM + MSC, EQM, and BoM. The images were taken with a magnification = 200 um.

Results

Effect of co-culture on the cumulus cells expansion

The degree of COCs expansion was significantly increased in the EqM + MSCs and BoM + MSCs groups compared to EqM and BoM or groups ($p < 0.05$) (Figures 2, 3). Furthermore, there was no significant difference between the EqM + MSCs and BoM + MSCs groups. The evaluation of the degree of cumulus cell expansion confirmed that co-culture with MSC showed a considerable increase in the proportion of COCs that showed cumulus expansion ($p < 0.05$).

Effect of different culture conditions on the efficacy of maturation of oocytes

Nuclear maturation was evaluated using DAPI staining and showed a similar percentage of metaphase II (M II) (Figure 4B) in all investigated groups, which ranged from 45% to 55% ($p > 0.05$). The co-culture system with MSCs did not affect the nuclear maturation of oocytes (Figure 4A).

Development of embryos derived from oocytes matured under different maturation conditions

As shown in Table 1, the percentage of the oocytes which cleaved was similar in the co-culture group EqM + MSCs and BoM + MSCs and higher ($p < 0.05$) than in the EqM and BoM group. The rate of the morula was similar among EqM, BoM and BoM + MSCs groups but higher in the group of oocytes matured in EqM + MSCs. The oocytes matured in EqM + MSCs showed the most promising development and the highest number of blastocysts compared with the BoM + MSCs, BoM and EqM groups. Thus, the use of MSC as a co-culture during oocyte maturation has an effect on the further development of feline embryos (Table 1).

Development of embryos cultured under different conditions

The rate of development of the resulting two-cell embryos was higher in the co-culture group of EqM + MSCs and BoM + MSCs compared with two-cell embryos that were cultured in BoM and EqM

($p > 0.05$). However, we also noticed that the blastocyst/morula rate was higher in pure EqM media when compared to non-conditioned Bo culture media ($p > 0.05$).

Discussion

In vivo, oocyte maturation occurs within the ovarian follicle, while fertilization and early embryo development occur in the fallopian tubes. When trying to recreate *in vitro* these physiological conditions, it is crucial to provide efficient culture systems (17); the first successful *in vitro* fertilization (IVF) in a domestic cat was achieved 45 years ago using *in vivo* matured oocytes and *in utero*-capacitated spermatozoa (18). Despite advances in culture conditions, media and protocols for oocyte maturation and embryo development, *in vitro* outcomes are still far from desirable compared with embryos produced *in vivo* (19, 20). In particular, recent studies have shown the beneficial effect of co-culture with MSCs (21), oviduct cells, and cumulus cells (22, 23) on the development of oocytes and embryos in various mammalian species, including cattle (24), horses (25), pigs (26) and canines (27). Therefore, culture conditions are crucial in determining the quality of *in vitro*-produced embryos. In the present study, we demonstrated for the first time the effect of feline Wharton's jelly-derived MSCs and different commercial media on the maturation of feline oocytes, cumulus cell expansion and embryo development.

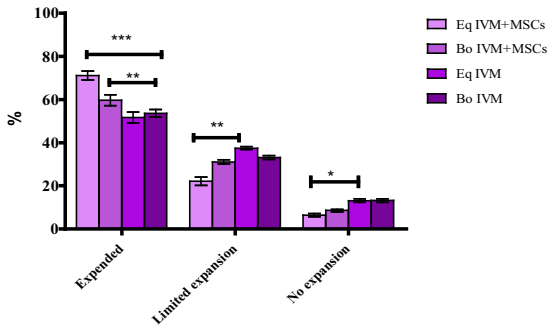

FIGURE 3
Effect of feline MSCs cells on cumulus expansion of oocytes after *in vitro* maturation. The cumulus expansion scoring system was as follows: limited expansion, no expansion, degenerated oocytes, denuded and expanded oocytes in four groups EqM + MSCs, BoM + MSCs, EQM, BoM. The Data are shown as the mean ± S.E.M.. ***, **, *Within the columns, values are significantly different ($p < 0.05$).

Oocytes with adequate nuclear and cytoplasmic maturation are more competent since many proteins and transcripts stored in their cytoplasm will be required for future embryo development. Therefore, in this study, we investigated whether the co-culture condition with MSCs as a feeder layer can influence oocyte maturation and their ability to develop into embryos. Based on the extrusion of the first polar body (metaphase II) in each experimental group, we did not observe the effect of co-culture with MSCs on the oocytes' nuclear maturation resulting in comparable percentages ranging between 45 and 55% of MII. Similarly to our results, Ascari et al. (28) showed that murine MSCs or embryonic fibroblasts did not affect the nuclear maturation rate of bovine oocytes. In contrast, the addition of the conditioned medium containing human bone marrow MSCs, as a supplement to enrich the IVM medium used for germinal vesicles in mice polycystic ovary syndrome (PCOS) significantly increased cytoplasmic and nuclear maturation of oocytes (13).

However, when analysing the treatment used during maturation, we observe the morphological difference in cumulus cells expansion after 24 h of maturation; the oocytes cultured in MSCs-conditioned

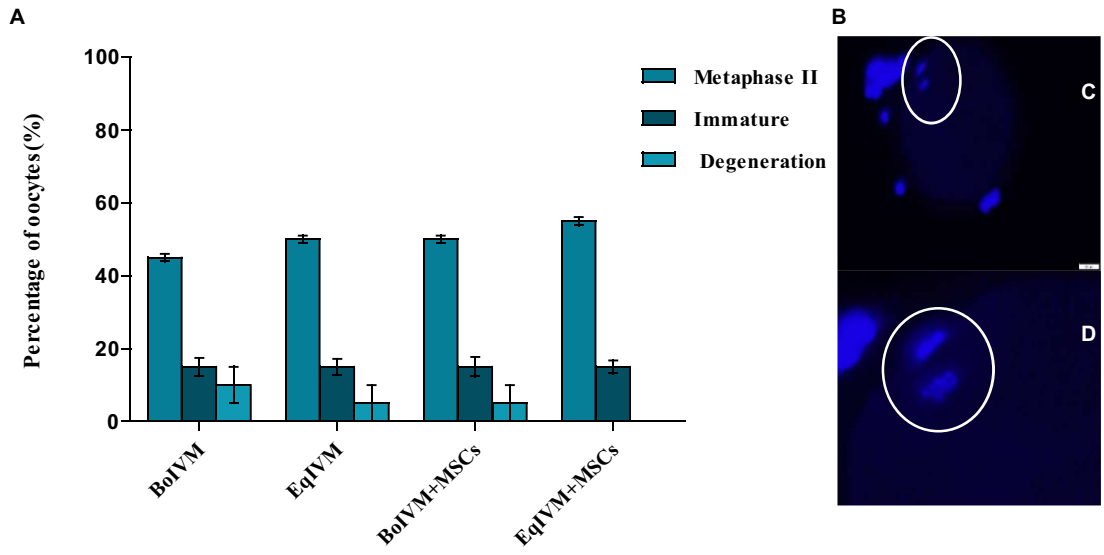

FIGURE 4
(A) Effect of different maturation conditions on nuclear maturation of feline oocytes. Metaphase II indicated oocyte maturation; the results are shown as the mean ± S.E.M. within the columns ($p < 0.05$). **(B)** Representative microscopy images of oocytes in metaphase II illustrating (C,D) polar body extrusion and nucleus stained with DAPI (Bar = 50 µm).

TABLE 1 Effect of different conditions during oocyte maturation on the subsequent embryo development.

Group	Oocytes number *n*, %	Cleavage *n*, %	Morula *n*, %	Blastocysts *n*, %
EqM + MSCs/EqM	109	78 (71.56)[a]	51 (46.79)[a]	28 (25.68)[a]
BoM + MSCs/BoM	124	84 (67.74)[c]	48 (38.71)[ab]	25 (20.16)[ab]
EqM/EqM	109	60 (55.04)[b]	40 (36.69)[ab]	18 (16.51)[b]
BoM/BoM	103	52 (50.48)[d]	35 (33.98)[b]	15 (14.56)[b]

EqM + MSCs/EqM, maturation in EqM + MSCs and embryo culture in EqM; BoM + MSCs/BoM, maturation in BoM + MSCs and embryos culture in BoM; EqM/EqM, maturation in EqM and embryo culture in EqM; BoM/BoM, maturation in BoM and embryo culture in BoM; ($p < 0.05$).

TABLE 2 Effect of different culture conditions on the development of the embryos from the oocytes matured in BoM or EqM.

Group	Oocytes number *n*, %	Morula *n*, %	Blastocysts *n*, %
EqM/EqM	109	40 (36.69)[ab]	18 (16.51)[c]
BoM/BoM	103	35 (33.98)[b]	15 (14.56)[b]
EqM/EqM + MSCs	142	59 (41.54)[a]	26 (20.31)[a]
BoM/BoM + MSCs	132	52 (39.39)[ab]	22 (19.82)[abc]

EqM/MSCs + EqM, maturation in EqM and embryo culture in EqM + MSCs; BoM/BoM + MSCs, maturation in BoM and embryo culture in BoM + MSCs; EqM/EqM, maturation in EqM and embryo culture in EqM; BoM/BoM, maturation in BoM and embryo culture in BoM); ($p > 0.05$).

media had significantly increased the cumulus cell expansion compared to oocytes cultured without MSC addition. Similar observations were reported for human adipose-derived stem cells (ASC) added to the medium (ASC-CM) that improved cumulus cell expansion with high transcript abundance of an expansion-related gene in porcine (29). It was also reported by Wang et al. (30) that human Wharton's jelly MSCs were used to treat mice with induced premature ovarian failure using a daily dose of intraperitoneal CTX injection (50 mg/kg) for 15 consecutive days; the results showed that MSCs reduced cumulus cell apoptosis in investigated mice. Other authors explored the use of human placental MSCs on human ovarian granulosa cells obtained from patients with premature ovarian insufficiency; the reported results showed that MSCs released epidermal growth factor (EGF) that reduced apoptosis and improved proliferation, and restored the oxidative enzyme levels of human granulosa and cumulus cells (31).

After fertilization, we observed differences between the oocytes that matured with and without MSCs. The embryos derived from the oocytes matured with MSCs: EqM + MSCs, BoM + MSCs showed a higher cleavage, morula, and blastocyst rate compared to the oocytes matured in classic BoM and EqM. We observed that the presence of MSCs during the maturation of oocytes did not affect nuclear maturation; it still affected the cleavage rate and blastocyst formation. As reported before, MSCs release several trophic factors, including EGF and cytokines. The trophic effects of these bioactive factors on preantral follicular growth and *in vitro* maturation of mouse oocytes have been shown (32); it was also reported that the conditioned medium containing human MSCs generated microenvironment that was more appropriate to induce oocyte maturation and increase embryo development of; they also described that high embryonic development rates might be associated with the quality of nuclear and cytoplasmic maturation (33).

The effect of co-culture with MSCs during embryo development was also evaluated in our study. In this part of the experiment, we noticed an improvement in embryo development (Table 2); the morula and blastocysts rate was higher in EqM + MSCs and BoM + MSCs than in BoM and EqM. It is interesting to point out that the embryos co-cultured with MSCs were previously maturated in classic BoM or EqM media. Our current findings are similar to the results of the previous study conducted by Jasmin et al. (34) using mice embryos; they observed that embryos co-cultured with MSCs for 4 days actually formed more blastocysts. Furthermore, our current data are similar to those shown by the same group using murine MSCs and embryonic fibroblast as a co-culture during embryo development (28).

In general, the co-culture with somatic cells has shown a positive impact on embryonic development *in vitro*. Most studies indicate a higher rate of blastocyst formation after culturing embryos with different types of somatic cells (35, 36). However, some studies did not show significant improvement in embryo development (37, 38), and some others indicated a negative effect of the co-culture system on preimplantation embryo development (39). However, the co-culture studies published to date used very different types and concentrations of cells ranging from 1×10^3 to 1×10^6 cells/mL (24, 40), whereas our study used 1×10^4 cells/mL, as a long with the main medium used, time points and oxygen concentrations, so comparisons are very hard and quite limited.

In fact, despite some similarities, each species may have different requirements regarding the substrates in the medium (41), which could explain the minor divergent results observed in our study. Here, we used the same source of mesenchymal cells, but we carried out the culture using two media dedicated to different species (cattle and horses). It is worth noticing that we noted more blastocysts with the use of equine media (EqM) compared to bovine (BoM). The latter could also have some impact on the results obtained during the co-culture experiment.

In summary, we investigated the potential of feline Wharton's jelly MSC to assist in feline oocyte maturation and embryo growth. With the addition of feline Wharton jelly MSC both to oocyte and embryo culture, we observed an improved embryo development. Furthermore, our results did not show a significant impact on the nuclear maturation process itself, but the addition of MSCs as a feeder layer during the

maturation or embryo culture still resulted in a higher rate of embryonic development. In particular, we found that the co-culture with MSCs was most effective during oocyte maturation, as the cleavage and blastocyst rates were higher when MSCs were added during oocytes maturation than during embryos development. These findings suggest that feline Wharton's jelly MSCs could be a promising tool for improving *in vitro* feline embryo development in the future.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

Ethical approval was not sought, as it is not required for studies on cells obtained from surgical waste tissues (Decision No. 004/2021). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

MO and MB: conceptualization, data collection, data evaluation, statistical analysis, and manuscript writing. PM: data evaluation and reviewing of the paper. YL: reviewing and editing the paper. WN: consulting and supervising the study and paper reviewing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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.

Publisher's note

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CHAPTER 7

GENERAL DISCUSSION AND CONCLUSION

Feline embryos produced in vitro are still of lower quality compared to those produced naturally. The laboratory process of growing embryos involves several steps, with *in vitro* oocyte maturation being the crucial stage. This stage significantly impacts the further success of preimplantation embryonic development and the following fetal growth[75]. Achieving successful oocyte maturation can substantially enhance embryo developmental efficiency, as oocytes that have attained the appropriate cytoplasmic and nuclear maturation levels are more capable of developing into future embryos. This is due to the fact that these oocytes contain numerous proteins and transcripts that play a crucial role in the growth and development of the embryo. Ensuring that oocytes are properly matured is a critical step in any fertility treatment or assisted reproductive technology[76].

Researchers are attempting to refine the culture conditions, including using coculture systems, but the outcomes still need improvement. One of the main challenges is that preimplantation embryos rely on various factors released by the oviduct, uterus, and embryos themselves[77]. It has been reported that MSCs have an antioxidant capacity through the secretion of superoxide dismutase. Additionally, MSCs release bioactive substances as growth factors and cytokines that support the proliferation, differentiation, and maintenance of other cells[78]. These growth factors and cytokines also play an important role in germ cell development, gamete maturation, and early embryo development[79]. While MSCs have already been isolated from different tissues in cats, including the umbilical cord, so far, no attempts have been made to isolate MSCs separately from individual sections of the umbilical cord.

Therefore, here we investigated for the first time the isolation and characterization of MSCs from the whole cord in comparison to separate isolation from vessels and Wharton jelly. Based on the obtained results, we were able to define the cell characteristics by their morphology, plastic adherence, proliferation, gene expression and trilineage differentiation as described in Chapter 5, and then Wharton jelly-derived MSCs were used as a feeder layer to enhance the oocyte's maturation and the embryo's development in vitro as presented in Chapter 6.

In this study, mesenchymal stem cells were successfully isolated from the whole feline umbilical cord and separately from the vessels and Wharton jelly and showed the characteristics of typical MSCs, including the plastic adherence of the cells, fibroblast-like morphology, differentiation potential, and surface markers.

To get a larger number of primary cells, we preferred to employ the enzymatic method rather than the explant attachment method; all of the isolated cells displayed a plastic-adherent fibroblast-like morphology, remained stable, and did not alter their morphology even after prolonged culturing periods. The latter feature - cellular stability, even with longer passages, is very promising because it enables obtaining numerous, good cell populations suitable for applications in tissue engineering. Establishing surface markers to define MSCs in animals has not yet been done in the same way as they were defined for humans. However, some studies have been conducted on feline MSCs, which have been isolated from various tissues, including bone marrow, amniotic membrane, adipose tissue, peripheral blood and umbilical cord. These studies suggested that CD44, CD105 and CD90 are expressed in feline MSCs, whereas they lack the expression of CD34, CD4, CD18 and MHC II. Other expression markers have varied in different studies[80]. Our study found that MSCs surface markers CD44 and CD90 were expressed in the cells we isolated from all parts of umbilical cords, whereas hematopoietic markers CD34 and MHC II were not expressed.

Further, the stemness of the isolated cells was confirmed by the expression of pluripotent markers NANOG, OCT-4 and SOX-2 at the mRNA levels, with significant differences between the investigated groups. Various studies have conclusively shown that specific pluripotency markers are indeed present in the early stages of UC-MSCs culture. However, it has also been noted that these markers tend to decrease as the culture is expanded. A comparison of pluripotency marker expression across different parts of UCs revealed that fWJ-MSCs exhibited a higher concentration of these markers when compared to other cord's parts[81,82]. According to another study, it was found that MSCs derived from the umbilical vein did not express SOX-2, but they did show expression of OCT4 and NANOG [83]. These results confirmed that when it comes to the expression of pluripotent markers, the WJ is the most favourable MSC source compared to other parts of the body. The presence of these markers signifies that the MSCs are in an undifferentiated state, which is crucial for maintaining their ability to renew themselves.

The differentiation competence also plays an important role in confirming the stemness of isolated cells. The MSCs from the three groups isolated in this study were differentiated toward adipogenic osteogenic and chondrogenic cells by inducing cells into appropriate lineage-specific culture conditions. In the present study, staining images of all the osteocytes, adipocytes, and chondrocytes differentiated UCV-MSCs and WUC-MSCs showed the same degree of

differentiation. However, the fWJ-MSCs isolated in our study showed a higher degree of differentiation for adipocytes and chondrocytes.

It was shown before in human-MSCs that the differentiation potential depends on the tissue origin and the concentration of inducible factors; similar to our study, the cells isolated from Wharton's jelly have the most significant differentiation potential, differentiating into various types of cells, including bone, fat, and cartilage, as well as cardiomyocytes, neurones, muscle cells, and hepatocytes comparing to the other part of the UC[84]. However, this may be simply due to the fact that Wharton's Jelly's has been more thoroughly studied and had the most extensive data compared to the other cord-derived cells.

Given the broad self-renewing and high proliferation properties of mesenchymal stem cells obtained from Wharton's jelly, as well as their high differentiation capacity, our data also suggested that Wharton's jelly is the best source of MSCs from the entire cat's umbilical cord. Moreover, it was shown that fWJ-MSCs secrete a variety of cytokines and growth factors[85]. These proprieties made Wharton's jelly an ideal candidate for the next step of the study. In this part fWJ-MSCs were used to evaluate their effects as a feeder layer on the oocyte maturation and embryo development in vitro.

In this second part of the study, we reported for the first time the effect of fWJ-MSCs in a co-culture system for in vitro production of feline embryos and oocyte maturation, which proved to be more effective than the pure media alone. These results are similar to the previous studies in other species that have demonstrated the positive impact of stem cells in co-culture with embryo culture. The study by Moshkdanian et al. [86] showed that the co-culture of mouse embryos with human umbilical cord mesenchymal cells (h-UCMS) improved embryo development after exposure to light stress. However, our study was slightly different because we utilized stem cells from the same species as the cultured oocytes and embryos.

The co-culture condition during the maturation phase did not influence the oocytes' nuclear maturation rate, but increased the cumulus expansion rate. Furthermore, after fertilization we observed that the groups matured in the presence of MSCs showed a higher cleavage, morula, and blastocyst rate compared to the oocytes matured in media without MSCs addition. Thus, it is possible that the co-culture with MSCs during maturation enhances the cytoplasmic maturation in oocytes and thereby contributes to its higher potential for embryo development. Similar to our

results, other studies demonstrated a lack of any beneficial effect of the co-culture on the nuclear maturation rate of immature ovine oocytes; simply removing oocytes from their follicles allowed the resumption of meiosis[87]. Indeed, many immature mammalian oocytes are capable of completing meiosis *in vitro* [88]. Still, only a small percentage of them are competent to continue development to the blastocyst stage and beyond, indicating that the IVM process may only partially be normal when carried out *in vitro*.

The expanded cumulus cells seen in the groups with a co-culture may also have an effect on the oocyte maturation process and the results obtained after fertilisation. It has already been shown that cumulus cells contributed to the cytoplasmic oocyte maturation and therefore improved fertilization rate through gap junction communication[89].

Ensuring the perfect culture conditions is crucial to avoid any negative effects on the embryo's physiology, divisions or metabolic functions. These repercussions can lead to severe complications at later stages, such as impaired fertilisation, embryo development, an increased risk of miscarriage, and variety of fetal health issues. Thus, it is highly recommended to maintain optimal *in vitro* embryo development conditions to prevent any harmful consequences[90]. Our study thoroughly evaluated the impact of co-culture with MSCs on the development of embryos to maintain good conditions for embryo development and to prevent any negative effects.

Our results revealed a positive impact on embryo development when embryos were co-cultured with MSCs *in vitro*. Specifically, the morula and blastocysts rate were higher in the groups that included the MSCs layer during culture compared to those without. It is noteworthy that the embryos that were co-cultured with MSCs had previously been matured using traditional media, which allowed us to evaluate the MSCs impact solely during the embryo development. These findings suggest that co-culture with MSCs may improve embryo developmental capacity. In agreement with our results, other research has shown that when horse MSCs were used in co-culture with bovine embryos the blastocyst formation rate increased [91]; in a similar study, bovine amniotic epithelial stem cells used in co-culture led to a better embryo development due to the presence of epidermal growth factor, which was suggested to be released by these cells[92]. Similarly, a conditioned medium of human MSCs has been used to culture bovine embryos, resulting in improved embryo development. Notably, the best results were observed when the conditioned medium groups were compared to their respective control groups

maintained without fetal bovine serum[93]. These findings above present an important implication for the use of stem cells and conditioned medium during embryo culture.

The positive effect of MSCs on embryo development in both cases during coculture with oocytes maturation or with the embryo's development reported in our study may be due to the paracrine modulation of the microenvironment and the ability of MSCs to release antioxidant and anti-inflammatory factors *in vitro* , which has been reported in several studies. Utilizing a stem cell-free medium may become the most favourable method for cell therapy in the coming years because of all released growth factors and exosomes. Several works have exhibited the advantages of solely using stem cell-conditioned medium for treatments, which can eliminate the potential danger of unpredictable cell divisions (such as tumors) in patients, along with cell membrane-related antigen reactions that could lead to cross-species treatments.[94]. Additionally, the conditioned media (CM) also increases cell viability and antioxidant enzyme activity while reducing reactive oxygen species (ROS) levels and thus apoptosis[95]. It has been shown that when CM is added to IVM or/and IVC, multiple growth factors/cytokines present in the CM positively regulate the mRNA/protein expression and IVM/IVC microenvironment supporting the oocyte development and enhancing the embryo quality *in vitro*[96–98].

Recent studies have also shown that exosomes: microvesicles containing growth factors, cytokines, and microRNAs may have a significant impact on cell therapy advancements[99]. These results suggest that the micro-modulation potential observed with stem cells could be attributed not only to stem cell differentiation properties, but also to the presence of MSCs-derived exosomes and microvesicles[72]. Thus, it may be hypothesized that the exosomes released by the MSCs could positively influence the oocytes and embryos in the culture, providing a valid explanation for the observed effects. However, additional research is necessary to validate these assumptions.

Conclusion

In the presenting study we have successfully isolated mesenchymal stem cells (MSCs) from the whole umbilical cord, as well as from the two distinct anatomical regions Wharton's Jelly and vessels. All the isolated cells shared the typical characteristics of MSCs, including self-renewal ability, fibroblast-like morphology, stem cell markers expression, and differentiation into three lineages. However, our findings revealed that Wharton's jelly-derived MSCs exhibited higher

expansion, better differentiation ability and the superior pluripotency markers expression. This highlights the significant potential of Wharton's Jelly as the optimal source of MSCs from the feline umbilical cords. Nonetheless, the whole umbilical cord and vessels also represent viable sources of MSCs.

Our study also explored the effect of feline Wharton's jelly MSCs during feline oocyte maturation and embryo culture *in vitro*. The addition of feline Wharton's jelly MSCs during oocyte maturation and embryo culture resulted in significant improvements in embryo developmental capacity. Although, the oocyte nuclear maturation process was not significantly impacted, the embryonic development was better when MSCs were used as a feeder layer during the maturation or embryo culture. Our results also showed that co-culture with MSCs was the most effective during oocyte maturation, leading to the higher cleavage and blastocyst rates compared to embryo development in pure media. These findings demonstrated the remarkable potential of feline Wharton's jelly MSCs in improving *in vitro* feline embryo development.

Unlocking the full potential of MSCs in promoting *in vitro* oocyte maturation and embryo development requires conducting more in-depth studies, including molecular and proteomic analyses. Further research on MSCs-derived exosomes and MSCs conditioned medium can offer valuable insights into their impact on oocyte maturation and embryo development, paving the way for advanced culture options.

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International Journal of Reproductive BioMedicine (IJRM) **2020**, doi:10.18502/ijrm.v18i12.8023.

98. Park, A.; Oh, H.J.; Ji, K.; Choi, E.M.; Kim, D.; Kim, E.; Kim, M.K. Effect of Passage Number of Conditioned Medium Collected from Equine Amniotic Fluid Mesenchymal Stem Cells: Porcine Oocyte Maturation and Embryo Development. *Int J Mol Sci* **2022**, *23*, 6569, doi:10.3390/ijms23126569.

99. Yin, K.; Wang, S.; Zhao, R.C. Exosomes from Mesenchymal Stem/Stromal Cells: A New Therapeutic Paradigm. *Biomark Res* **2019**, *7*, 8, doi:10.1186/s40364-019-0159-x.

Scientific achievements

PUBLICATIONS

- Lynda Bourebaba., Izabela Michalak., Meriem Baouche., Katarzyna Kucharczyk., Krzysztof Marycz.

Cladophora glomerata methanolic extract promotes chondrogenic gene expression and cartilage phenotype differentiation in equine adipose-derived mesenchymal stromal stem cells affected by metabolic syndrome.

Stem Cell Research & Therapy

December 2019 | journal-article

doi:10.1186/s13287-019-1499-z

- Lynda Bourebaba., Izabela Michalak., Meriem Baouche., Katarzyna Kucharczyk .,Andrzej M., Krzysztof Marycz.

Cladophora glomerata enriched by biosorption with Mn(II) ions alleviates lipopolysaccharide-induced osteomyelitis-like model in MC3T3-E1, and 4B12 osteoclastogenesis.

June 2020 | journal-article

Journal of Cellular and Molecular Medicine

doi: 10.1111/j cmm .152 94

- Meriem Baouche.; Agnieszka Krawczenko.; Maria Paprocka.; Aleksandra Klimczak.; Pascal Mermillod.; Yann Locatelli.; Małgorzata Ochota.; Wojciech Niżański.

Feline Umbilical Cord Mesenchymal Stem Cells: Isolation and in Vitro Characterization from Distinct Parts of the Umbilical Cord.

Theriogenology

December 2022 | journal-article

doi:10.1016/j.theriogenology.2022.11.049.

- Meriem Baouche.; Małgorzata Ochota.; Yann Locatelli.; Pascal Mermillod.; Wojciech Niżański.

Mesenchymal Stem Cells: Generalities and Clinical Significance in Feline and Canine Medicine.

Animals

June 2023 | review

doi.org/10.3390/ani13121903.

- Aleksandra Synowiec.; Agnieszka Dąbrowska.; Magdalena Pachota.; Meriem Baouche.; Katarzyna Owczarek.; Wojciech Niżański.; Krzysztof Pyrc.

Feline herpesvirus 1 (FHV-1) enters the cell by receptor-mediated endocytosis.

Journal of Virology

July 2023 | journal-article

doi:10.1128/jvi.00681-23.

- Meriem Baouche.; Małgorzata Ochota.; Pascal Mermillod.; Yann Locatelli and Wojciech Nizanski.

Feline Wharton's Jelly-derived mesenchymal stem cells as a feeder layer for oocytes maturation and embryos culture in vitro.

Frontiers in veterinary sciences

2023 | journal-article

doi: 10.3389/fvets.2023.1252484.

PARTICIPATION IN PROJETS

- The effect of bioactive algae enriched by biosorption on certain minerals such as Cr(lII), Mg (II) and Mn (II) on the status of glucose in the course of metabolic syndrome horses. Evaluation in vitro and in vivo" (2015 / 18 / E / NZ9 / 00607)

- Inhibition of tyrosine phosphatase as a strategy to enhance insulin sensitivity through activation of chaperone-mediated autophagy and amelioration of inflammation and cellular stress in the liver of equine metabolic syndrome (EMS) horses" (No 2018/29 / B/NZ7/02662)

- International multicentric platform as a key element for the effective scientific research" financed by the Polish National Agency for Academic Exchange (NAWA) within the program Academic International Partnerships PPI/APM/2019/1/00044

- „Dlaczego najądrze kota domowego się kurczy? Badania wstępne na poziomie receptorowym.", no. 2019/03/X/NZ3/00704 (project MINIATURA 3,NCN), UPWr 2.0 project

- UPWr 2.0 project International and interdisciplinary development programme of development of Wroclaw University of Environmental and Life Sciences, cofinanced by the European Social Fund under the Operational Program Knowledge Education Development Program 2014–2020: Axis III Higher education for the economy and development; Action 3.5. Comprehensive university programs; for schools of higher education (POWR.03.05.00–00-Z062/18).

WORKSHOPS and TRAINING

- Specialist summer school as a bridge to a career at UPWr doctoral school, IMPK2 dog and cat in interdisciplinary scientific research Jun (2021,2022,2023).

- 5th INTERNATIONAL WORKSHOP within APM NAWA - Clinical Aspects of Feline Reproduction September 16, 2022.

- 4th INTERNATIONAL WORKSHOP within APM NAWA project ScienceNet Spermatozoa Function and Conservation in Large and Small Animals July 22, 2022.

- 3rd INTERNATIONAL WORKSHOP within APM NAWA project ScienceNet Assisted reproduction: Comparative aspects between Large and Small Animals March 12, 2022.

- Training on the extracellular vesicles from the medium of bovine embryos on 7-14 Jun 2022 at the Department of Obstetrics, reproduction and herd health, Ghent University Belgium.

- 1st INTERNATIONAL WORKSHOP within APM NAWA project ScienceNet entitled: 'ART in the rescue of endangered animals' July 17, 2021.

CONGRESSES

- Poster at 1st ESAR congress (European Symposium on Animal Reproduction), held in Nantes, France, on 21st, 22nd and 23rd September 2023.

- Oral presentation at the 9th Quadrennial INTERNATIONAL SYMPOSIUM ON CANINE AND FELINE REPRODUCTION in a Joint Meeting with the 24th European Veterinary Society for Small Animal Reproduction Congress ISCFREVSSAR 2020+2 held at the University of Milan, Italy.

- Oral presentation at Still e-live... 23rd EVSSAR Congress that took place virtually on October 1-2, 2021.

- Oral presentation at the webinar entitled “L'importance des méthodes moléculaires en microbiologie” that was held in KBS on July 24, 2021.

INTERNSHIP

- Institut National de Recherche pour l’Agriculture, l’Alimentation et l'Environnement (INRAE) Centre Val de Loire Nouzilly, Tours, France

One year internship with Dr Pascal Mermillod's team, Physiology of Reproduction and Behavior (PRC) in collaboration with Dr Yann Locatelli Museum National d’Histoire Naturelle, Réserve Zoologique de la Haute Touche, Obterre, France

- ✓ Exosomes isolation
- ✓ Exosomes characterization
- ✓ routine of *in vitro* embryo production in cat

SCHOLARSHIPS

- Ph.D. scholarship for academic excellence to foreigners, under the STER project International Doctoral School at UPWr for the year 2020

- Ph.D. scholarship for academic excellence to foreigners, under the STER project International Doctoral School at UPWr for the year2020/2021

Wroclaw September 27, 2023

Meriem Baouche

Department of Reproduction and Clinic for Farm Animals
Wroclaw University of Environmental and Life Sciences pl.
Grunwaldzki 49,
50-366 Wroclaw, Poland

STATEMENT

I declare that in the paper:

Baouche M* , Ochota M, Locatelli Y, Mermillod P, Niżański W. Mesenchymal stem cells: generalities and clinical significance in feline and canine medicine. Animals. (2023) 13:1903. doi: 10.3390/ani13121903.

As the corresponding and first author, I collected the necessary literature, established the manuscript's concept, wrote the initial and final drafts, submitted it to the journal, and applied all revisions requested by the reviewers during the revision process before acceptance.

27.09.2023

Date and signature

Wroclaw September 27, 2023

Meriem Baouche

Department of Reproduction and Clinic for Farm Animals
Wroclaw University of Environmental and Life Sciences pl. Grunwaldzki 49,
50-366 Wroclaw, Poland

STATEMENT

I declare that in the paper:

Baouche M, Krawczenko A, Paprocka M, Klimczak A, Mermillod P, Locatelli Y, Ochota M* , Niżański W*. Feline umbilical cord mesenchymal stem cells: isolation and in vitro characterization from distinct parts of the umbilical cord. Theriogenology. (2023) 201:116-25. doi:10.1016/j .theriogen010gy.2022.11.049.

As the first author, I collected and evaluated the data, performed statistical analysis, and wrote the manuscript. Additionally, I made thechanges requested by the reviewers during the revision process before acceptance.

27.09.2023

Date and signature

Wroclaw September 27, 2023

Meriem Baouche
Department of Reproduction and Clinic for Farm Animals
Wroclaw University of Environmental and Life Sciences pl.
Grunwaldzki 49,
50-366 Wroclaw, Poland

STATEMENT

I declare that in the paper:

Baouche M*, Ochota M* , Mermillod P, Locatelli Y, Niżański W* Feline Wharton's Jelly derived mesenchymal stem cells as a feeder layer for oocytes maturation and embryos culture *in vitro*. Front. Vet. Sci. (2023) vol. 10. doi: 10.3389/fvets.2023.1252484

As the first author, I collected and evaluated the data, performed statistical analysis, and wrotethe manuscript. Additionally, I made the changes requested by the reviewers during the revisionprocess before acceptance.

27.09.2023

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