

Article

Isolation, Expansion and Characterization of Mesenchymal Stromal Cells from Fresh and Cryopreserved Umbilical Cord Tissues in Horse and Dog

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Simple Summary

Umbilical cord tissues are a valuable source of mesenchymal stromal cells (MSCs) for regenerative medicine because of their ability to support tissue repair and healing. This study evaluated the feasibility of whole umbilical cord cryopreservation in canine and equine species using different freezing solutions. MSCs were successfully isolated from both fresh and cryopreserved tissues and maintained their main biological and functional properties, including proliferative capacity, migration ability, multilineage differentiation, and mesenchymal marker expression. Overall, cryopreservation did not significantly affect cell characteristics, supporting the use of cryopreserved umbilical cord tissues for veterinary biobanking and future regenerative medicine applications.

Abstract

Mesenchymal stromal cells (MSCs) derived from perinatal tissues represent a promising resource for regenerative medicine due to their proliferative, immunomodulatory, and differentiation properties. Among these tissues, the umbilical cord is considered an accessible and ethically acceptable source of MSCs. This study evaluated the feasibility of whole umbilical cord cryopreservation in canine and equine species and assessed the biological properties of MSCs isolated from cryopreserved tissues compared with fresh samples. Equine Wharton's jelly and canine umbilical cord tissues were processed either immediately or after cryopreservation using two different freezing media: 10% DMSO + 10% FBS (DF) and 0.05 M glucose + 0.05 M sucrose + 1.5 M ethylene glycol (GSE). Isolated MSCs were characterized through proliferation, clonogenicity, adhesion and migration assays, trilineage differentiation, and molecular marker analysis. MSCs were successfully isolated from all experimental groups and maintained the typical morphology, plastic adherence, proliferative capacity, multilineage differentiation potential, and mesenchymal marker expression associated with MSC populations. Overall, cryopreservation did not significantly affect the main biological and functional properties of the cells. These findings support the use of cryopreserved umbilical cord tissues for veterinary biobanking and regenerative medicine applications.

Keywords: mesenchymal stem/stromal cells; umbilical cord; Wharton's jelly; cryopreservation; regenerative medicine; veterinary medicine; equine; canine; biobanking; perinatal tissues



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1. Introduction

Over the past decade, mesenchymal stromal cell (MSC)-based therapies have emerged as a promising approach in both human and veterinary medicine, owing to the capacity of MSCs for in vitro expansion, their immunomodulatory properties, and their ability to secrete a wide range of bioactive molecules that support tissue repair and regeneration [1]. The therapeutic potential of MSCs is largely attributed to their paracrine activity, mediated by a complex secretome composed of cytokines, growth factors, and extracellular vesicles, which play a key role in modulating inflammation and promoting tissue regeneration [2].

In veterinary medicine, MSC-based therapies have been increasingly applied in canine and equine patients for the treatment of various conditions, including musculoskeletal, inflammatory, and degenerative diseases, showing encouraging outcomes in terms of functional recovery and immunomodulation [3]. MSCs can be isolated from multiple sources, including adult tissues such as bone marrow [4] and adipose tissue [5], as well as perinatal tissues, including placenta [6], amniotic membrane [7], Wharton's jelly [8], and amniotic fluid [9]. Perinatal tissues represent a particularly attractive source of MSCs due to their non-invasive collection, minimal ethical concerns, and reduced donor morbidity. In addition, MSCs derived from these tissues exhibit enhanced biological properties compared with their adult counterparts, including higher proliferative capacity, lower immunogenicity, and reduced oncogenic risk [10,11].

Among perinatal sources, the umbilical cord (UC), and particularly Wharton's jelly (WJ), represents a rich and accessible source of MSCs [12]. Umbilical cord-derived MSCs (UC-MSCs) are characterized by high proliferative potential [13], strong immunomodulatory activity [14], low immunogenicity [15], and a favorable safety profile [16]. In addition, these cells show long-term genetic stability and resistance to senescence [17], and express pluripotency-associated markers such as OCT4, NANOG, SOX2, KLF-4, and SSEA-4, suggesting a more primitive phenotype compared with adult-derived MSCs [18,19]. These features make UC-MSCs particularly suitable for regenerative applications and for stem cell banking strategies.

When biobanking is considered, isolating and expanding UC-MSCs can be laborious, time-consuming, and costly, particularly when procedures must comply with current Good Manufacturing Practice (cGMP) standards for clinical applications. Cryopreservation of the whole umbilical cord immediately after collection represents an attractive alternative, allowing long-term storage with minimal manipulation. Advances in cryobiology have enabled the preservation of both isolated cells and intact tissues, with evidence suggesting that maintaining cells within their native microenvironment may enhance post-thaw recovery [20–24]. Nevertheless, cryopreservation can still negatively affect cell viability and function, potentially reducing therapeutic efficacy and inducing structural or molecular alterations [25–28].

Previous studies in human medicine have demonstrated that MSCs isolated from cryopreserved umbilical cord or placental tissues retain key biological properties, including proliferative capacity and differentiation potential [29–32]. However, limited information is available regarding the effects of whole tissue cryopreservation on UC-derived MSCs in veterinary species, particularly in dogs and horses. Furthermore, variability in cryopreservation protocols, including differences in cryoprotective agents and storage conditions, may significantly influence cell recovery and functionality.

Considering these considerations, optimizing cryopreservation strategies for UC tissues is essential for the development of standardized and reliable biobanking approaches in veterinary regenerative medicine.

Therefore, the aim of the present study was to evaluate the feasibility and efficiency of whole UC cryopreservation in canine and equine species using different freezing media. MSCs derived from cryopreserved tissues were compared with those obtained from fresh samples in terms of growth characteristics, clonogenic potential, stemness profile, functional properties, and differentiation capacity, to assess the impact of cryopreservation on their biological features.

This study demonstrates the feasibility of whole UC cryopreservation as an effective strategy for the long-term storage of perinatal tissues in canine and equine species, enabling the recovery of MSCs that retain key biological and functional properties comparable to those derived from fresh tissues.

2. Materials and Methods

2.1. Experimental Design

Equine and canine UCs were collected and processed to isolate MSCs.

For equine samples, Wharton's jelly (eWJ) was isolated and subjected to two different approaches: (i) direct MSC isolation from fresh tissue (EQ-C) and (ii) cryopreservation prior to cell isolation using two different freezing media, namely 10% DMSO + 10% FBS (EQ-DF) or 0.05 M glucose + 0.05 M sucrose + 1.5 M ethylene glycol (EQ-GSE). Cryopreserved samples were first stored at -80°C overnight and subsequently transferred to liquid nitrogen before MSCs isolation.

Similarly, canine umbilical cords (cUCs) were processed either immediately for MSC isolation from fresh tissue (CN-C) or cryopreserved using 10% DMSO + 10% FBS (CN-DF), followed by storage at -80°C overnight and long-term preservation in liquid nitrogen prior to cell isolation.

Isolated MSCs from all experimental groups were subjected to a panel of functional and molecular assays, including population doubling time (PDT) analysis, colony-forming unit (CFU) assay, adhesion and migration assays, *in vitro* differentiation, and molecular characterization.

A schematic representation of the experimental workflow is provided in Figures 1 and 2.

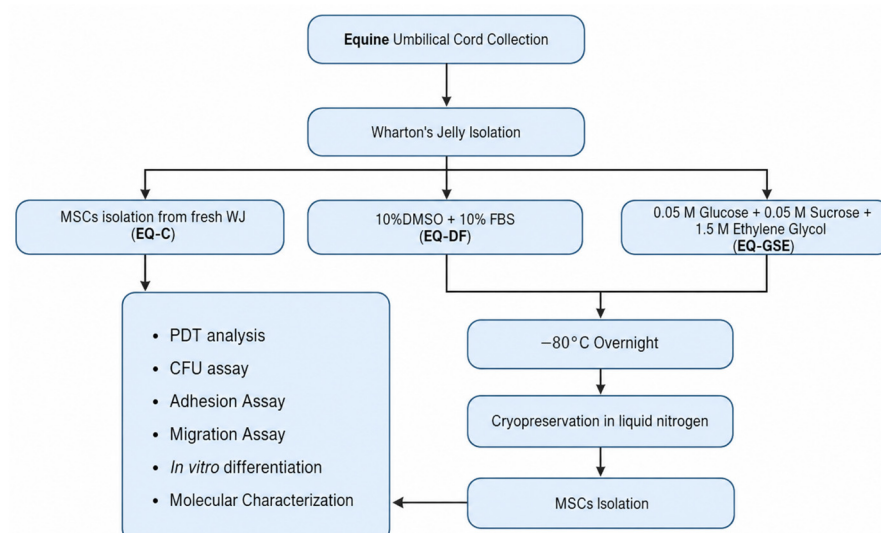


Figure 1. Schematic overview of the experimental design for equine umbilical cord samples, including Wharton's jelly isolation, fresh or cryopreserved tissue processing, and subsequent MSC isolation and characterization.

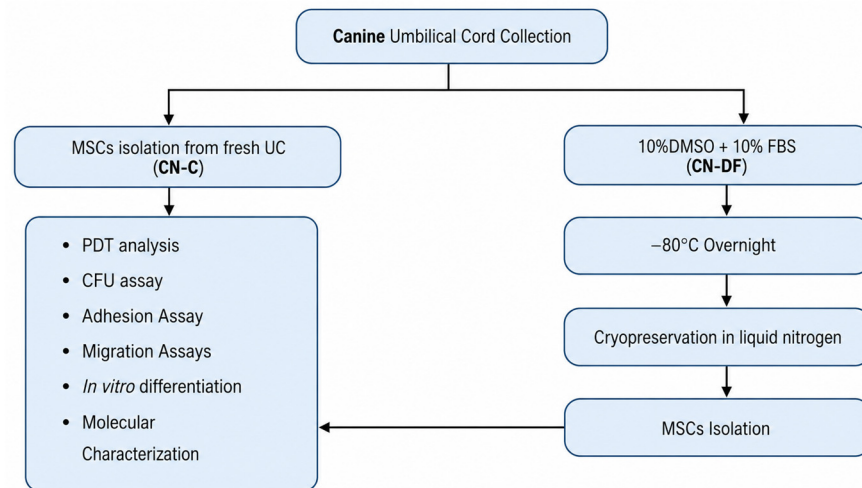


Figure 2. Schematic overview of the experimental design for canine umbilical cord samples, including fresh or cryopreserved tissue processing and subsequent MSC isolation and characterization.

2.2. Materials

Unless otherwise specified, all reagents and media used were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and GIBCO (Thermo Fisher Scientific, Waltham, MA, USA). Plastics were obtained from Sarstedt (Sarstedt AG & Co. KG, Nümbrecht, Germany) and Falcon (Corning Inc., New York, NY, USA).

2.3. Equine Umbilical Cord Collection

Equine UCs ($n = 16$) were collected immediately after delivery from Standardbred mares aged 6–15 years, hospitalized at the Equine Perinatology and Reproduction Unit (Equine Clinical Service, Department of Veterinary Medical Sciences, University of Bologna, Italy), for routine assisted parturition. All mares had normal pregnancies that concluded with uncomplicated deliveries, resulting in foals with Apgar scores of 10 at birth [33]. Mares were housed in 4×4 m straw-bedded boxes and fed hay ad libitum, with concentrates provided twice daily.

Immediately following foal–mare separation and spontaneous UC rupture, the portion of the cord closest to the foal, which is enriched in WJ, was excised using sterile scissors and rinsed under running water to remove straw or fecal contaminants. Samples were then placed in an antibiotic solution (DPBS supplemented with 100 IU/mL penicillin and 100 µg/mL streptomycin) and stored at +4 °C for a maximum of 12 h prior to transport to the Laboratory of Animal Reproduction and Biotechnologies (Equine Clinical Service, Department of Veterinary Medical Sciences, University of Bologna, Italy). All procedures were conducted in accordance with the guidelines approved by the University of Bologna Animal Welfare Committee (CoBA; protocol no. 4630/23), and written informed consent was obtained from the owners for the use of excised tissues for research purposes.

2.4. Canine Umbilical Cord Collection

Healthy adult mixed-breed dogs ($n = 9$) were referred to the Small Animal Reproduction Unit, Department of Veterinary Medical Sciences, University of Bologna (Italy) for scheduled caesarean section. Dogs were premedicated with acepromazine maleate (0.1 mg/kg; Prequillan, Fatro Spa, Metropolitan, Italy), and anesthesia was induced intravenously with thiopental sodium (15 mg/kg; Pentothal). Anesthesia was maintained with isoflurane (AErrane, Baxter, Mississauga, ON, Canada). All procedures were performed under sterile conditions.

Umbilical cord tissues were collected from discarded placental remnants following neonatal resuscitation, without manipulation of the neonatal umbilical stump. Samples were placed in DPBS supplemented with antibiotics (100 IU/mL penicillin and 100 µg/mL streptomycin) and stored at 4 °C until transfer to the Laboratory of Animal Reproduction and Biotechnologies. Written informed consent was obtained from the owners for the use of excised tissues for research purposes.

2.5. Umbilical Cord Matrix Cryopreservation

2.5.1. Equine Samples

Equine UCs samples were washed at least four times in DPBS supplemented with antibiotics (100 IU/mL penicillin and 100 µg/mL streptomycin). To further reduce the risk of microbial contamination, equine UCs were briefly immersed in 70% ethanol and subsequently washed again at least four times in DPBS containing antibiotics.

Following washing, eWJ was aseptically isolated using sterile scissors, minced into approximately 0.5 cm fragments, and weighed. Each sample was then divided into three equal portions: one processed immediately for cell isolation, and two cryopreserved using different solutions [34]:

- DPBS supplemented with 10% dimethyl sulfoxide (DMSO) and 10% fetal bovine serum (FBS) (EQ-DF group).
- DPBS supplemented with 0.05 M glucose, 0.05 M sucrose, and 1.5 M ethylene glycol (EQ-GSE group).

For cryopreservation, eWJ fragments were transferred into 1.5 mL cryogenic vials containing 1 mL of cryoprotectant solution. Samples were equilibrated at +4 °C for 10 min, then placed in a MrFrosty® freezing container (Nalgene, Rochester, NY, USA) at −80 °C for 24 h, and finally stored in liquid nitrogen (−196 °C) for up to 3 months.

For thawing, vials were removed from liquid nitrogen, briefly exposed to air for 10 s, and immersed in a 37 °C water bath until complete ice dissolution. Tissues were then transferred to 50 mL tubes for cryoprotectant removal. Twenty milliliters of culture medium (DMEM-F12 supplemented with antibiotics and 10% FBS) were added dropwise, followed by centrifugation at $1881 \times g$ for 30 min. After supernatant removal, two additional washes were performed with DPBS supplemented with 10% FBS ($470 \times g$, 10 min).

Following washing, samples were processed for cell isolation alongside fresh (control) tissues. All procedures were carried out under a laminar flow hood in compliance with ISO 9001 standards (UNI EN ISO 9001:2015; <https://www.uni.com>, accessed on 22 April 2026).

2.5.2. Canine Samples

Samples were processed following the same laboratory standards described above. After washing at least four times in DPBS + antibiotics, tissues were minced into 0.5 cm pieces and weighed. Due to limited tissue quantity, samples were divided into two portions of equal weight: one used immediately for cell isolation (CN-C) and one cryopreserved in DPBS + 10% DMSO + 10% FBS (CN-DF group). Unlike equine Wharton's jelly, which can be readily distinguished from the surrounding placental tissues based on its macroscopic appearance, the Wharton's jelly in canine umbilical cords cannot be reliably delineated and separated because of the small size of the cord and the close association of the matrix with the umbilical vessels. Therefore, the entire canine umbilical cord was processed for cell isolation.

Cryopreservation, thawing, washing, and preparation for cell isolation were performed as described for equine samples.

2.6. Cell Isolation and In Vitro Culture

As previously described [35,36], eWJ and cUC fragments were transferred into 50 mL tubes containing 0.1% type IV collagenase in DPBS (1 mL solution per 1 g of tissue). Samples were vigorously agitated and incubated at 37 °C for at least 30 min.

Following enzymatic digestion, collagenase activity was neutralized by adding DPBS supplemented with 10% FBS (1:1). The resulting suspension was filtered to remove undigested tissue and centrifuged at $1881 \times g$ for 10 min at 25 °C. The cell pellet was resuspended in 5 mL of culture medium and centrifuged again under the same conditions ($1881 \times g$, 10 min).

Cells were then resuspended in culture medium (equine: DMEM-F12 supplemented with 10% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin; canine: DMEM-F12 supplemented with 20% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin), seeded into 25 cm² flasks, and incubated at 38.5 °C in a humidified CO₂ atmosphere. This stage was defined as Passage 0 (P0).

The culture medium was first replaced after 48 h and subsequently every 3 days until cells reached 80–90% confluence. At this point, cells were detached using a 0.25% Trypsin-EDTA (ethylenediaminetetraacetic acid) solution, and the enzymatic reaction was neutralized with DPBS supplemented with 10% FBS (1:2). Cells were then centrifuged at $470 \times g$ for 6 min and subsequently counted using an improved Neubauer chamber.

Cells were reseeded in 25 cm² flasks at a density of 5000 cells/cm² and designated as Passage 1 (P1). Cells at Passage 3 (P3) were used for subsequent in vitro assays.

2.7. Characterization of eWJ- and cUC-MSCs

At 80–90% confluence at Passage 2 (P2), cells from all equine and canine samples were detached, counted, and assessed for viability using a 0.4% Trypan blue staining solution. Cells were subsequently seeded to evaluate their growth kinetics through population doubling time (PDT) analysis and for characterization by colony-forming unit (CFU) assay, adhesion and migration assays, and tri-lineage in vitro differentiation.

In addition, CD marker expression was analyzed by RT-PCR. All experiments were performed in triplicate for each sample.

2.7.1. Population Doubling Time (PDT) Analysis

To determine growth rate, eWJ- and cUC-MSCs derived from both fresh and cryopreserved tissues were seeded in 6-well plate at a cell density of 5000 cells/cm². Cell number was assessed every day for 7 days in triplicate. The population doubling time (PDT) was calculated from the log phase of the growth curve using the equation described by Bezerra et al. (2022) [37]:

$$\text{PDT} = (t \times \log 2) / \log (Nt/N0) \quad (1)$$

where t represents the period of cultivation in days, $N0$ represents the initial number of cells, and Nt represents the number of cells in a specific period of culture.

2.7.2. CFU (Colony-Forming Unit) Assay

For determining the ability of cells to form colonies, 1×10^2 cells were cultured for 4 weeks in a 6-well plate. Colonies were then fixed in 4% paraformaldehyde at RT for 1 h and stained with Giemsa 0.1% stain (30 min). Using an inverted light microscope (Eclipse TE 2000-U, Nikon Instruments Inc., Tokyo, Japan), the operator counted colonies formed by at least 16–20 nucleate cells.

2.7.3. Adhesion and Migration Assays

To determine whether eWJ- and cUC-MSCs derived from both fresh and cryopreserved tissues preserved their adhesion capability, a spheroid formation assay was performed. Differently from the cell-substratum adhesion, performed on monolayer cultures adherent to rigid substrates, this test gives information about the direct cell–cell adhesion architecture found in normal tissues. Cells were cultured through the ‘hanging-drop’ method in Corning spheroid microplates (96-well, black/clear bottom round, Ultra-Low Attachment surface, Corning Inc., New York, NY, USA, 5000 cells/25 µL drop). Bright-field images were acquired by a CCD camera (DS-Fi2, Nikon, Tokyo, Japan) mounted on an inverted light microscope (Eclipse TE 2000-U, Nikon Instruments Inc., Tokyo, Japan) using 4× magnification 24 h after seeding. Starting from the binary masks obtained by Fiji software (ImageJ, v. 1.8.0_345/1.54g), the volume of each spheroid was computed using ReViSP (<https://sourceforge.net/projects/revisp/>, v. 2.3, accessed on 22 April 2026) [38], a software specifically designed to accurately estimate the volume of spheroids and to render an image of their 3D surface.

To assess cell migration potential, a scratch assay (or wound-healing assay) was carried out, as previously described by Liang et al. [39]. Briefly, P3 cells were seeded (5000 cells/cm²) in a 6-well plate. At 80 to 90% confluence the cell monolayer was scraped using a 200 µL pipette tip. The cells were then washed twice with DPBS to remove any detached cells, and the plate was incubated under standard culture conditions. Images were captured at the same two locations of each well, both immediately after the tip-scratch (time 0 = t₀) and every 24 h over a 48 h period (0, 24, 48 h). The distances of each scratch closure were measured by Fiji software (ImageJ, v. 1.8.0_345/1.54g), and the migration percentages (*Wound Closure %*) were calculated using the following formula [40]:

$$[(\text{distance at } t_0 - \text{distance at } t_n) \times 100] / \text{distance at } t_0 \quad (2)$$

where t_n corresponds to the distance at each specific time point analyzed.

2.7.4. Multi-Lineage In Vitro Differentiation

In vitro differentiation potential toward osteogenic, adipogenic, and chondrogenic lineages of eWJ- and cUC-MSCs derived from both fresh and cryopreserved tissues was also evaluated. Cells were seeded in 24-well plates at a density of 5000 cells/cm² and cultured until sub-confluency. Then, the culture medium was replaced, and cells were cultured for 3 weeks under specific induction media (Table 1) [41]. As a negative control, an equal number of cells was cultured in expansion medium. The specific induction media and expansion medium were replaced twice a week. To assess differentiation, cells were fixed with 4% paraformaldehyde at RT for 1 h. Differentiation was visualized using Oil Red O for adipogenic vacuoles, Alcian Blue for sulfated proteoglycans, and Alizarin Red S for calcium deposits. Bright-field images were captured using a CCD camera (DS-Fi2, Nikon, Tokyo, Japan) mounted on an inverted light microscope (Eclipse TE 2000-U, Nikon Instruments Inc., Tokyo, Japan) at 10× magnification.

Quantification of positively stained areas was performed using Fiji software (ImageJ, v. 1.8.0_345/1.54 g) following the methodology described by Heyman et al. (2022) [42]. The “ColourDeconvolution2” plugin was applied to separate the stain and background channels, which were then converted to 8-bit grayscale images and binarized using auto-thresholding. The positively stained areas were measured, and the differentiation ratio (DR) was calculated as the ratio between the areas of the stain-positive signal and the background. To compare differentiation across samples, a normalized DR was determined for each sample by dividing the mean DR of the differentiated wells by the mean DR of the non-induced controls.

Table 1. Specific induction media compositions.

Adipogenic	Chondrogenic	Osteogenic
DMEM	DMEM	DMEM
10% FBS	1% FBS	10% FBS
1 μ M DXM ² (removed after 6 days)	0.1 μ M DXM ²	0.1 μ M DXM ²
0.5 mM IBMX ¹ (removed after 3 days)	50 nM AA2P ⁴	50 μ M AA2P ⁴
10 μ g/mL insulin	6.25 μ g/mL insulin	10 mM BGP ⁵
0.1 mM indomethacin	10 ng/mL hTGF- β 1 ³	

¹ IBMX: isobutylmethylxanthine, ² DXM: dexamethasone, ³ hTGF- β 1: human transforming growth factor- β 1,

⁴ AA2P: ascorbic acid 2-phosphate, ⁵ BGP: beta-glycerolphosphate.

2.7.5. Molecular Characterization

For the molecular characterization of eWJ- and cUC-MSCs derived from both fresh and cryopreserved tissues, total RNA was extracted from snap-frozen cells using the NucleoSpin[®] RNA Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol. Residual genomic DNA was removed by treating the RNA with Amplification-Grade DNase I (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA (cDNA) was synthesized from the purified RNA using the RevertAid Reverse Transcriptase Kit (Thermo Fisher Scientific, Waltham, MA, USA).

The resulting cDNA was directly subjected to PCR using the 2 $\times$ EpiArt[™] HS Taq Master Mix (Vazyme Biotech Co., Ltd., Nanjing, China). The expression of genes encoding mesenchymal stromal cell markers (equine: *CD90*, *CD73*; canine: *CD90*, *CD44*), hematopoietic markers (equine: *CD45*, *CD34*; canine: *CD45*, *CD34*), and major histocompatibility complex markers (equine: *MHC-I*, *MHC-II*; canine: *DLA-DRA1*, *DLA-DQA1*) was analyzed. To ensure the proper expression of samples, *GAPDH* was used as a housekeeping gene. The specific primers employed for amplification are listed in Table 2 [43–45].

PCR products were resolved on a 2% agarose gel stained with ethidium bromide. Electrophoresis was conducted to verify the expected band sizes, and gels were visualized and photographed under ultraviolet light using a UVP PhotoDoc-It Imaging System (UVP, LLC, Upland, CA, USA). The presence of discrete bands corresponding to the expected sizes confirmed positive expression, while the absence of bands indicated a negative result. A 50 bp DNA Ladder (Thermo Fisher Scientific, Waltham, MA, USA) was used as a molecular size marker.

2.8. Statistical Analysis

Data are presented as mean $\pm$ standard deviation (SD). Statistical analyses and graph generation were performed using GraphPad Prism software (version 9.0.0). Data normality was assessed using the Shapiro–Wilk test. For equine samples, population doubling times (PDTs) and colony-forming unit (CFU) counts were analyzed using one-way ANOVA, followed by Tukey's post hoc test for multiple comparisons. Cell yield, wound closure percentages and spheroid area and volume measurements were analyzed using the Kruskal–Wallis test, followed by Dunn's multiple comparisons test. For canine samples, cell yield, PDTs, CFU counts, and spheroid area and volume measurements were analyzed using the Mann–Whitney test, whereas wound closure percentages and normalized DR were analyzed using an unpaired *t*-test. Differences were considered statistically significant at *p* < 0.05.

Table 2. Primers sequences for PCR analysis.

Primers		References	Sequences (5' → 3')	bp
MSC marker				
Equine	CD90	[43]	F: TGCGAACCTCCGCCTCTCT R: GCTTATGCCCTCGCACTTG	93
	CD73	[43]	F: GGGATTGTTGGATACACTTCAAAAG R: GCTGCAACGCAGTGATTCA	90
Canine	CD90	[46]	F: CAGCATGACCCGGGAGAAAAAG R: TGGTGGTGAAGCCGGATAAGTAGA	134
	CD44	[47]	F: GCCCTGAGCGTGGGCTTTGA R: TCTGGCTGTAGCGGGTGCCA	268
Hematopoietic markers				
Equine	CD34	[43]	F: CACTAAACCCTCTACATCATTTTCTCCTA R: GGCAGATACCTTGAGTCAATTTCA	101
	CD45	[43]	F: TGATTCCCAGAAATGACCATGTA R: ACATTTTGGGCTTGTCTCTGTAAC	101
Canine	CD34	[47]	F: GCCTGCTCAGTCTGCTGCCC R: TGGTCCCAGGCGTTAGGGTGA	255
	CD45	[47]	F: CTCACGCACACAGGCTCGCA R: CCCACCCACTGGCACTGCTG	159
MHC markers				
Equine	MHC-I	[44]	F: GGAGAGGAGCAGAGATACA R: CTGTCACTGTTTGCAGTCT	218
	MHC-II	[44]	F: TCTACACCTGCCAAGTG R: CCACCATGCCCTTTCTG	178
Canine	DLA-DRA1	[47]	F: CGCTCCAACCACACCCCGAA R: GGCTGAGGGCAGGAAGGGGA	246
	DLA-DQA1	[47]	F: GCACTGGGGCCTGGATGAGC R: ACCTGAGCGCAGGCCTTGGA	163
Housekeeping				
Equine	GAPDH	[45]	F: GTCCATGCCATCACTGCCAC R: CCTGCTTACCACCTTCTTG	262
Canine	GAPDH	[48]	F: GGTCAACCAGGGCTGCTTT R: ATTTGATGTTGGCGGGAT	209

3. Results

3.1. Isolation Efficiency and Morphological Characterization of eWJ- and cUC-MSCs

Mesenchymal stromal cells (MSCs) were successfully isolated from equine Wharton's jelly (eWJ) and canine umbilical cord (cUC) tissues in all experimental groups, including fresh (C) and cryopreserved samples (DF and GSE), based on their ability to adhere to plastic.

Representative phase-contrast images of the isolated cells are shown in Figure 3. Phase-contrast microscopy revealed a homogeneous population of spindle-shaped, fibroblast-like cells across all groups, consistent with the typical MSC morphology. Adherent cells were detectable as early as 24 h post-seeding, forming discrete colonies that progressively expanded until reaching confluence.

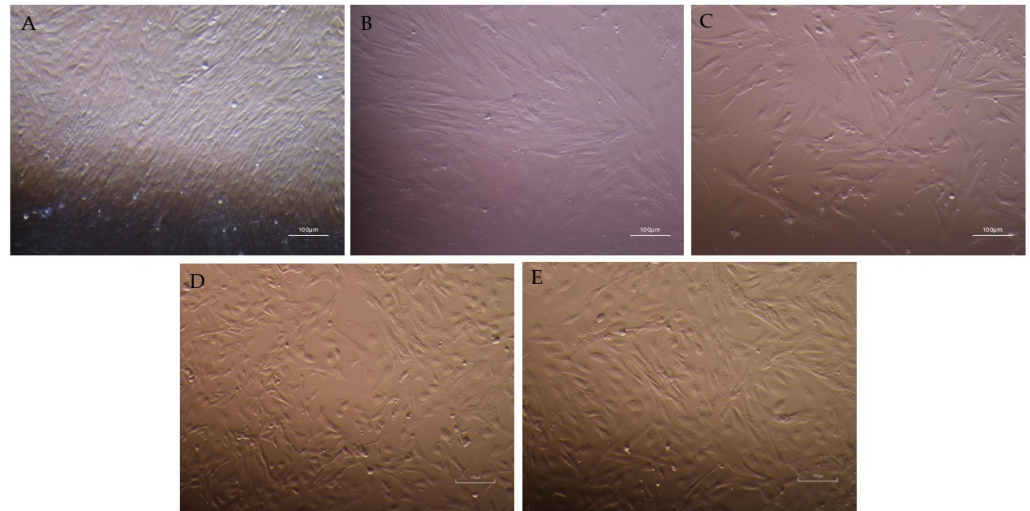


Figure 3. Representative phase-contrast images of MSC monolayers isolated from fresh and cryopreserved tissues. (A) EQ-C; (B) EQ-DF; (C) EQ-GSE; (D) CN-C; and (E) CN-DF. Magnification 100 $\times$.

Quantitative analysis of cell yields at confluence revealed differences between species and experimental conditions. In equine samples, freshly processed tissues (EQ-C) yielded between 0.07 and 1.95×10^6 cells. Similar ranges were observed in samples cryopreserved in DMSO/FBS (EQ-DF), with yields between 0.03 and 1.75×10^6 cells. Lower cell numbers were obtained from samples cryopreserved using the glucose–sucrose–ethylene glycol solution (EQ-GSE), ranging from 0.01 to 0.11×10^6 cells. Moreover, cell yield was significantly lower in the EQ-GSE group compared with fresh tissues (EQ-C) ($p < 0.05$).

In canine samples, MSCs were successfully isolated from both fresh and cryopreserved tissues. Fresh cUC samples (CN-C) yielded between 0.11 and 0.475×10^6 cells, whereas cryopreserved samples (CN-DF) showed cell yields ranging from 0.6 to 1.68×10^6 cells. Cell yield was significantly higher in the CN-DF group compared with the CN-C group ($p < 0.05$).

3.2. Characterization of eWJ- and cUC-MSCs

3.2.1. Population Doubling Time (PDT) Analysis

To assess the proliferative capacity of eWJ- and cUC-MSCs derived from fresh and cryopreserved tissues, population doubling times (PDTs) were calculated.

In equine samples, no significant differences in PDT values were observed among the experimental groups. Specifically, PDT values were 1.21 ± 0.58 days for fresh samples (EQ-C), 1.34 ± 0.52 days for DMSO/FBS cryopreserved samples (EQ-DF), and 1.28 ± 0.51 days for GSE cryopreserved samples (EQ-GSE).

Similarly, in canine samples, comparable PDT values were observed between fresh and cryopreserved groups, with CN-C cells showing a PDT of 1.28 ± 0.41 days and CN-DF cells a PDT of 1.44 ± 0.10 days, with no statistically significant differences between the groups.

3.2.2. CFU (Colony-Forming Unit) Assay

The CFU assay was performed to evaluate the clonogenic capacity of eWJ- and cUC-MSCs.

In equine samples, MSCs derived from cryopreserved tissues showed higher CFU numbers compared to fresh samples. Specifically, the EQ-DF group generated the highest number of colonies (126 ± 23.51), followed by the EQ-GSE group (117 ± 13.23), with no significant differences between the two cryopreserved conditions. In contrast, MSCs from fresh tissues (EQ-C) formed a lower number of colonies (89 ± 8.54); however, this difference did not reach statistical significance.

Similarly, in canine samples, MSCs isolated from cryopreserved tissues (CN-DF) showed a slightly higher clonogenic capacity compared to fresh tissues (CN-C), forming 140 ± 31.3 and 124.4 ± 34 colonies, respectively, although no statistically significant differences were observed between the groups.

3.2.3. Adhesion and Migration Assays

At Passage 3 (P3), using a hanging drop culture system, all tested MSC populations formed three-dimensional spheroid-like structures after 24 h of culture, indicating the ability to establish cell–cell interactions. Bright-field images were acquired at 24 h and analyzed using ReViSP (<https://sourceforge.net/projects/revisp/>, v. 2.3, accessed on 22 April 2026), an open-source voxel-based 3D image analysis tool that estimates spheroid volume from binary masks and generates three-dimensional surface reconstructions. Quantitative analysis revealed differences in spheroid size among experimental groups. In equine samples, EQ-DF spheroids displayed significantly greater area and volume compared to EQ-C (** $p < 0.01$, Figure 4A,B). In canine samples, CN-DF spheroids showed significantly lower area and volume compared to CN-C (**** $p < 0.0001$, Figure 4C,D).

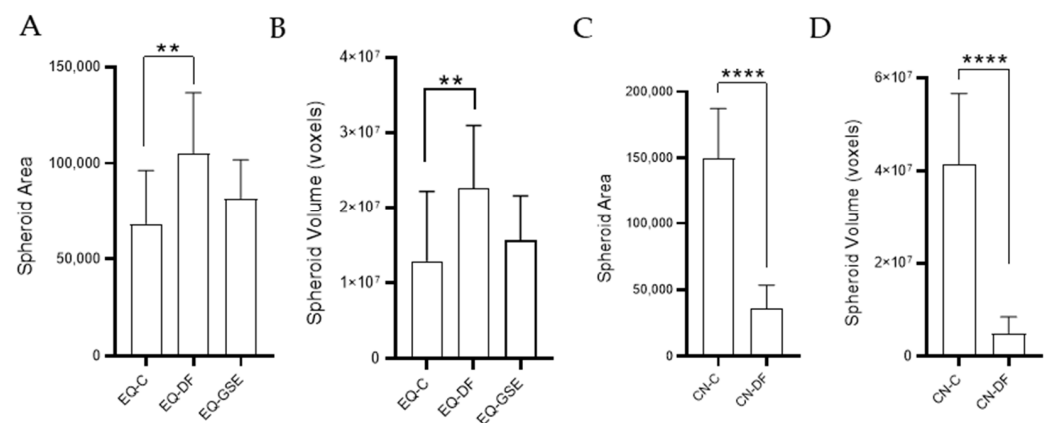


Figure 4. Quantitative analysis of spheroid area and volume in eWJ- and cUC-MSCs after 24 h of hanging drop culture. (A) Spheroid area and (B) spheroid volume of equine MSCs derived from fresh (EQ-C) and cryopreserved tissues (EQ-DF and EQ-GSE). (C) Spheroid area and (D) spheroid volume of canine MSCs derived from fresh (CN-C) and cryopreserved tissues (CN-DF). Data are expressed as mean $\pm$ SD. Statistical analysis: Kruskal–Wallis test followed by Dunn’s multiple comparisons test for equine samples and Mann–Whitney test for canine samples. Significant differences are indicated as follows: ** $p < 0.01$; **** $p < 0.0001$. Measure units: pixels for area and voxels for volume.

To evaluate the migratory potential of eWJ- and cUC-MSCs, wound closure percentage was measured at 48 h. Quantitative analysis revealed no significant differences among the experimental groups. In equine samples, wound closure percentages were similar between fresh (EQ-C, 44.78 ± 15.07) and cryopreserved groups (EQ-DF, 44.99 ± 2.63 ; EQ-GSE, 45.39 ± 12.76) (Figure 5A). Likewise, in canine samples, comparable values were observed between fresh (CN-C, 30.94 ± 15.96) and cryopreserved conditions (CN-DF, 44.78 ± 15.07) (Figure 5B).

3.2.4. Multi-Lineage In Vitro Differentiation

eWJ- and cUC-MSCs were assessed for their trilineage differentiation potential (adipogenic, chondrogenic, and osteogenic) in accordance with ISCT guidelines.

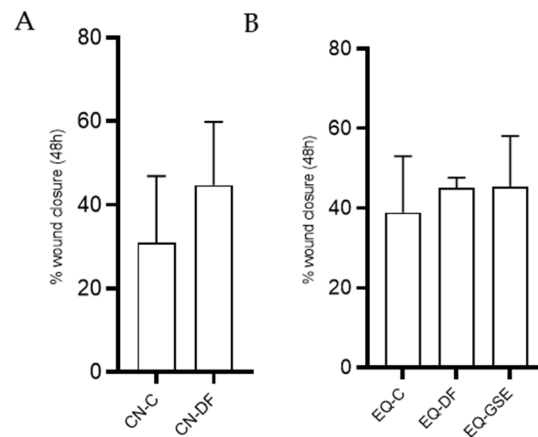


Figure 5. Quantitative analysis of wound closure percentage in eWJ- and cUC-MSCs after 48 h. (A) Wound closure percentage of equine MSCs derived from fresh (EQ-C) and cryopreserved tissues (EQ-DF and EQ-GSE). (B) Wound closure percentage of canine MSCs derived from fresh (CN-C) and cryopreserved tissues (CN-DF). Data are expressed as mean $\pm$ SD. Statistical analysis: Kruskal–Wallis test followed by Dunn’s multiple comparisons test for equine samples and unpaired *t*-test for canine samples. No statistically significant differences were observed among the experimental groups.

After 3 weeks of adipogenic induction, cells from all experimental groups exhibited morphological changes from a spindle-shaped to a rounded or oval morphology, along with the presence of intracytoplasmic lipid droplets. Lipid accumulation was confirmed by positive Oil Red O staining (Figure 6). In contrast, control cells maintained a fibroblast-like morphology and showed no staining.

Chondrogenic differentiation was observed after 21 days of induction in all groups, as indicated by the formation of rounded cell aggregates and extracellular matrix deposition. The presence of sulfated proteoglycans was confirmed by positive Alcian Blue staining (Figure 5), whereas control samples showed no staining.

Following osteogenic induction, all groups displayed calcium deposition within the extracellular matrix, as demonstrated by positive Alizarin Red S staining (Figure 5). No mineralization was observed in control cultures.

No significant differences in trilineage differentiation were observed among equine groups (EQ-C, EQ-DF, and EQ-GSE). In canine samples, however, quantitative analysis of adipogenic differentiation revealed significantly higher lipid accumulation in CN-C (normalized DR: 2.47 ± 1.14) cells compared with CN-DF (normalized DR: 0.91 ± 0.29) cells ($p < 0.01$). Representative images of adipogenic, osteogenic, and chondrogenic differentiation are shown in Figure 5, while the complete panel of images for all experimental groups is reported in the Supplementary Materials.

3.2.5. Molecular Characterization

Gene expression analysis at P3 was performed to characterize eWJ- and cUC-MSCs derived from fresh and cryopreserved tissues, evaluating mesenchymal markers (*CD90* and *CD73*), hematopoietic markers (*CD34* and *CD45*), and immunogenic markers (*MHC-I* and *MHC-II* in equine samples; *DLA-DRA1* and *DLA-DQA1* in canine samples), with *GAPDH* used as housekeeping gene.

Qualitative PCR analysis confirmed that all MSC populations consistently expressed the mesenchymal markers *CD90* and *CD73*, while no expression of the hematopoietic markers *CD34* and *CD45* was detected.

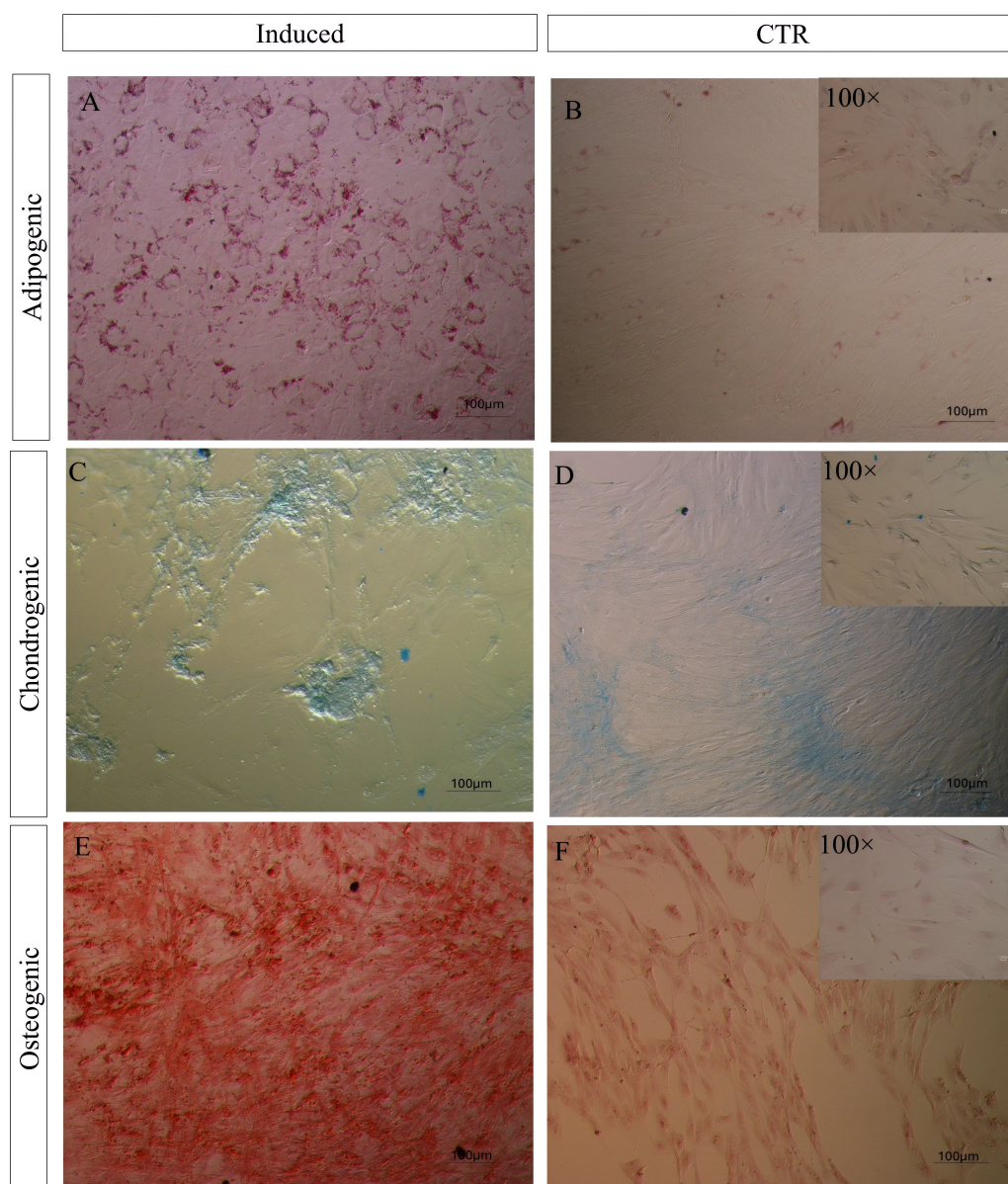


Figure 6. Representative images of trilineage differentiation of eWJ- and cUC-MSCs after 21 days of induction. (A) Adipogenic differentiation was confirmed by Oil Red O staining of intracellular lipid droplets; (C) chondrogenic differentiation by Alcian Blue staining of sulfated proteoglycans within the extracellular matrix; and (E) osteogenic differentiation by Alizarin Red S staining of calcium deposits. Control cultures (B,D,F) maintained in standard growth medium showed no positive staining. Representative images for all experimental groups are reported in the Supplementary Materials. Magnification: 40× and 100×.

Regarding immunogenic markers, in equine samples, *MHC-II* expression was undetectable across all groups. *MHC-I* expression was detected in only one out of three samples, and exclusively in the cryopreserved conditions (DF and GSE), while it was not detected in fresh samples. In canine samples, *DLA-DRA1* was not detected in any group, while *DLA-DQA1* expression was observed only in one CN-DF sample.

The results are summarized in Table 3.

Table 3. Results of PCR analysis of MSCs derived from six colostrum samples at P3.

Sample	MSC Marker			Hematopoietic Markers		MHC Markers			
	CD90	CD73	CD44	CD34	CD 45	MHC-I	MHC-II	DLA-DRA1	DLA-DQA1
EQ-C	+	+	NA	—	—	—	—	NA	NA
EG-DF	+	+	NA	—	—	+ / —	—	NA	NA
EQ-GSE	+	+	NA	—	—	+ / —	—	NA	NA
CN-C	+	NA	+	—	—	NA	NA	—	—
CN-DF	+	NA	+	—	—	NA	NA	—	+ / —

NA: Not Applicable.

4. Discussion

Perinatal tissues, including the umbilical cord (UC) and Wharton's jelly (WJ), are increasingly recognized as a valuable and readily accessible sources of mesenchymal stromal/stem cells (MSCs) for regenerative medicine applications in both human and veterinary medicine [49,50]. These tissues provide a non-invasive and ethically acceptable source of cells characterized by high proliferative potential, low immunogenicity, and strong immunomodulatory properties [50,51]. In this context, the development of reliable biobanking strategies for perinatal tissues is of growing interest. The present study demonstrates that whole umbilical cord cryopreservation represents a feasible strategy for preserving equine and canine perinatal tissues while maintaining the biological and functional characteristics of the derived mesenchymal stromal cells. MSCs isolated from cryopreserved tissues retained their characteristic morphology, proliferative capacity, clonogenic potential, migratory ability, multilineage differentiation capacity, and molecular profile typically associated with MSC populations. These findings support whole-tissue cryopreservation as a practical approach for veterinary biobanking, allowing MSC isolation only when clinically or experimentally required. Interest in whole-tissue cryopreservation has increased because it minimizes tissue manipulation immediately after collection while preserving the native extracellular matrix, which may provide structural and biochemical protection to resident stem cells during freezing and thawing. Previous studies in human medicine have reported successful recovery of MSCs from cryopreserved Wharton's jelly and umbilical cord tissues [29–32,52–54], whereas information in veterinary species remains limited. The present work extends these observations to equine and canine tissues, showing that long-term cryostorage does not compromise the ability to recover functionally competent MSCs.

MSCs were successfully isolated from all fresh and cryopreserved tissues, and all cell populations exhibited the typical spindle-shaped fibroblast-like morphology and plastic adherence defined by the International Society for Cell & Gene Therapy (ISCT) criteria [55]. These findings are consistent with previous studies showing that MSCs recovered from cryopreserved adult [56–60] and perinatal [29–32,54,60–70] tissues retain their typical morphology, plastic adherence, viability, and in vitro expansion capacity, including when alternative cryoprotective formulations based on sucrose, ethylene glycol, glycerol, or propylene glycol are used [31,63,70].

Although MSCs were successfully isolated from all experimental groups, cell yield varied according to species and cryopreservation protocol. Equine tissues cryopreserved using the conventional DMSO/FBS-based protocol (EQ-DF) yielded cell numbers comparable to those obtained from fresh (EQ-C) samples, whereas lower cell yields were observed in the EQ-GSE group. This finding suggests that, under the experimental conditions adopted in the present study, the alternative cryoprotective solution provided less effective protection during freezing and thawing. Reduced recovery, however, should not necessarily be inter-

puted as reduced cell quality. Cryopreservation may differentially affect the proportion of viable cells recovered after tissue digestion without altering the biological properties of surviving MSCs. Similar findings were reported by Shivakumar et al. [31] for human Wharton's jelly, where lower isolation efficiency did not impair proliferation or differentiation of the recovered cells. Conversely, canine cryopreserved tissues (CN-DF) showed higher cell yields than fresh (CN-C) samples. Although this unexpected finding should be interpreted cautiously because of the limited sample size and the intrinsic heterogeneity of umbilical cord tissues [71,72], several factors may have contributed to this observation. Cryopreservation has been reported to alter tissue architecture and extracellular matrix organization, which may facilitate enzymatic tissue dissociation and enhance cell release during the isolation procedure [73,74]. In addition, canine umbilical cords display considerable structural heterogeneity, particularly because the entire cord, rather than isolated Wharton's jelly, was processed in the present study. Consequently, variability in connective tissue composition among donors may therefore have contributed to differences in digestion efficiency and final cell recovery. It is also possible that cryopreservation partially disrupted the extracellular matrix, thereby improving collagenase accessibility and facilitating the release of stromal cells during tissue processing. Considering the limited number of biological replicates, these findings should be interpreted cautiously and warrant confirmation in larger cohorts.

Despite differences in initial cell recovery, cryopreservation did not affect the intrinsic proliferative behavior of MSCs. Population doubling times (PDT) were comparable among fresh and cryopreserved groups in both species, indicating that *in vitro* growth kinetics were preserved following freeze–thaw procedures. These findings suggest that cryopreservation primarily affects the efficiency of the initial cell isolation process rather than the intrinsic proliferative capacity of the surviving MSCs. This observation indicates that cells able to survive cryopreservation retain their ability to expand *in vitro*, supporting the hypothesis that whole tissue cryostorage does not compromise the biological fitness of recovered MSC population. These results are consistent with previous reports showing that MSCs isolated from cryopreserved perinatal tissues preserve their capacity for extensive *in vitro* expansion [29,54,59,62,63,65,67,69].

Similarly, clonogenic capacity was preserved after cryopreservation. The slightly higher CFU numbers observed in cryopreserved groups, although not statistically significant, may reflect the preferential survival or recovery of MSC subpopulations displaying greater clonogenic potential, rather than a true stimulatory effect of cryopreservation itself. Alternatively, the freeze–thaw process may have selectively eliminated more vulnerable cells, resulting in cultures relatively enriched in progenitor cells with higher colony-forming ability, as reported in other MSC sources, where cryopreservation selectively preserves cells displaying greater stress resistance without altering their stem cell phenotype [54,75]. However, this interpretation deserves further investigation.

Functional analyses further demonstrated that cryopreservation preserved essential MSC properties involved in tissue repair. The ability of MSCs to form spheroids within 24 h was maintained in all experimental groups, consistent with previous studies [35,76,77]. These findings confirm that cryopreservation does not impair the capacity of MSCs to establish cell–cell interactions and self-organize into three-dimensional aggregates, a property that has been associated with enhanced secretion of trophic and immunomodulatory factors and, consequently, improved regenerative potential. Interestingly, cryopreservation induced species-dependent differences in spheroid morphology. Specifically, equine MSCs formed larger spheroids, whereas canine MSCs generated smaller and more compact aggregates. These opposite responses likely reflect intrinsic differences in extracellular matrix composition, cell adhesion molecules or cytoskeletal organization between species

rather than a direct detrimental effect of cryopreservation. Because spheroid size does not necessarily correlate with biological functionality, these morphological differences should be interpreted cautiously. The preservation of spheroid-forming ability itself is likely more relevant than absolute spheroid dimensions, as it indicates maintenance of cell–cell communication and tissue-like organization after cryostorage.

Likewise, migration assays demonstrated that cryopreservation did not affect MSC migratory capacity. After 48 h, no significant differences in wound closure were observed among experimental groups in either species, with an overall closure rate of approximately 42%. Cell migration is considered one of the key functional properties of MSCs, as it contributes to tissue homing and localization at sites of injury following transplantation. Therefore, the preservation of migratory ability after cryopreservation represents an important functional indicator supporting the potential therapeutic applicability of cryopreserved tissue-derived MSCs. These results are consistent with previous data generated in our laboratory, reporting comparable migration efficiencies in canine UC-MSCs ($49.0 \pm 24.2\%$) [35] and equine WJ-MSCs ($38.20 \pm 2.88\%$) [78]. Together, the spheroid formation and migration assays indicate that cryopreservation preserved not only the proliferative characteristics of MSCs, but also functional properties closely associated with their regenerative potential.

The trilineage differentiation assay further demonstrated that both equine WJ- and canine UC-derived MSCs preserved adipogenic, chondrogenic and osteogenic potential after cryopreservation, fulfilling the minimal ISCT criteria for MSC characterization [70]. No differences were observed among equine groups (EQ-C, EQ-DF, and EQ-GSE), indicating that neither cryopreservation protocol affected eWJ-MSC multilineage potential, in agreement with previous reports on human perinatal tissues [29–31,54,65,66,75]. In canine samples, chondrogenic and osteogenic differentiation were comparable between fresh and cryopreserved cells, whereas adipogenic differentiation was reduced in MSC from cryopreserved tissues. This selective effect suggests that individual differentiation pathways may display different sensitivities to cryopreservation-induced stress rather than reflecting a generalized impairment of stem cell function. Previous studies have likewise reported that adipogenesis is often the lineage most susceptible to cryopreservation, probably because it relies on early metabolic reprogramming, cytoskeletal remodeling, and lipid metabolism, processes that may be particularly vulnerable to osmotic and oxidative stress during freezing and thawing [56]. Although the biological mechanisms underlying this selective reduction remain unclear, it is noteworthy that the maintenance of the overall multipotent phenotype indicates that cryopreservation did not compromise the fundamental biological identity of the recovered MSCs but rather induced only a modest modulation of one specific differentiation pathway.

Gene expression analysis provided additional evidence that cryopreservation preserved the molecular identity of MSCs. All cell populations expressed the expected mesenchymal markers while lacking hematopoietic markers expression, confirming successful isolation of stromal cell populations irrespective of tissue preservation. These findings agree with previous studies reporting the immunophenotypic stability of MSCs derived from cryopreserved umbilical cord and Wharton's jelly tissues [31,54,64–66,68,75]. Particular attention should be paid to the immunogenic markers. *MHC-II* and *DLA-DRA1* transcripts were undetectable in all samples, whereas *MHC-I* expression in equine MSCs and *DLA-DQA1* expression in a single canine sample were detected only sporadically in isolated cryopreserved samples. Because these findings were inconsistent across donors and were not associated with a specific cryopreservation protocol, they do not support a systematic effect of tissue cryostorage on MSC immunogenicity. Instead, several biological and methodological factors may explain this variability. First, MSC populations derived from perinatal tissues are intrinsically heterogeneous, and donor-to-donor variability in

basal expression of immune-related genes has been widely documented. Consequently, in the stromal subpopulations recovered after tissue digestion may have contributed to the occasional detection of these transcripts. Second, cryopreservation may induce transient osmotic or oxidative stress responses capable of modulating the transcription of immune-related genes without necessarily producing stable phenotypic or functional alterations. Finally, because marker expression was assessed by conventional end-point RT-PCR, weak amplification of low-abundance transcripts close to the analytical detection threshold cannot be excluded. Therefore, sporadic gene expression should not be interpreted as evidence of increased immunogenicity but rather as the combined result of normal biological variability and the intrinsic sensitivity of the analytical method. This interpretation is further supported by previous studies demonstrating that equine and canine MSCs constitutively express very low levels of major histocompatibility molecules and generally upregulate these markers only after exposure to inflammatory stimuli such as interferon- γ [79–83]. Nevertheless, some limitations should be acknowledged. Molecular characterization relied on conventional RT-PCR and therefore provided qualitative rather than quantitative assessment of gene expression without confirmation at the protein level. In addition, the limited availability of validated antibodies for several equine MSC markers currently restricts comprehensive immunophenotypic characterization by flow cytometry, making the adoption of the same molecular approach for both species the most appropriate strategy to ensure methodological consistency. Furthermore, functional immunomodulatory assays were beyond the scope of the present study and should be included in future investigations to determine whether whole-tissue cryopreservation also preserves the immunoregulatory activity of veterinary MSCs.

Overall, despite these limitations and the relative limited number of biological replicates determined by the tissue availability, the present findings provide compelling evidence that whole umbilical cord cryopreservation effectively preserves the principal biological characteristics of equine and canine MSCs. Although the conventional DMSO/FBS-protocol provided the highest recovery efficiency, the glucose–sucrose–ethylene glycol-formulation also enabled successful MSC isolation while maintaining the essential stem cell properties of recovered cells. Collectively, these findings support the implementation of whole perinatal tissue biobanking as a practical and reliable strategy for veterinary regenerative medicine and provide a foundation for future optimization of clinically translatable cryopreservation protocols.

5. Conclusions

This study demonstrates that whole umbilical cord cryopreservation is a reliable approach for the long-term preservation of equine and canine perinatal tissues. MSCs isolated from cryopreserved tissues, using both the conventional DMSO/FBS-based protocol and the alternative glucose–sucrose–ethylene glycol formulation, retained the key biological and functional characteristics of fresh-derived cells, including proliferative capacity, clonogenic potential, migratory ability, multilineage differentiation, and expression of mesenchymal markers. Although the conventional DMSO/FBS protocol provided the highest cell recovery, both cryopreservation strategies enabled the successful isolation of functionally competent MSCs. Collectively, these findings support the implementation of whole umbilical cord biobanking as a practical strategy for veterinary regenerative medicine and provide a foundation for the further optimization of clinically translatable cryopreservation protocols.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/ani16152349/s1>: Figure S1: Representative images of adipogenic,

chondrogenic, and osteogenic differentiation of MSCs isolated from fresh equine Wharton’s jelly (EQ-C); Figure S2: Representative images of adipogenic, chondrogenic, and osteogenic differentiation of MSCs isolated from equine Wharton’s jelly cryopreserved with DMSO/FBS (EQ-DF); Figure S3: Representative images of adipogenic, chondrogenic, and osteogenic differentiation of MSCs isolated from equine Wharton’s jelly cryopreserved with glucose–sucrose–ethylene glycol solution (EQ-GSE); Figure S4: Representative images of adipogenic, chondrogenic, and osteogenic differentiation of MSCs isolated from fresh canine umbilical cord (CN-C); Figure S5: Representative images of adipogenic, chondrogenic, and osteogenic differentiation of MSCs isolated from canine umbilical cord cryopreserved with DMSO/FBS (CN-DF).

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Institutional Review Board Statement: All sample collection procedures were performed in accordance with the guidelines approved by the University of Bologna’s Animal Welfare Committee (CoBA; protocol no. 4630/23, approved 12 January 2023) for equine samples.

Informed Consent Statement: Animals participants: written informed consent has been obtained from the owner of the animals involved in this study.

Data Availability Statement: The datasets generated and/or analyzed during the current study are publicly available in the AMS Acta Institutional Repository of the University of Bologna at: <https://doi.org/10.6092/unibo/amsacta/9016>.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

MSC	Mesenchymal Stromal Cell
UC	Umbilical Cord
WJ	Wharton’s Jelly
UC-MSCs	Umbilical cord-derived MSCs
OCT4	Octamer-binding Transcription Factor 4
NANOG	Homeobox Protein NANOG
SOX2	SRY-Box Transcription Factor 2
KLF-4	Kruppel-like Factor 4
SSEA-4	Stage-specific embryonic antigen-4
cGMP	Current Good Manufacturing Practice
eWJ	Equine Wharton’s Jelly
EQ-C	Equine Fresh Control Group
DMSO	Dimethyl Sulfoxide
FBS	Fetal Bovine Serum
EQ-DF	Equine DMSO/FBS Cryopreserved Group
EQ-GSE	Equine Glucose–Sucrose–Ethylene Glycol Group

cUC	Canine Umbilical Cord
CN-C	Canine Fresh Control Group
CN-DF	Canine DMSO/FBS Cryopreserved Group
PDT	Population Doubling Time
CFU	Colony-Forming Unit
DPBS	Dulbecco's Phosphate-Buffered Saline
DMEM F-12	Dulbecco's Modified Eagle Medium F-12
EDTA	Ethylenediaminetetraacetic Acid
P	Passage (number of cell subcultures)
CD	Cluster of Differentiation
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
RT	Room Temperature
DXM	Dexamethasone
IBMX	Isobutylmethylxanthine
AA2P	Ascorbic acid 2-phosphate
BGP	Beta-glycerophosphate
hTGF- β 1	Human transforming growth factor- β 1
cDNA	Complementary DNA
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
MHC	Major Histocompatibility Complex
DLA-DRA1	Dog Leukocyte Antigen—DR Alpha 1
DLA-DQA1	Dog Leukocyte Antigen—DQ Alpha 1
SD	Standard deviation
CPAs	Cryoprotective agents
ISCT	International Society for Cell & Gene Therapy

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