

**INVITED REVIEW**

# Adult multipotent stromal cell cryopreservation: Pluses and pitfalls

Wei Duan, MS, PhD<sup>1</sup> | Mandi J. Lopez, DVM, MS, PhD, DACVS<sup>1</sup> | Kevin Hicok, MSc<sup>2</sup>

<sup>1</sup>Department of Veterinary Clinical Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana

<sup>2</sup>Research and Development, VetStem Biopharma Inc, Poway, California

**Correspondence**

Mandi J. Lopez, Department of Veterinary Clinical Sciences, School of Veterinary Medicine, Louisiana State University, 1909 Skip Bertman Drive, Baton Rouge, LA 70803.  
Email: mlopez@lsu.edu

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**Abstract**

Study and clinical testing of adult multipotent stromal cells (MSCs) are central to progressive improvements in veterinary regenerative medicine. Inherent limitations to long-term culture preclude use for storage. Until cell line creation from primary isolates becomes routine, MSC stasis at cryogenic temperatures is required for this purpose. Many protocols and reagents, including cryoprotectants, used for veterinary MSCs are derived from those for human and rodent cells. Dissimilarities in cryopreservation strategies play a role in variable MSC behaviors. Familiarity with contemporary cryopreservation reagents and processes is essential to an appreciation of their impact on MSC survival and post-cryopreservation behavior. In addition to these points, this review includes a brief history and description of current veterinary stem cell regulation.

## 1 | INTRODUCTION

Adult multipotent stromal cells (MSCs) are increasing as standard therapy for a multitude of diverse pathologic conditions. Isolation of adequate cells for several therapeutic doses with minimally invasive tissue harvest is a perpetual struggle.<sup>1</sup> Cell dosage varies widely among applications and is not established for any single treatment.<sup>2</sup> Specific MSC immunophenotype subpopulations require extensive culture expansion due to low cell numbers,<sup>3</sup> and genetic alterations and contamination risk increase with culture time.<sup>4,5</sup> In addition to allogeneic immunogenicity concerns,<sup>6</sup> MSC quality varies with age and health status.<sup>7</sup> Thermally dependent metabolic processes do not occur below  $-120^{\circ}\text{C}$ , so MSCs are in metabolic stasis at liquid nitrogen temperature, around  $-196^{\circ}\text{C}$ .<sup>8</sup> Cell aliquots can be maintained for later administration immediately upon revitalization or after short-term expansion.<sup>9</sup> Cryopreservation also increases MSC availability as

frozen cells can be delivered over long distances.<sup>10</sup> Despite prevalent MSC cryopreservation, relatively little focus has been directed toward cell effects.

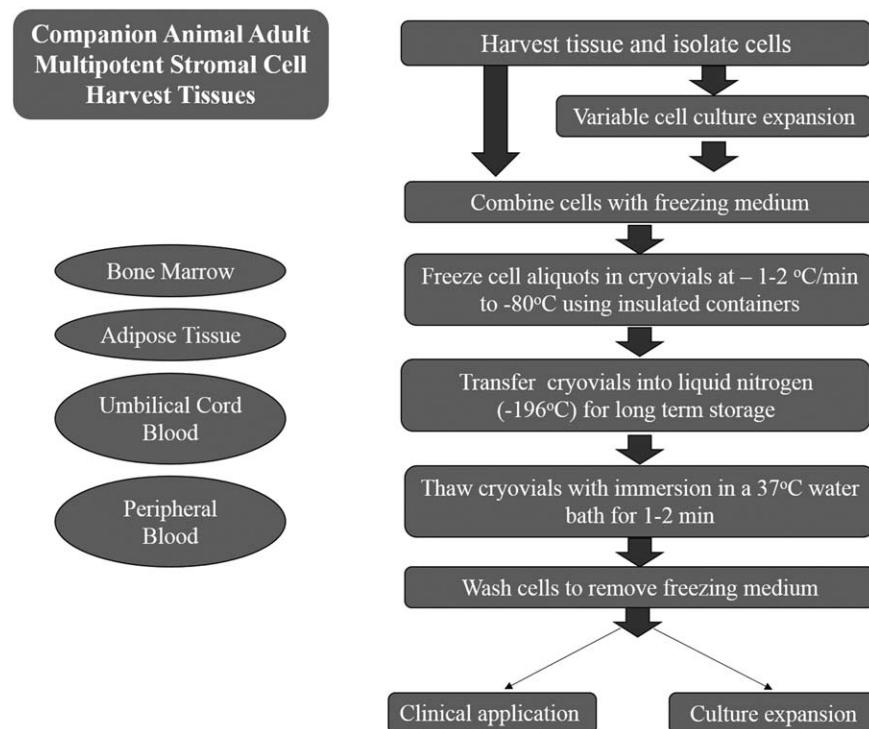
There is a growing awareness of differences between fresh and cryopreserved MSCs.<sup>11-13</sup> Most veterinary MSC cryopreservation techniques are derived from human and murine protocols<sup>12,14</sup> and use cryopreservation medium that contains cryoprotectants (CP) and exogenous serum.<sup>15</sup> Cells are cooled to about  $-80^{\circ}\text{C}$  before transfer to liquid nitrogen.<sup>16</sup> For revitalization, cells are thawed and then rinsed prior to culture.<sup>8</sup> Each step, as well as cryopreservation duration, can impact MSC survival and attributes (Figure 1, Tables 1 and 2).

## 2 | FREEZING

The cell freezing rate must be fast enough to avoid solute and electrolyte imbalances that cause cell dehydration and damage and slow enough to prevent extracellular and

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**FIGURE 1** Schematic of adult multipotent stromal cell cryopreservation

intracellular ice crystal formation.<sup>25</sup> Cryoprotectants reduce the freezing point of the medium, so the mixture of cells, medium, and CPs is a eutectic system because the combined freezing point is lower than the individual components. During the freezing process, fluid moves from lower solute concentrations in unfrozen cells into partially frozen medium while plasma membranes prevent entrance of extracellular ice crystals. Slow freezing permits fluid to move out of the cells at a rate that results in a balanced osmotic pressure between cells and medium by the time the medium freezes. If the rate is too slow, cells are fatally dehydrated or their plasma membranes irreversibly damaged.<sup>8</sup> If the rate is too high, there is insufficient fluid migration to maintain the high solute concentration that prevents fatal cell freezing.<sup>26</sup> Cooling at a rate of  $-1^{\circ}\text{C}/\text{min}$ <sup>27</sup> with microprocessor-controlled freezers or freezing containers with a heat transfer interface (isopropyl alcohol or insulation) reportedly has minimal effect on MSCs.<sup>28</sup> Slow freezing of sterile specimens within sealed vials also minimizes contamination risk.<sup>29</sup> Limitations of this cooling mechanism include cell dehydration and membrane damage, intracellular ice formation, and exposure to CPs.<sup>8</sup>

Vitrification is a form of MSC cooling that involves extremely rapid ( $>-1,000^{\circ}\text{C}/\text{s}$ ) cooling of cells immersed in CPs within open storage vessels.<sup>30</sup> The process prevents fluid crystallization but requires potentially cytotoxic CP concentrations.<sup>30</sup> Samples must always be at a cryogenic temperature, and open containers are a potential source of contamination.<sup>31</sup>

Vitrification is rarely used for veterinary MSC cryopreservation, so this review is focused on slow cooling.

### 3 | THAWING

Cells pass through a temperature range for ice crystal formation,  $-15^{\circ}\text{C}$  to  $-60^{\circ}\text{C}$ , during freezing and thawing.<sup>8</sup> Rapid thawing,  $90-100^{\circ}\text{C}/\text{min}$ , by immersion in a  $37^{\circ}\text{C}$  water bath is often employed to prevent fluid crystallization.<sup>32</sup> Murine hematopoietic progenitor cell survival is higher when cells are thawed rapidly at  $900^{\circ}\text{C}/\text{min}$  versus slowly at  $2^{\circ}\text{C}/\text{min}$ .<sup>33</sup> Recovery rates of human erythroid progenitor cells are the same when they are thawed at  $37^{\circ}\text{C}$  or  $20^{\circ}\text{C}$ .<sup>34</sup> The ideal thawing rate prevents ice formation and prolonged exposure to CPs and likely varies among both cells and CPs. Freezing and thawing processes should be customized and consistently utilized for a given species and MSC type.

### 4 | CRYOPROTECTANTS

Cryoprotectants prevent cell damage during freezing and thawing.<sup>35,36</sup> Formulation and concentration varies among species, MSCs, and cooling techniques.<sup>27,36</sup> Relatively low CP concentrations, 1-2 molar, used for slow freezing are associated with toxicity that differs among cell types and increases with time, temperature, concentration, and metabolic activity.<sup>36</sup> There are 2 major CP categories, cell

**TABLE 1** Adult canine multipotent stromal cell freezing medium components, conditions, and post-cryopreservation behaviors

Harvest tissue	Passage	Cell aliquot (cells/mL)	Freezing medium	Freezing rate	Cooling process	Thawing process	Effects	Reference
Adipose	0	$\sim 2 \times 10^6$	DMEM, 10% FBS, 10% DMSO	$-1^\circ\text{C}/\text{min}$	Insulated container at $-80^\circ\text{C}$ for 24 hours before liquid nitrogen	N/A	N/A	17
	0	$1 \times 10^6$	Serum-free medium, 80% FBS, 10% DMSO	$-1^\circ\text{C}/\text{min}$	Insulated container at $-80^\circ\text{C}$ overnight before liquid nitrogen	$37^\circ\text{C}$ water bath for 2-3 min	None	18,19
	0	N/A	Low glucose DMEM, 30% FBS, 5% DMSO	N/A	N/A	N/A	N/A	20
	1	$3 \times 10^6$	90% FBS, 10% DMSO	$-1^\circ\text{C}/\text{min}$	Insulated container at $-80^\circ\text{C}$ for 1 week before liquid nitrogen	$37^\circ\text{C}$ water bath for 1-2 min followed 10% FBS, 90% DMEM wash	Lower proliferation and telomerase	15
Bone marrow	0	$1.0 \times 10^6$	10% DMSO, 10% FBS, $\alpha$ -MEM	$-1^\circ\text{C}/\text{min}$	Insulated container at $-80^\circ\text{C}$ for 7 days	$37^\circ\text{C}$ water bath for 1 min	Lower viability, proliferative capacity	21

Abbreviations:  $\alpha$ -MEM, minimum essential medium eagle (alpha modification); DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; N/A, not applicable.

**TABLE 2** Adult equine multipotent stromal cell (MSC) freezing medium components, conditions, and post-cryopreservation behaviors

Harvest tissue	Passage	Cell aliquot (cells/mL)	Freezing medium	Freezing rate	Cooling process	Thawing process	Effects	Reference
Adipose	N/A	$\sim 5 \times 10^5$	20% FBS, 10% DMSO, DMEM	$-1^\circ\text{C}/\text{min}$	$-80^\circ\text{C}$ before liquid nitrogen	$37^\circ\text{C}$ water bath for 2 min 20% FBS, 70% DMEM wash	Decreased proliferation rate	22
Bone marrow	N/A	$\sim 1 \times 10^6$	10% FBS, 10% DMSO, DMEM	$-1^\circ\text{C}/\text{min}$	Insulated container for 24 hours at $-80^\circ\text{C}$ before liquid nitrogen	N/A	N/A	16
		$\sim 10 \times 10^6$	20% serum, 10% DMSO, $\alpha$ -MEM or 95% serum, 5% DMSO	$-1^\circ\text{C}/\text{min}$	Isopropyl alcohol container at $-80^\circ\text{C}$ for 24 hours before liquid nitrogen	$35^\circ\text{C}$ water bath until ice gone	Lower MSC numbers	23
Peripheral blood	2-3	$2 \times 10^6$	90% FBS, 10% DMSO	$-1^\circ\text{C}/\text{min}$	Insulated container at $-80^\circ\text{C}$ for 1 week before liquid nitrogen	$37^\circ\text{C}$ water bath for 1-2 min 10% FBS, 90% DMEM wash	Lower proliferation rate	14
Umbilical cord blood	N/A	$1 \times 10^6$	10% DMSO, 70% FBS, DMEM	N/A	$-20^\circ\text{C}$ for 1 hour, $-80^\circ\text{C}$ overnight before liquid nitrogen	$37^\circ\text{C}$ water bath for 3 min 60% FBS, 40% DMEM wash	Lower cell viability that decreased rapidly with passage	24

Abbreviations:  $\alpha$ -MEM, minimum essential medium eagle (alpha modification); DMEM, Dulbecco's Modified Eagle's medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; N/A, not applicable.

membrane permeable and impermeable.<sup>36</sup> Those with high permeability tend to be most cytotoxic.<sup>8</sup> Combining permeable CPs like dimethyl sulfoxide (DMSO), ethylene glycol, methanol, propylene glycol, and dimethylacetamide with less permeable CPs like polyvinylpyrrolidone, hydroxyethyl starch (HES), polyethylene glycol, and dextran reduces permeable CP concentrations.<sup>8,36</sup> DMSO and fetal bovine serum (FBS) are among the most common CPs used for companion animal MSC cryopreservation.<sup>14,37</sup> The carcinogenic properties of DMSO<sup>38</sup> and xenogeneic proteins in FBS may alter the cells and impact postimplantation behavior.<sup>12</sup> Some functions and limitations of these as well as alternative CPs follow.

#### 4.1 | Dimethyl sulfoxide

One of the most popular CPs, DMSO, stabilizes cell proteins<sup>39</sup> and displaces intracellular fluid to equilibrate intracellular and extracellular electrolyte concentrations.<sup>40</sup> Protein stabilization is mediated via hydrophobic interactions between DMSO and positively charged proteins, including cell membrane phospholipids.<sup>39</sup> Additionally, DMSO forms high energy hydrogen bonds with water molecules to prevent ice formation.<sup>41</sup>

DMSO can cause MSC chemical toxicity and osmotic shock.<sup>40,42</sup> Hydrophobic interactions that protect proteins during cryopreservation can also denature and deactivate them.<sup>43</sup> Increasing DMSO concentrations (5%-20%) in the freezing medium is associated with lower survival and increased apoptotic gene expression in porcine bone marrow-derived stromal cells (BMSCs).<sup>13</sup> Neurotoxicity can occur from 10% DMSO, a typical concentration in cryopreservation medium.<sup>44</sup> Serious side effects including hypotension and anaphylactic shock have been attributed to DMSO in cell suspensions administered intravenously to humans.<sup>44</sup> Washing cells after thawing to reduce DMSO concentration results in cell loss and lower colony forming units,<sup>45</sup> and complete DMSO removal is complex and time consuming.<sup>46</sup> These points, among others, support continued efforts to identify replacements for DMSO in cryopreservation medium.

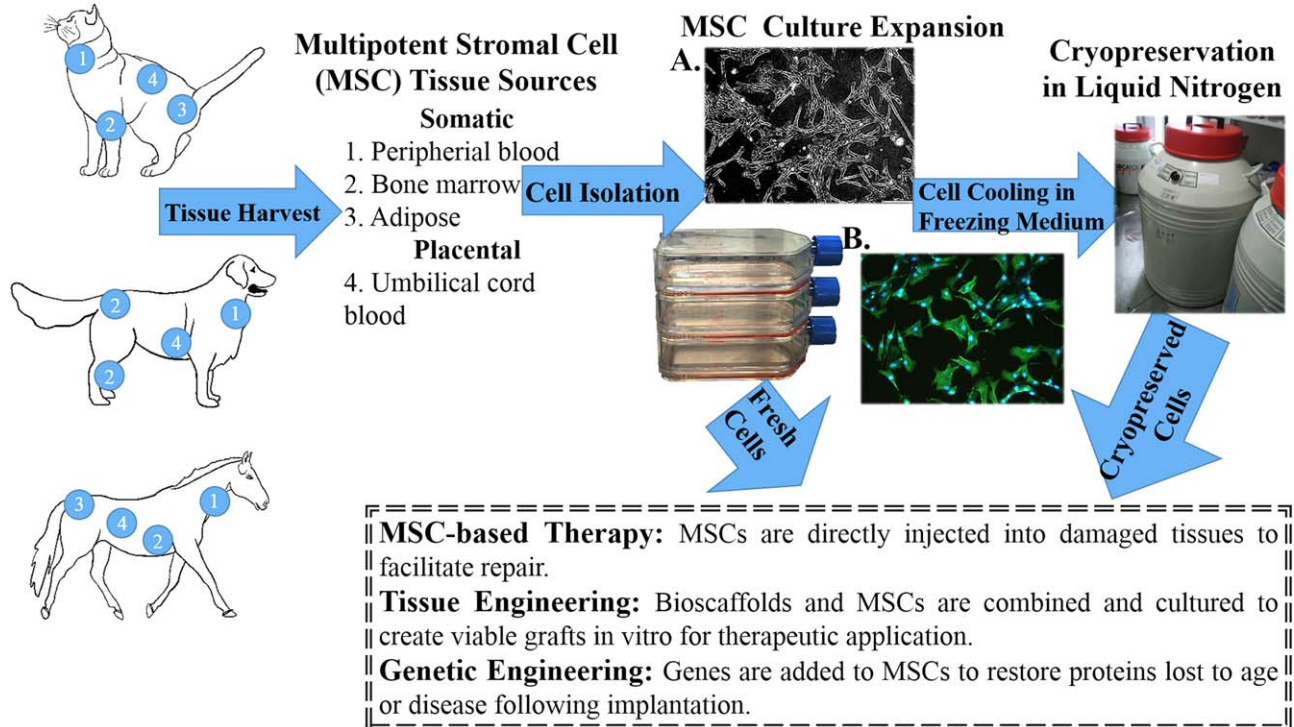
#### 4.2 | Fetal bovine serum

FBS collected at different gestational stages<sup>47</sup> is a common culture medium ingredient that provides growth factors, nutrients, and hormones for cell proliferation and adhesion.<sup>40</sup> It is also thought to act as a CP through protection of cell proteins and stabilization of osmotic pressure.<sup>40,48</sup> In addition to ethical, zoonosis, and xenogeneic protein concerns, variation in composition among FBS lots contributes to inconsistent cell culture performance.<sup>47</sup> A recent finding that cryopreserved canine adipose-derived multipotent stromal cells (ASCs) have increased CD44 expression compared to fresh cells was attributed to FBS in the freezing medium.<sup>12</sup> The US Food and Drug

Administration (FDA) does not permit use of FBS in products intended for humans or animals owing to potential immunogenicity.<sup>49</sup> Autologous and allogeneic serum in MSC freezing media reportedly compares favorably to FBS for MSC viability, morphology, and plasticity.<sup>23</sup> A study to assess different media effects on equine BMSCs included 2 freezing media composed of 20% serum, 10% DMSO, 70% DMEM or 95% serum and 5% DMSO, both with autologous serum, commercial pooled equine serum, or FBS.<sup>23</sup> There was no difference in post-thaw cell viability, morphology, or growth kinetics among different freezing media, and 95% autologous serum with 5% DMSO was recommended for short-term (2-3 days) cryopreservation.<sup>23</sup> Serum-free MSC cryopreservation medium has been shown to have similar or superior post-cryopreservation outcomes compared to FBS-containing media.<sup>48,50</sup> Increasing availability of FBS-free freezing media may be important to improving consistency in MSC pre-cryopreservation and post-cryopreservation characteristics.

#### 4.3 | Impermeable cryoprotectants

Methylcellulose (MC) is a high molecular weight polymer in MSC freezing medium.<sup>27,50</sup> Human ASC post-thaw cell viability with freezing medium containing MC in Dulbecco's modified Eagle's medium (DMEM) is greater than DMEM alone, but lower than that containing 80% serum, 10% DMSO, and 10% DMEM.<sup>35</sup> Another popular CP is polyvinylpyrrolidone (PVP), a nontoxic, high molecular weight polymer.<sup>51,52</sup> The concentration of dissolved PVP increases in extracellular fluid as ice forms at  $-10^{\circ}\text{C}$  to  $-20^{\circ}\text{C}$  to create an osmotic gradient that draws fluid out of cells.<sup>53</sup> Despite lower intracellular fluid, intracellular ice formation may occur at low PVP levels, and high concentrations of 20%-40% can cause excessive cellular dehydration, cell necrosis, and membrane damage.<sup>51,52</sup> Human ASC viability and plasticity appears to be maintained with 10% PVP.<sup>51</sup> HES is a synthetic polymer CP that absorbs water molecules (0.5 g water per 1 g HES) and maintains them in a solid state without crystallization during the cooling process.<sup>54</sup> The polymer, widely used as a plasma volume substitute, is metabolized by glycolytic enzymes *in vivo*, so it does not have to be removed from thawed cells.<sup>54</sup> A "6&5 solution" of physiologic saline, 6% HES, 5% DMSO, and 4% human serum albumin appears to maintain better viability, recovery rates and plasticity of human peripheral blood progenitors, cord blood stem cells, peripheral blood cells, and BMSCs compared to 10% DMSO in Roswell Park Memorial Institute 1640 medium.<sup>54</sup> Similar findings are reported for canine BMSCs cryopreserved in the same solution.<sup>55</sup> Novel cryopreservation solutions that support cell stasis without impacting inherent features will continue to promote availability and standardization of cell therapies across species.



**FIGURE 2** Schematic of companion animal adult multipotent stromal cell (MSC) processing. A, Polarized light photomicrograph of canine adipose-derived multipotent stromal cells cultured in stromal medium (P2), 5×. B, Fluorescent photomicrograph of cells in A with cytoskeleton (actin, green) and nuclear (DNA, blue) staining, 5×

## 5 | CRYOPRESERVATION OF MESENCHYMAL STEM CELLS FROM DIFFERENT SPECIES

Human and veterinary MSCs are typically identified by cell surface antigens (Tables 1 and 2, Figure 2). Based on a recent review of FDA Investigational New Drug submissions for MSC-based products, the top 7 surface antigens proposed as criteria for lot identity and purity are CD45, CD105, CD90, CD73, CD34, CD14, and the human leukocyte antigen class II (HLA Class II).<sup>56</sup> Most submissions use a subset of the markers to confirm MSC identity. According to current International Society for Cellular Therapy standards, MSCs from bone marrow and adipose tissue have shared and distinct surface antigens.<sup>57</sup> Over 70% of MSCs from both tissue sources are positive for CD13.<sup>58</sup> The majority of BMSCs are CD45 positive versus less than 2% of ASCs. Most ASCs express CD73, CD90, and CD105, and 2%-30% of BMSCs are positive. Surface antigen criteria are equally variable in veterinary species. Use of human MSC antigen panels in animals has been limited by species-specific antigens.<sup>59</sup> This is exemplified by a report that showed differences in surface antigen expression in fresh equine MSCs from bone marrow and adipose tissue determined with human-specific antibodies and potential expression detected with real time polymerase chain reaction (RT-PCR).<sup>60</sup> At present, there are no established criteria for MSCs in any

veterinary species. Identification of a panel of surface markers for universal MSC characterization will contribute to reliable and safe clinical translation.

Some of the earliest cryopreservation descriptions were for spermatozoa, ova, and embryos.<sup>61</sup> Cell cryopreservation has expanded with the evolution of cell-based regenerative medicine. It is increasingly apparent that cryopreservation should be customized for species and cell type, especially with established differences among immunophenotypes in primary cell isolates.<sup>12,15</sup> Despite numerous variables, conflicting outcomes and inconsistent cryopreservation effects, common findings have emerged and are summarized below.

Comparisons among fresh and cryopreserved cells provide important information about storage and behavior to guide therapeutic and research applications and assessments. Existing literature documents similar, although not identical, changes in cell plasticity and expansion potential across species. Canine ASCs from subcutaneous and intra-articular adipose tissues have lower sex determining region Y-box 2 (SOX2) protein expression and distinct ultrastructure and immunophenotype compared to fresh ASCs following 30 days of cryopreservation in 80% FBS, 10% DMSO, and 10% DMEM.<sup>12</sup> Two reports indicate that while canine ASCs and BMSCs maintain their fibroblast-like morphology, alkaline phosphatase (ALP) activity, and plasticity following cryopreservation, there are significant differences from fresh

cells.<sup>15,21</sup> Specifically, after 12 months of cryopreservation in freezing medium composed of 90% FBS and 10% DMSO, canine ASCs have lower proliferation and telomerase activity than fresh cells,<sup>15</sup> and, separately, canine BMSCs have lower viability and proliferative capacity following 7 days of cryopreservation in freezing medium with 10% DMSO and 10% FBS.<sup>21</sup> In contrast to these studies, another found lower ALP activity and plastic adhesion in canine BSMCs cryopreserved for 1 month.<sup>62</sup> Equine peripheral blood MSCs cryopreserved in 90% FBS and 10% DMSO have faster proliferation but lower telomerase activity and myogenic plasticity compared to unfrozen cells.<sup>14</sup> The *in vitro* osteogenic differentiation of fresh and cryopreserved rat, lapin, and porcine ASCs are reportedly similar on direct comparison.<sup>63</sup> Human ASCs have lower proliferation and adipocytic and osteoblastic plasticity following cryopreservation in 90% FBS and 10% DMSO, and cryopreserved cells less effectively enhance calvarial healing in an athymic mouse model compared to fresh.<sup>11</sup> These findings support the need to definitively characterize cell isolates before and after cryopreservation and to establish and maintain cryopreservation procedures for consistency.

Another finding common among species is more rapid loss of progenitor cell expansion and multipotentiality with passage and changes in surface antigen expression following cryopreservation. Canine BMSCs cryopreserved in 80% FBS, 10% DMSO, and 10% DMEM for 1 month had lower fibroblastic and osteoblastic colony forming unit frequencies than fresh cells with increasing passage.<sup>18</sup> In one report, fresh canine P1 and P3 ASCs had significantly higher expression of CD29 than the same passages after cryopreservation in 80% FBS, 10% DMSO, and medium, and fresh cells had lower percentages of P1 ASCs that expressed CD44.<sup>12</sup> Notably, CD29 and CD44 expression decreased with increasing passage more rapidly in cryopreserved versus fresh cells.<sup>12</sup> In contrast to the former report, canine ASCs cryopreserved in 90% FBS and 10% DMSO were reported have similar expression of CD29, CD44, CD140a, CD117, CD34, and CD45 as fresh cells in cell passages P3 to P6, although cryopreserved cells had slower proliferation.<sup>15</sup> Feline ASCs cryopreserved in identical freezing medium for 1 month had lower CD9 and CD105 expression compared to fresh cells, and the proliferation rate and osteoblastic capability decreased to a greater extent with increasing passage in cryopreserved versus fresh cells.<sup>1</sup> The cell proliferation rate of equine ASCs frozen in 20% FBS, 70% DMEM-high glucose, and 10% DMSO significantly declined at P12 while fresh cells did not show a similar decline until P15.<sup>22</sup> Separately, the expression of CD90 and CD44 remained high after cryopreservation of equine peripheral blood MSCs but CD13 expression decreased slightly (61%) from that of fresh cells (78%).<sup>14</sup> The potential aging effects of cryopreservation on MSCs that contribute to more rapid waning of cell expansion and plasticity

compared to fresh cells is an important area of continued discovery to anticipate both *in vitro* and *in vivo* cell potential.

## 6 | CELL PASSAGE NUMBER, CONCENTRATION, AND CRYOPRESERVATION DURATION

The amount of cell expansion prior to and duration of cryopreservation is an established factor in post-cryopreservation cell traits. There is a direct relationship between equine umbilical cord blood MSC viability and pre-cryopreservation passage number following cryopreservation in 20% DMEM, 70% FBS, and 10% DMSO for 8 weeks. Cell viability decreases from about 80.4% for P1 to 51.2% for P10.<sup>24</sup> A study to assess cooling rate, end temperature, hold time, and thawing rate on human ASC cell membrane integrity showed a significant effect of thawing rate on only P3 and P4 while the interaction between cooling rate and end temperature was significant for P0-P4.<sup>27</sup>

Results of studies to investigate effects of cell concentration on post-cryopreservation viability vary. Human dental pulp MSCs cryopreserved in aliquots of 0.5, 1, 1.5, and  $2 \times 10^6$  cells/mL in 10% DMSO and 90% medium had an average post-thaw viability of 93%.<sup>64</sup> In contrast, human adult ASC aliquots of  $0.5 \times 10^6$  cells/mL cryopreserved in 10% DMSO, 10% medium, and 80% FBS had higher post-cryopreservation viability than 0.25, 1, or  $2 \times 10^6$  cells/mL.<sup>65</sup> It is possible that cells are damaged from inadequate expansion space during the freezing process at high cell concentrations.<sup>65</sup>

The impact of cryopreservation duration may be most detectable following short-term storage. Human BMSCs reportedly maintain trilineage differentiation capacity after 7 years of cryopreservation.<sup>32</sup> However, total cell recovery is reportedly significantly lower after 5 (80%) versus 1 (90%) months of cryopreservation in 10% fetal calf serum, 10% DMSO, and 30% bovine serum albumin.<sup>66</sup> These findings convey the importance of consistent cell expansion and aliquot concentration as well as consideration of the cryopreservation period when preparing MSCs for potential clinical application.

## 7 | CELL TRANSPORTATION

Cell delivery from current Good Manufacturing Processes facilities to patient administration sites requires maintenance of frozen cells for variable time periods despite external temperature fluctuations.<sup>10</sup> Vitrified cells are transported at cryogenic temperatures in dry shippers with liquid nitrogen in absorbent materials to avoid sample contact with liquid.<sup>46</sup> Slow cooled samples can be shipped frozen in approved,

polystyrene containers.<sup>67</sup> Cryovials are often wrapped with precooled, absorbent material, and placed in a leak-proof, biohazard grade container to prevent direct contact of samples with dry ice placed on top of the container within the polystyrene shipping box. Good practices include permanently labeled samples, inclusion of clear guidelines for sample handling and administration, a detailed inventory, signage on shipping containers, filing of all necessary shipping manifests, and package tracking.<sup>46</sup> Contemporary travel makes it possible to transport cryopreserved samples globally, but national and international laws and regulations must be observed.

## 8 | REGULATION OF CRYOPRESERVED REGENERATIVE CELLS FOR VETERINARY USE

Regulation of veterinary medicine in the United States has a complicated history with some products regulated by the US Department of Agriculture and others by the Food and Drug Administration Center for Veterinary Medicine (CVM). The Federal Food, Drug, and Cosmetic Act of 1938 established federal government regulation of animal health products. Veterinarians were allowed to prescribe human drugs and extra-label use of veterinary drugs for animals under specific circumstances by the Animal Medicinal Drug Use Clarification Act of 1994. The Animal Drug Availability Act added moderation to the animal drug approval process, including flexible labeling and more direct communication between drug sponsors and the FDA in 1996. During early adult stem cell discoveries in 2002, the FDA announced a current good manufacturing practice initiative to create focus on the greatest public health risks of manufacturing procedures and ensure that process and product quality standards did not impede innovation. In 2004, passage of the Minor Use and Minor Species Animal Health Act encouraged development

of treatments for species that may otherwise attract little interest. Collectively, these laws provided veterinarians reasonable discretion and freedom to use emergent drugs and medical devices. There was no specific guidance surrounding development and use of regenerative cell therapies for veterinarians before 2015.

The Guidance for Industry Publication #218, Cell-Based Products for Animal Use, was published by the CVM in June 2015 to clarify regulation of cell therapies. Nonprimary cells that are culture expanded and intended to treat patients other than the donor are considered to be “drugs” and must go through FDA drug licensing and approval processes.<sup>68</sup> Autologous cryopreserved cells to treat injury or disease in the donor are classified as either type I or type II cell-based products (Table 3). Both are regulated as drugs and must undergo all indicated safety and efficacy testing and receive CVM authorization for use similar to allogeneic cell therapies. For type II classification, autologous cells must be minimally manipulated, for homologous use and for nonfood-producing animals. They cannot be combined with anything other than water, crystalloids or sterilizing, preserving, or storage agents that do not raise additional safety concerns, or combined with or modified by a drug or a device. Prior to the advent of stem cell products, the term “type II autologous cells” was generally understood to mean whole or fractionated peripheral, umbilical cord, or marrow-derived blood cells intended for transplantation, cells within cryopreserved mesenchymal tissues like fat, bone, ligament, and tendon, cartilage grafts, or  $\beta$  cell pancreatic islets.

Improved methods of cell preservation with ingredients beyond serum and DMSO complicate cell classification since commercial cryopreservation solution components may be considered drugs under some circumstances. Use of cryopreserved cells in combination with popular blood derivatives like platelet rich plasma occupies a nebulous area in the classification scheme. The speed of discovery in the stem cell arena exceeds development of regulations governing their

**TABLE 3** Autologous cell-based therapy classification

<b>Autologous type I cell therapy criteria (one must be true)</b>	<b>Autologous type II cell therapy criteria (all must be true)</b>
<i>More than minimally</i> manipulated (Extended period of in vitro culture)	<i>Minimally</i> manipulated (Centrifugation only)
For <i>nonhomologous</i> use	For <i>homologous</i> use
For use in a <i>food-producing</i> animal	For use in <i>nonfood-producing</i> animals
Effects dependent on metabolic activity of cells	No statement regarding metabolic activity
Manufacture <i>involves combination</i> of cells with another article (Except water, crystalloids, or a sterilizing, preserving, or storage agent with no new product safety concerns)	Manufacture <i>does not involve combination</i> of cells with another article (Except water, crystalloids, or a sterilizing, preserving, or storage agent with no new product safety concerns)



use. Similarly, definitions of “homologous use” and “minimally manipulated” do not entirely capture current knowledge of stem cell functionality. Academic and industrial scientists continue to work with regulatory authorities to achieve and maintain contemporary language that is consistent with intended and practical use.

In summary, CVM consultation should be sought prior to manufacture and use of cryopreserved cells as a commercial treatment, especially since cells transported across state lines are automatically within federal regulatory jurisdiction. Additionally, state requirements for reporting and licensing a Good Tissue Practice cell banking facility must be observed. Use of cell processing and banking services provided by veterinary regenerative medicine companies appears to be acceptable as the long the provider has implemented appropriate quality and safety standards.

## 9 | CONCLUSION

Cryopreservation of adult MSCs is central to their development, availability, and use. The practice is relatively new in veterinary medicine, and its use will continue to grow.<sup>35,52</sup> It is clear that fresh and frozen MSCs are not identical, although differences are not fully established. Efforts to discover and standardize cryopreservation protocols based on species, tissue, and cryostasis duration will continue to advance therapeutic efficacy and safety of cryopreserved cells.

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest related to this report.

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