Comparison of Freshly-Cultured versus Cryopreserved Mesenchymal Stem Cells in Animal Models of Inflammation: A Pre-Clinical Systematic Review

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1 **Comparison of Freshly-Cultured versus Cryopreserved Mesenchymal Stem Cells in Animal**

2 **Models of Inflammation: A Pre-Clinical Systematic Review**

3

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16
17
18 **Abstract**

19 **Background:** Mesenchymal stem cells (MSCs) are multipotent cells that demonstrate therapeutic potential for the treatment of acute and
20 chronic inflammatory-mediated conditions. Although controversial, some studies suggest that MSCs may lose their functionality with
21 cryopreservation which could render them non-efficacious.

22 **Objective:** In comparative pre-clinical models of inflammation, to determine if there are differences in *in-vivo* measures of pre-clinical
23 efficacy (primary outcomes) and *in-vitro* potency (secondary outcomes) between freshly-cultured and cryopreserved MSCs.

24 **Methods:** A systematic search on OvidMEDLINE, EMBASE, BIOSIS, and Web of Science (until June 5, 2020) was conducted. The primary
25 outcome included measures of *in-vivo* pre-clinical efficacy; secondary outcomes included measures of *in-vitro* MSC potency. Risk of bias was
26 assessed by the SYRCLE “Risk of Bias” assessment tool for pre-clinical studies.

27 **Results:** 15 studies were included. A total of 194 *in-vivo* pre-clinical efficacy experiments represented 72 distinct outcome measures. 2.6%
28 (5/194) of these outcomes were significantly different at the 0.05 level or less; 2 favoured freshly-cultured and 3 favoured cryopreserved

MSCs. A total of 63 *in-vitro* experiments represented 26 different potency measures; 11.1% (7/63) of the experiments were significantly different at the 0.05 level or less, with 5 experiments favouring freshly-cultured MSC and 2 favouring cryopreserved MSCs.

Conclusions: The majority of preclinical *in-vivo* outcomes and *in-vitro* potency outcomes did not detect significant differences ($p < 0.05$) in pre-clinical efficacy between freshly-cultured and cryopreserved MSCs. Our systematic summary of the current evidence base may provide MSC research scientists as well as regulatory bodies additional rationale and justification for considering a cryopreserved MSC product in their clinical trials.

Registration: Protocol has been registered with PROSPERO (CRD42020145833).

Introduction

Mesenchymal stromal cells (mesenchymal stem cells; MSCs) are multipotent stem cells that can be isolated from many adult tissues (e.g. bone marrow, adipose tissue) [1]. MSCs have been studied in clinical trials for almost two decades [2], and have since been implicated in use for diverse conditions [3]. MSCs release growth factors and cytokines along with extracellular vesicles to activate cell proliferation, prevent apoptosis, and ultimately improve regenerative response [1]. MSCs may also modulate the immune response by decreasing inflammation, reducing scar formation, increasing pathogen clearance, altering endothelial permeability, and improving mitochondrial dysfunction as demonstrated in different pre-clinical models of inflammation [4–9]. The mechanism for how MSCs modulate inflammation and promote

healing is not yet completely understood; however, the observed effect may be mediated by both the direct contact with immune cells and release of soluble factors [10,11]. Given their potent immunomodulatory effects, MSCs are particularly attractive for use in infectious as well as acute and chronic inflammatory conditions. There are a growing number of studies that demonstrate the efficacy of MSC therapy in a variety of pre-clinical models, such as acute lung injury [12–16], sepsis [17,18], acute myocardial infarction [19], multiple sclerosis [20], graft-versus-host disease [21–23], osteoarthritis [24–28], and inflammatory bowel disease (IBD) [29,30]. Moreover, as of June 1, 2021, 1004 active trials involving MSCs were registered (www.clinicaltrials.gov). Although MSCs have potential to treat many clinical conditions, a major limitation with nearly all studies is the constrained real-world applicability, where it is vital to have an intervention that is readily available and administered in a time-sensitive manner. For this to occur the MSCs must overcome the logistical challenges of *in-vitro* isolation and culture, effective cryopreservation methodology, and a route for rapid accessibility to the bedside. Future real-world therapeutic applications of MSCs will need to be ready for immediate use as off-the-shelf products in urgent medical situations [31,32].

To date, a majority of preclinical MSC research employ freshly-cultured MSCs and in a recent systematic review of 55 randomized clinical trials, only 15 studies (27%) used cryopreserved cells [33], potentially due to the concern that cryopreserved MSCs may lose some of their functionality [34]. However, there are now studies that suggest cryopreservation may not negatively impact the functionality of MSCs [35–38].

To evaluate evidence currently available in the literature, our team conducted a systematic synthesis of all pre-clinical comparative studies that examined freshly-cultured versus cryopreserved MSCs on surrogate measures of *in-vivo* efficacy (primary outcomes) and *in-vitro*

potency (secondary outcomes) in animal models of inflammation. The protocol for our systematic review is published in *Systematic Reviews* (<https://doi.org/10.1186/s13643-020-01437-z>) and registered in PROSPERO (CRD42020145833).

Materials and Methods

Search Strategy

We conducted electronic search strategies without language restriction of Ovid platform, Ovid MEDLINE®, OvidMEDLINE® In-Process & Other Non-Indexed Citations, Embase Classic plus Embase, and BIOSIS and Web of Science using Web of Knowledge until June 5, 2020. Given the non-standard terminology associated with MSCs, a number of terms were used, and the electronic and manual search strategies were developed and tested through an iterative process by an experienced medical information specialist in consultation with the research team (S1 Fig). Six target articles provided by an expert in the field of preclinical research (SM) that were known prior to the search were included in the search criteria to help capture all potential studies. No additional filters were employed to ensure the largest number of relevant studies are captured. We followed the PRISMA guidelines (S2 Table) for reporting our systematic review.

Assessment of Risk of Bias

Risk of bias was assessed independently by two reviewers (CD and AM), and disagreements were resolved via consensus, or by a third reviewer when necessary. All studies were assessed as high, low, or unclear for the 10 domains of bias adapted from the SYRCLE “Risk of Bias” assessment tool for pre-clinical *in-vivo* studies [69]. This tool has been adapted from the Cochrane Collaboration Risk of Bias tool employed in clinical studies, with an aim to incorporate key elements that are relevant for *in-vivo* animal studies. The 10 risk of bias domains are provided in Table 7.

Study Eligibility

Pre-clinical studies of *in-vivo* models of inflammation that directly compared freshly-cultured to cryopreserved MSC products (randomized, quasi-randomized, and non-randomized designs) were included. To be defined as cryopreserved, MSCs could have been cryopreserved for any duration of time and/or be placed in culture for less than 24 hours post-thaw prior to use in the given experiment. MSCs were considered freshly-cultured when the cells were either in continuous culture or cryopreserved but then thawed and placed in culture for at least 24 hours prior to use in experiments. We used this 24-hour culture time as a cut-off as previous experiments have demonstrated that cryopreserved MSCs may require 24 hours of culture to recover their functionality [70]. The study must have included an *animal* model of inflammation where the intervention and comparison groups examined the administration of cryopreserved and freshly-cultured MSCs,

91 respectively, delivered by any route, and derived from the same MSC origin (ex. bone marrow, adipose tissue, umbilical cord, or other) and
92 source (xenogeneic, syngeneic, autologous, or allogeneic). MSCs that were pre-treated, pre-conditioned, genetically altered, or co-
93 administered with other experimental interventions were included if the same alteration was applied to both the freshly-cultured and
94 cryopreserved MSCs.

95 Studies that administered MSCs before or during the induction of the experimental pre-clinical model (i.e., prevention studies), that
96 included immunocompromised animals (SCID), treatments to immunosuppress the animals or that examined the effects of MSCs on
97 implantation and tissue regeneration (e.g. bone regeneration), that compared differentiated MSCs (e.g. differentiated into a myocyte),
98 Mesenchymal Progenitor Cells (MPCs), Mononuclear Cell (MNC) fraction, stem cells that were not described as MSCs, and studies that only
99 reported *in-vitro* experiments comparing freshly-cultured to cryopreserved MSC products were excluded.

100
101 **Outcomes**

102 The primary outcomes were surrogate measures of *in-vivo* pre-clinical efficacy that were relevant to specific acute and chronic
103 inflammatory animal models and defined by two outcome domains: 1) The Function and Composition of Tissues (e.g. organ dysfunction,
104 histopathological damage); and 2) Protein Expression and Secretion (e.g. cytokine levels, immunohistochemistry analysis).

106 Secondary outcomes included measures of *in-vitro* MSC potency (that were described as additional experiments in the included *in-vivo*
107 studies). Ideally, potency should represent the MSCs' mechanism of action; however, MSCs have complex and multiple mechanisms of
108 action, all of which are not yet fully characterized or reported [34]. In accordance with the International Society for Cellular Therapy
109 perspective paper on this topic [34], MSC potency was based on an assay matrix (collection of assays) that included a combination of *in-vitro*
110 analytical and/or biological assays (e.g. the cellular secretome by ELISA [enzyme-linked immunosorbent assay], or functional cell-based
111 assays [*in-vitro* assay culturing MSCs with responder immune cells] respectively). Hence, the two main secondary *in-vitro* potency outcome
112 domains were: 1) Co-culture assays; and 2) Protein Expression and Secretion (ex: cytokine levels).

113

114 **Study Selection and Data Collection**

115 The titles and abstracts were screened independently by two members (CD, ED). The full-text of all potentially eligible studies were
116 retrieved and reviewed for eligibility, independently, by two members of the team using the *a priori* eligibility criteria described above.
117 Disagreements between reviewers were resolved by consensus or by a third member of the systematic review team (LM, SM). Data were
118 extracted independently by two members of the research team into standardized, pilot-tested excel sheet forms (S3 Table). Authors were
119 contacted for data clarification or for additional data when required.

120

121 **Data analysis**

122 Meta-analyses were planned as per protocol, if sufficient data were available and if appropriate: two or more studies with similar disease
123 models for an *in-vivo* pre-clinical efficacy outcome, with the same outcome definition. Data reported in non-standard format (e.g. mean $\pm$
124 standard error, median and range) was converted to mean $\pm$ standard deviation. Given the complexity and variety of results, the results
125 were summarized in tabular format and visualized presented as number of experiments that reached statistical significance at the 0.05
126 level.

127

128

129 **Results**

130 **Search Results and Study Characteristics**

131 The search strategy yielded 2156 potential studies; and after applying the eligibility criteria and full text review, 15 studies were deemed
132 eligible for inclusion (Fig 1) [35,36,46–50,37,39–45].

133

134

Fig 1. Literature search and study inclusion.

Six studies used mice for their experiments (35,37,39,40,42,50), six studies used rats [36,41,43,46,48,49], one study used both mice and rats [44], one study used beagle dogs [47], and one study used guinea pigs [45]. Nine studies included a 'vehicle only' as an additional control arm [36,37,39–41,45,46,49,50], while four studies employed a sham animal model, where disease negative animals received MSCs or vehicle [42–44,48]. One study directly compared cryopreserved and freshly-cultured MSCs without an additional control arm [47] and one study employed a sham model, vehicle, and cryopreserved and freshly-cultured fibroblasts as controls [35].

Of the 15 included studies, six studied models of preclinical lung injury and sepsis [36,42,43,46,48,50], one an allergic airway inflammatory disease model [35], three a wound healing model [39,41,45], one an acute and chronic inflammatory model of colitis [40], one employed models of wound healing and chronic inflammatory arthritis [44], and three studies employed models of neurological or ocular disease, specifically, one study assessed corneal transplantation [49], one study employed a retinal ischemia/reperfusion model [37], and one study employed a spinal cord injury model [47]. Complete reporting of inflammatory models, MSC origins and characteristics can be found in Tables 1 and 2.

151

152 **Description of Cryopreservation and Thaw Process for Cryopreserved MSCs**

153 The duration of cryopreservation for cryopreserved MSCs prior to use in experiments was not reported in seven studies [36,40,42–
154 44,49,50], five studies cryopreserved the MSCs for at least 1 month [39,41,45,46,48], one study cryopreserved for 2–3 weeks [47], another
155 between 1 and 4 weeks [37], and one study cryopreserved their MSCs for 9 days [35].

156 Nine studies used 10% DMSO (dimethyl sulfoxide) as part of their cryopreservation solution [35,36,39–41,44,45,47,49], two studies used
157 CryoStor Cell Preservation Media (Sigma-Aldrich) [37,48], one study used MSC Freezing media (Biological Industries) [42], and three studies
158 did not report the solution used for cryopreservation [36,43,50]. Four studies did not report on their method of cryopreservation
159 [36,40,46,48], three studies employed a controlled-rate freezer to achieve cryopreservation [42–44], while seven studies used liquid
160 nitrogen at -80°C [37,39,41,44,45,49,50] for storage, and one study gradually cryopreserved the MSCs with decremental temperature over
161 24 hours, followed by storage at -150°C [47].

162 Seven studies did not report their thawing protocol [35,36,41,44,45,48,50], and the remaining eight studies used a 37°C hot water bath to
163 thaw the cryopreserved MSCs [37,39,40,42,43,46,47,49]. Two studies thawed MSCs on the day of administration for their experiments
164 [43,50], while eight studies reported thawing MSCs either immediately or within 1 hour of use in experimentation (Bárcia et al., 2017; Cruz
165 et al., 2015; Devaney et al., 2015; Gramlich et al., 2016; Horie, Masterson, et al., 2020; Khan et al., 2019; Lohan et al., 2018; Tan et al., 2019).

166 Five studies did not report time from thaw to use in experimentation [39–41,45,48]. Eight studies suspended thawed MSCs in phosphate

167 buffered saline (PBS, vehicle for experiments) [35,36,41,43,44,46,47], while one study re-suspended them in ringer's lactate supplemented
168 with 3% Dimethyl sulfoxide (DMSO) [50], one used MSCs suspended in 0.9% NaCl+ 3.6% HSA (Human Serum Albumin) [40], one used PBS
169 with 5% HSA [42], and four studies did not report their resuspension solution [39,45,48,49].

170

171 **Description of Cryopreservation and Culture Process for Freshly-Cultured MSCs**

172 Freshly-cultured MSCs were not cryopreserved at any point after harvest from source in 11 studies (range of total culture time: 4 to 28 days)
173 [35,36,49,37,39–41,45–48]. In four studies, the MSCs were cryopreserved and then culture-expanded for more than 24 hours prior to use in
174 experimentation [42–44,50].

175 Further details related to MSC culture, including medium, passage, concentration, and route of administration can be found in Table 2.

176

177 **Risk of Bias**

178 Of the 15 included studies, none of the studies met low-risk of bias criteria for all 10 domains and all studies demonstrated unclear risk of
179 bias in atleast 2 domains. Seven did not have any features that would confer a high-risk of bias in the one of the 10 domains [35,42,43,45–
180 48]. Five studies demonstrated high-risk of bias in one domain [36,39–41,50], and the remaining three studies demonstrated high-risk of
181 bias in two or more domains [37,44,49]. The complete reporting of the risk of bias domains is presented in Table 3.

182

183 **Primary and Secondary Outcomes**

184 Across the 15 included studies, a total of 271 experiments and 99 distinct outcome measures were reported on our primary and secondary
185 outcomes and are summarized below. Data extraction of outcomes from included studies yielded significant amounts of data given the
186 extensive and varied inflammatory disease models and their specific outcomes. A description of all primary *in-vivo* pre-clinical efficacy and
187 secondary *in-vitro* potency outcomes are reported in Tables 4 and 6 respectively. Similar pre-clinical animal inflammatory models that
188 reported a similar outcome at similar timepoints are reported in Table 5 but were not sufficient to conduct meaningful meta-analyses.

189

190

191 **Primary Outcomes**

192 **In-Vivo Pre-Clinical Efficacy Outcomes**

193 The 15 studies reported a total of 194 experiments and 72 distinct outcome measures related to our *in-vivo* pre-clinical efficacy primary
194 outcomes. Fourteen assessed composition of tissues [35,36,47–50,37,39–45], and 9 assessed organ dysfunction [35–37,40,43,44,46–48].

195 Ten of the 15 studies assessed protein expression and secretion [35,36,40,42,43,46–50] (Table 2).

196 Of the 194 experiments, five outcomes were significantly different at the 0.05 level or less, with two that favoured freshly-cultured and
197 three that favoured cryopreserved MSCs (Table 4).

198 ***In-vivo Pre-Clinical Efficacy: Function and Composition of Tissue***

199 Fourteen studies reported organ dysfunction and/or composition of tissue outcomes and a total of 116 different experiments were reported
200 across the studies. Of the 116 experiments, only one reported a significant difference at the 0.05 level or less between the freshly-cultured
201 and cryopreserved MSC groups which favoured the cryopreserved group (Fig 2).

202

203 **Figure 2. Primary *in-vivo* outcomes.** All the outcomes related to function and composition of tissues are presented below. Number of
204 experiments represent the number of separate comparisons between fresh and cryopreserved MSCs on surrogate measures of in-vivo
205 efficacy.

206

207

208 ***In-vivo Pre-Clinical Efficacy: Protein (Cytokine) Expression and Secretion***

209 Ten studies reported protein expression and secretion outcomes, with total of 78 experiments reported across the studies. Four of the 78
210 experiments reported a statistically significant difference between freshly-cultured and cryopreserved MSCs that were derived from one
211 study [35]. Of the four experiments that demonstrated a significant difference at the 0.05 level or less, two favoured freshly-cultured and
212 two favoured cryopreserved MSCs (Fig 3).

213

Figure 3. Primary *in-vivo* outcomes. All the outcomes related to protein (cytokine) expression and secretion are presented below. Number of experiments represent the number of separate comparisons between fresh and cryopreserved MSCs on surrogate measures of *in-vivo* efficacy.

Secondary Outcomes

In-Vitro Potency Outcomes

Thirteen studies reported *in-vitro* potency outcomes [35,36,47–50,37,39,41–46] with 63 experiments and 26 different outcome measures.

All reported *in-vitro* outcomes can be found in Table 6. Of the 63 experiments (26 distinct outcome measures), 7 were significantly different at the 0.05 level or less, with 5 that favoured freshly-cultured and 2 that favoured cryopreserved MSCs (Fig 4).

Figure 4. *In-vitro* potency outcomes. All the *in-vitro* reported outcomes are displayed below. Number of experiments represent the number of separate comparisons between fresh and cryopreserved MSCs on surrogate measures of *in-vivo* efficacy.

231 ***In-vitro Potency: Protein (Cytokine) Expression and Secretion***

232 A total of three studies [37,45,47] reported *in-vitro* protein (cytokine) expression and secretion outcomes. Of the 28 experiments, five
233 demonstrated a significant difference at the 0.05 level or less, with two favouring cryopreserved and three favouring freshly-cultured MSCs
234 (Table 5).

235

236 ***In-vitro potency: Co-culture assays***

237 Three studies reported *in-vitro* co-culture assay outcomes (7 separate experiments) to assess the impact of MSCs on responder cell
238 proliferation [37,42,44]. All three studies used PBMCs (peripheral blood mononuclear cell) activated with CD3 and CD28 as the responder
239 cells. The studies employed variable MSC:Responder cell ratios and duration of culture. All three studies found no significant difference in
240 potency for cryopreserved as compared to freshly-cultured MSCs at varying concentrations of MSCs to responder cells [37,42,44] (Table 7).

241

242

243 **Viability**

244 Of the 10 studies [35–37,39,41,42,44–46,48] that reported post-thaw viability of cryopreserved MSCs, the range was from 60%–97% across
245 various time points. The viability of freshly-cultured MSCs ranged from 91%–97%, assessed at various time points. Only 4 studies had

246 experiments (total of 22) involving both freshly-cultured and cryopreserved MSCs [37,39,42,44]. Of the 22 experiments, 9 (41%) favoured
247 freshly-cultured MSCs (Fig 5).

248

249 **Figure 5. Comparison of Viability.** Experiments where viability at varying time points of freshly-cultured and cryopreserved MSCs were
250 compared directly are presented below. The figures represent

251

252

253 Discussion

254 This study is the first comprehensive pre-clinical systematic review to examine the effect of cryopreservation on the *in-vivo* efficacy and *in-*
255 *vitro* potency of MSCs in animal models of inflammation. Across the 15 included studies, our review found that 189 out of 194 (97%) of the
256 *in-vivo* pre-clinical efficacy outcomes demonstrated no statistically significant differences between cryopreserved and freshly-cultured MSCs
257 at a p value of <0.05. When evaluating the results of a large, heterogeneous group of studies comparing freshly-cultured versus
258 cryopreserved MSCs for efficacy and potency, it is useful to compare the results to what one would expect to see if (a) there were truly no
259 difference or if (b) there truly were a difference. In the former case, where all differences would be due exclusively to Type I error, we would
260 expect to see roughly 5% of the p-values being statistically significant. Furthermore, when a difference was statistically significant, we would

261 expect it to be equally likely to favor fresh versus frozen or vice versa. In the latter case, where there truly is a difference, we would expect
262 to see more than 5% of the p-values being statistically significant and a strong concordance in the sense that most would favor the same
263 group. We argue that our results for efficacy are entirely consistent with pure Type 1 error (2.6% were statistically significant with half
264 favoring freshly-cultured and half favoring cryopreserved MSCs). For *in-vitro* potency, the results are somewhat less clear cut. We found
265 11% (95% CI: 3%-19%) were statistically significant; 5 of these favored freshly-cultured and 2 favored cryopreserved MSCs. Given that the
266 confidence interval for the rate of statistical significance does not exclude 5% and that 2 of the 7 significant results favored cryopreserved
267 MSCs, this does not represent strong evidence of a difference in potency. In terms of viability, the evidence appears to support reduced
268 viability in cryopreserved versus freshly-cultured MSCs, but the viability (60%-93% as opposed to 91%-97%) of cryopreserved MSCs still
269 appears to be acceptable for experimentation and clinical research [51].

270 Cryopreservation under safe and quality-controlled conditions remains critical for future real-world applications of MSC therapies [52] by
271 easing the logistical burden of supplying freshly-cultured MSCs, enabling quality control and standardization of the cell preparation, and to
272 facilitate the logistical transport of cellular products to acute care hospitals [53]. Some studies have shown that cryopreservation does not
273 negatively impact MSCs; even if stored in cryopreservation for up to 23–24 years [54–56]. However, other studies have demonstrated mixed
274 effects with both short-term and long-term cryopreservation [57,58]

275 A recent systematic review of 41 *in-vitro* studies that examined bone marrow-derived MSCs (BM-MSCs) demonstrated that MSC cell
276 morphology, marker expression, proliferation potential and tri-lineage differentiation capability were unaffected by stresses imposed by
277 freezing and thawing; whereas viability, attachment to plasticware and migration, genomic stability and paracrine function of MSCs
278 demonstrated conflicting results [59]. Out of their included 41 studies, only eight of them studied BM-MSCs immune function (88%
279 conducted co-culture assays) post-thaw with four studies concluding damaging effect and four concluding no effect of cryopreservation on
280 MSC *in-vitro* immune function. Interestingly, this review found that the immediate post-thaw viability varied from about 50% to 100%
281 among the included studies; 16 studies reported no change in viability immediately after thawing and 10 studies reported significantly lower
282 viability [59]. These latter findings align with our findings, namely, that the viability of cryopreserved MSCs as compared to freshly-cultured
283 MSCs may be lower.

284 Recently, a phase III randomized clinical trial that examined the addition of either a cryopreserved MSC product, PROCHYMAL
285 (Remestemcel-L), or placebo to standard second line therapies in children with acute graft-versus-host disease (aGVHD) showed that high
286 risk patients were more likely to have a partial response at 28 days with Remestemcel [60]. Furthermore, a recently published systematic
287 review that examined 55 randomized trials which used a MSC product versus control/usual care not only suggested evidence for safety of
288 cryopreserved MSCs but also efficacy. Of the 15 trials that studied a cryopreserved product, 5 of them (33%) found significant differences
289 favoring cryopreserved MSCs in either the primary or secondary endpoints [33]. Our study contributes to the growing evidence that
290 cryopreserved MSCs are a viable and efficacious option for further study, including clinical trials.

291 It is known that cryopreserved MSCs have a higher percentage of apoptotic cells than MSCs from fresh cultures [61]. Many factors could
292 contribute to the diminished viability and functionality of cryopreserved MSCs, including the source of MSCs, rate of cooling, storage
293 temperature and period, method of recovery from cryopreservation, and the cryoprotectants used [58]. Cryopreserved MSCs are commonly
294 frozen in 5%–10% DMSO and fetal bovine serum (FBS) [62,63], but there are disadvantages of using these agents. DMSO is toxic at higher
295 concentrations and adverse events have been reported in patients [64,65], and the use of animal proteins theoretically increases the risk of
296 transferring infectious agents or stimulating unwanted immunological responses. Despite the continued search for the most optimal
297 cryoprotectant, no consensus has been developed on the safest type and concentration of cryoprotectant to use [66]. Optimizing the rate of
298 cooling is important as is the thawing process, both of which can further contribute to cell injury. Apoptotic and necrotic pathways are
299 activated in these cells 6–48 h post-thaw in response to low temperature exposure [67]. Remarkably, many studies demonstrate that MSCs,
300 isolated from diverse sources, cryopreserved using various cooling rates, in the presence of different cryoprotectants, stored for various
301 lengths of time, and at various sub-zero temperatures still retain their biological properties post-thaw except for viability [58]. For better or
302 worse, viability of MSCs remains an evocative indicator of cryopreservation success where at least 90% viability for fresh MSC product and
303 70% viability for cryopreserved MSC product are considered the benchmark for pre-clinical application [51].

304 There are several strengths in this current systematic review. First, we have published our protocol which includes a transparent search
305 strategy, pre-defined definitions for cryopreserved and freshly-cultured MSCs and outcome measures, and minimal exclusion criteria. Ours
306 is the first comprehensive systematic review assessing the *in-vivo* efficacy of cryopreserved MSCs when directly compared to freshly-

307 cultured MSCs in models of inflammation. Despite variability in reporting, all variables and experiment details were collected systematically.

308 Given the breadth and variety of *in-vivo* and *in-vitro* outcome measures, we report our data in a specific way by considering each

309 experiment where cryopreserved and freshly-cultured MSCs are compared as an individual hypothesis test. Our review provides the totality

310 of the existing pre-clinical evidence and will help assist researchers, funders and regulators address further evidence gaps as well as guide

311 future research in this area [68].

312 Our study did have some limitations. Given our emphasis on including studies that examined MSC *in-vivo* efficacy, we excluded all studies

313 that only conducted *in-vitro* studies. This led to a significant number of studies being excluded and hence, our *in-vitro* outcome reporting

314 may be incomplete. However, when considering whether cryopreserved MSCs may be efficacious from the pre-clinical perspective, pre-

315 clinical efficacy outcomes might be more convincing than *in-vitro* studies. Most of the preclinical studies did not provide sufficient

316 information to adequately conduct the SYRCLE risk of bias assessment, resulting in unclear reporting in at least three bias domains or more

317 in all but one study. Due to the breadth of different primary *in-vivo* outcomes measured, we could not examine for publication bias.

318 Similarly, our ability to conduct meta-analyses was significantly limited by the heterogeneity of animal models and breadth of outcome

319 measures. Finally, it is possible that other important *in-vivo* pre-clinical efficacy or *in-vitro* potency outcomes were not reported in our

320 review and are deserving of further study.

321

322

323 **Conclusions**

324 Our study provides a comprehensive systematic review of pre-clinical studies comparing cryopreserved versus freshly-cultured MSCs in
325 animal models of inflammation. Our findings suggest that for the majority of outcomes measured in this review, cryopreservation does not
326 impact *in-vivo* efficacy or *in-vitro* potency of MSCs. With our systematic summary of the current evidence base, we hope it may provide MSC
327 research scientists as well as regulatory bodies additional rationale and justification for considering a cryopreserved MSC product in their
328 clinical trials.

329

330

331 **Authors' contributions**

332 LM, SM, AM, and CD conceived the study design. CD, AM, and LM were responsible for initial drafting and manuscript revisions. CD, CH, AM,
333 and LM are responsible for the data collection. KS, JC, SM, and LM will provide critical revisions and statistical support. SM, AM, and LM
334 provided translational biology expertise, and provided oversight for planned eligibility criteria and outcome measures. All authors read and
335 approved the final manuscript.

336

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339 scientific, comprehensive search strategy, and Diana Wolfe for her contribution to the development of the protocol for this systematic
340 review.

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342 Daniel O’Toole, and James Ankrum.

343
344 **Competing Interests**

345 No competing interests to declare.

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350

351 **Tables**

352 **Table 1. Models of inflammation and characteristics of included studies.**

First Author (Year)	Animal Inflammatory Model	Country	Language of Publication	Species	Strain	Gender	Sample size	Age (range)	Weight (grams)
Barcia (2017)	1) Chronic adjuvant- induced arthritis (AIA) model 2) Hindlimb ischemia model	Portugal	English	1) Rat 2) Mouse	1) Winstar 2) C57BL/6	1) Male 2) Female	1) 18 2) 36	1) NR 2) 12 weeks	1) 365-480g 2) NR
Cruz (2015)	Allergic Airways Inflammation induced by Aspergillus hyphal extract (AHE) exposure in immunocompetent mice	USA	English	Mouse	C57BL/6	Male	72	8-12 weeks	NR

Curley (2017)	Acute respiratory distress syndrome by intratracheal instillation of E. coli	Canada	English	Rat	Sprague-Dawley (specific pathogen-free)	Male	NR	NR	350-450g
Devaney (2015)	Acute lung injury induced by E.coli pneumonia	Ireland	English	Rat	Sprague-Dawley (specific pathogen-free)	Male	40	NR	350-450g
Gramlich (2016)	Retinal Ischemia/Reperfusion Injury Model	USA	English	Mouse	C57BL6/J	Male and Female	37	2 months	NR
Lohan (2018)	Corneal Transplantation	Ireland	English	Rat	Lewis	Male	NR	8-14 weeks	NR
Salmenkari (2019)	Colitis (3% DSS)	Finland	English	Mouse	balb/c	Male	NR	8 weeks	NR
Somal (2017)	Wound healing	India	English	Rat	Wistar	Male	27	NR	NR

Bharti (2020)	Wound healing	India	English	Guinea pigs	Dunkin Hartley	Male	25	NR	NR
Horie and Laffey (2020)	Ventilator-induced Lung Injury	Ireland	English	Rats	NR	NR	NR	NR	NR
Horie and O'Toole (2020)	E. coli-induced lung injury	Ireland	English	Rats	Pathogen-free sprague Dawley	Male	NR	NR	300-450g
Khan (2019)	Spinal Cord Injury induced through a balloon compression method	Korea	English	Dog	Beagle	NR	12	1.2+/-0.2yrs	12+/-3kg
Rogulska (2019)	Wound healing	Ukraine	English	Mouse	Balb/C	Male	27	5-6 months	25-30g
Tan (2019)	Polymicrobial sepsis induced by cecal-	Canada	English	Mouse	C57BL6/J	Female	NR	8 weeks	17-21g

	ligation-and-puncture (CLP)								
Perlee (2019)	K. pneumoniae induced pneumosepsis	Netherlan ds	English	Mouse	Pathogen free C57BL/6	Female	NR	8-10 weeks	NR

*NR= Not Reported.

Table 2. MSC characteristics of included studies.

First Author (Year)	Species and Tissue Source	Compatibili ty with Animal	ISCT Criteria Met	Route of Administration	Vehi cle	Timing of MSCs post- disease induction	Fresh MSCs		Cryopreserved MSCs		
							Cryopres erved at any point?	Duration of Culture	Method	Durati on	Time from Thaw to Experiment
Barcia (2017)	Human Umbilical Cord	Xenogenic	Yes	1) Intra- articular 2) Intra- muscular	PBS	1) 7 days 2) 5 hours	No	>5 days	Controlled Rate Freezer	NR	Immediately
Cruz (2015)	Human and Murine Bone Marrow	Syngenic and Xenogenic	Yes	Intravenous	PBS	14 days	Yes	NR	-80°C for 48 hours then liquid nitrogen	9 days	15 minutes
Curley (2017)	Human Umbilical Cord and Bone Marrow	Xenogenic	Yes	Intravenous	PBS	NR	No	4 days	Controlled Rate Freezer	NR	Day of administrati on

Devaney (2015)	Human Bone Marrow	Xenogenic	Yes	Intravenous	PBS	0.5 hours	Yes	NR	NR	NR	30 minutes
Gramlich (2016)	Human	Xenogenic	Yes	Intra-ocular	PBS	2 hours	Yes	>7 days	Controlled Rate Freezer	7-30 days	<1 hour
Lohan (2018)	Rat Bone Marrow	Allogenic	NR	Intravenous	PBS	1 and 7 days prior	Yes	NR	-80°C for 24 hours then liquid nitrogen	NR	Immediately
Salmenkari (2019)	Human Bone Marrow	Xenogenic	NR	Intravenous	0.9% NaCl + 3.6% HAS	3 and 5 days	Yes	NR	NR	NR	NR
Somal (2017)	Gravid caprine AF (amniotic fluid), AS (amniotic sac), WJ	Xenogenic	NR	Subcutaneously	PBS	7, 14, 21, 28 days	Yes	NR	-80°C overnight	Atleast 1 month	NR

	(Wharton's jelly), CB (cord blood)								then liquid nitrogen		
Bharti (2020)	Dog Bone Marrow	Xenogenic	NR	Surgically placed over wound	Poly prop ylen e mes h	NR	Yes	NR	-80°C overnight then liquid nitrogen	1 month	NR
Horie and Laffey (2020)	Human Bone Marrow	Xenogenic	NR	Intravenous	PBS	6 hours	Yes	NR	NR	NR	NR
Horie and O'Toole (2020)	Human Bone Marrow and Umbilical Cord	Xenogenic	NR	Intra-tracheal	PBS	30 minutes	Yes	NR	NR	NR	Immediately
Khan (2019)	Dog Adipose Tissue	Allogenic	NR	Intravenous	Hart man n's	Immediate ly	Yes	NR	4°C for 1h, - 20°C for 2h, - 80°C for 24 h, then -150°C	2-3 weeks	Immediately

					Solut ion						
Rogulska (2019)	Human Adipose Tissue	Xenogenic	NR	Implantation into wound	3D gel	Immediate ly	Yes	NR	-80°C the liquid nitrogen	1 month	NR
Tan (2019)	Human Bone Marrow	Xenogenic	Yes	Intravenous	5% Hum an Albu min in Plas maLy te	6 hours	No	>24 hours	Controlled Rate Freezer	NR	Immediately

Perlee (2019)	Human Adipose Tissue	Xenogenic	Yes	Intravenous	Ring er's Lacta te	1 or 6 hours	No	24 hours	Liquid nitrogen	Until requir ed	Day of administrati on
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377 **Table 3. Risk of Bias assessments for the included in-vivo studies using SYRCLE Tool.**

Selection Bias			Performance Bias			Detection Bias		Attrition Bias	Reporting Bias	Other Bias
Author (year)	Adequate randomization	Baseline characteristics given	Evidence of adequate concealment of groups	Evidence of random housing of animals	Evidence of caregivers blinded to intervention	Evidence of random selection for assessment	Evidence of assessor blinded	Explanation of missing animal data	Free of selective reporting based on methods/results	Free of other high bias risk
Barcia (2017)	Unclear	Yes (Low Risk)	Unclear	Yes (Low Risk)	No (High Risk)	Unclear	No (High Risk)	Yes (Low Risk)	Yes (Low Risk)	No (High Risk)
Bharti 2020	Unclear	Unclear	Unclear	Yes (Low Risk)	Unclear	Unclear	Unclear	Unclear	Yes (Low Risk)	Yes (Low Risk)
Cruz (2015)	Unclear	Yes (Low Risk)	Unclear	Yes (Low Risk)	Unclear	Unclear	Yes (Low Risk)	Unclear	Yes (Low Risk)	Yes (Low Risk)
Curley (2017)	Unclear	Yes (Low Risk)	Unclear	Unclear	Unclear	Unclear	Yes (Low Risk)	Unclear	Yes (Low Risk)	Yes (Low Risk)
Devaney (2015)	Unclear	Yes (Low Risk)	Unclear	Unclear	Unclear	Unclear	No (High Risk)	Yes (Low Risk)	Yes (Low Risk)	Yes (Low Risk)
Gramlich (2016)	No (High Risk)	Yes (Low Risk)	Unclear	Unclear	Unclear	Unclear	Yes (Low Risk)	Unclear	Yes (Low Risk)	No (High Risk)
Horie and Laffey (2020)	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Yes (Low Risk)	Unclear	Yes (Low Risk)	Yes (Low Risk)
Horie and O'Toole (2020)	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Yes (Low Risk)	Yes (Low Risk)	Yes (Low Risk)
Khan 2019	Unclear	Yes (Low Risk)	Unclear	Unclear	Yes (Low Risk)	Unclear	Yes (Low Risk)	Yes (Low Risk)	Yes (Low Risk)	Yes (Low Risk)
Lohan (2018)	No (High Risk)	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	No (High Risk)	Yes (Low Risk)
Perlee 2019	No (High Risk)	Unclear	Unclear	Yes (Low Risk)	Unclear	Unclear	Yes (Low Risk)	Unclear	Yes (Low Risk)	Yes (Low Risk)
Rogulska 2019	Unclear	Yes (Low Risk)	Unclear	Yes (Low Risk)	Unclear	Unclear	Yes (Low Risk)	Unclear	Yes (Low Risk)	No (High Risk)

Salmenkari (2019)	No (High Risk)	Yes (Low Risk)	Unclear	Yes (Low Risk)	Unclear	Unclear	Yes (Low Risk)	Yes (Low Risk)	Yes (Low Risk)	Yes (Low Risk)
Somal (2017)	No (High Risk)	Unclear	Unclear	Yes (Low Risk)	Unclear	Unclear	Unclear	Unclear	Yes (Low Risk)	Yes (Low Risk)
Tan 2019	Yes (Low Risk)	Yes (Low Risk)	Yes (Low Risk)	Unclear	Yes (Low Risk)	Unclear	Yes (Low Risk)	Yes (Low Risk)	Yes (Low Risk)	Yes (Low Risk)

Table 4. All *in-vivo* outcomes where freshly-cultured vs. cryopreserved MSCs have been compared directly are reported.

Study	Animal Model	Outcome	Number (n)	Type and Source of MSCs	Duration of Culture Post-Thaw (hr)	Concentration of MSCs	Pre-Treatment of MSCs	Negative Control (NC)	Positive Control (PC)	p-value for Fresh MSCs vs. control	p-value for Frozen MSCs vs. control	Fresh or Frozen MSC more effective ?	p-value for Fresh vs. Frozen comparison
Acute Lung Injury and Sepsis													
Devaney (2015)	Acute lung injury induced by <i>E.coli</i> pneumonia in rats	Arterial oxygenation	10	Human Bone Marrow	0	1×10 ⁷ hMSCs/kg	N/A	N/A	PBS	<0.05	<0.05	↔	NS
		Lung compliance	10	Human Bone Marrow	0	1×10 ⁷ hMSCs/kg	N/A	N/A	PBS	<0.05	<0.05	↔	NS
		BAL protein	10	Human Bone Marrow	0	1×10 ⁷ hMSCs/kg	N/A	N/A	PBS	<0.05	<0.05	↔	NS
		BAL neutrophils	10	Human Bone Marrow	0	1×10 ⁷ hMSCs/kg	N/A	N/A	PBS	<0.05	<0.05	↔	NS
		BAL <i>E. coli</i> bacterial load	10	Human Bone Marrow	0	1×10 ⁷ hMSCs/kg	N/A	N/A	PBS	<0.05	<0.05	↔	NS
		BAL IL-6	10	Human Bone Marrow	0	1×10 ⁷ hMSCs/kg	N/A	N/A	PBS	<0.05	<0.05	↔	NS
		BAL IL-10	10	Human Bone Marrow	0	1×10 ⁷ hMSCs/kg	N/A	N/A	PBS	<0.05	<0.05	↔	NS
Cruz (2017)	Allergic Airways Inflammation induced by <i>Aspergillus</i> hyphal extract (AHE)	Large Airway Resistance	10 (Fresh) and 7 (Frozen)	Human Bone Marrow	0	1×10 ⁶ viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS
		Large Airway Resistance	6	Murine Bone Marrow	0	1×10 ⁶ viable MSC cells	Frozen MSCs washed 3 times	Naïve (PBS model)	AHE + PBS, Human Lung	<0.05	<0.05	↔	NS

	exposure in mice.						prior to use		Fibroblasts				
		Overall Tissue Resistance	10 (Fresh) and 7 (Frozen)	Human Bone Marrow	0	1x10^6 viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS
		Overall Tissue Resistance	6	Murine Bone Marrow	0	1x10^6 viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS
		Lung Elastance	10 (Fresh) and 7 (Frozen)	Human Bone Marrow	0	1x10^6 viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS
		Lung Elastance	6	Murine Bone Marrow	0	1x10^6 viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS
		Inflammation Score	10 (Fresh) and 7 (Frozen)	Human Bone Marrow	0	1x10^6 viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS

		Inflammation Score	6	Murine Bone Marrow	0	1x10 ⁶ viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS
		BALF Total Cell Number	10 (Fresh) and 7 (Frozen)	Human Bone Marrow	0	1x10 ⁶ viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS
		BALF Total Cell Number	6	Murine Bone Marrow	0	1x10 ⁶ viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS
		BAL Neutrophils	10 (Fresh) and 7 (Frozen)	Human Bone Marrow	0	1x10 ⁶ viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS
		BAL Neutrophils	6	Murine Bone Marrow	0	1x10 ⁶ viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS
		BAL Eosinophils	10 (Fresh) and 7 (Frozen)	Human Bone Marrow	0	1x10 ⁶ viable MSC cells	Frozen MSCs washed 3 times	Naïve (PBS model)	AHE + PBS, Human Lung	<0.05	<0.05	↔	NS

						prior to use		Fibroblasts					
	BAL Eosinophils	6	Murine Bone Marrow	0	1x10^6 viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS	
	BAL Macrophages	10 (Fresh) and 7 (Frozen)	Human Bone Marrow	0	1x10^6 viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS	
	BAL Macrophages	6	Murine Bone Marrow	0	1x10^6 viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS	
	BAL Lymphocytes	10 (Fresh) and 7 (Frozen)	Human Bone Marrow	0	1x10^6 viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS	
	BAL Lymphocytes	6	Murine Bone Marrow	0	1x10^6 viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	Frozen better	<0.05	

		BAL IL-1a	10 (Fresh) and 7 (Frozen)	Human Bone Marrow	0	1x10 ⁶ viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Huma n Lung Fibrobl asts	<0.05	<0.05	↔	NS
		BAL IL-1a	6	Murine Bone Marrow	0	1x10 ⁶ viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Huma n Lung Fibrobl asts	<0.05	<0.05	↔	NS
		BAL IL-3	10 (Fresh) and 7 (Frozen)	Human Bone Marrow	0	1x10 ⁶ viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Huma n Lung Fibrobl asts	<0.05	<0.05	↔	NS
		BAL IL-3	6	Murine Bone Marrow	0	1x10 ⁶ viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Huma n Lung Fibrobl asts	<0.05	<0.05	↔	NS
		BAL IL-4	10 (Fresh) and 7 (Frozen)	Human Bone Marrow	0	1x10 ⁶ viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Huma n Lung Fibrobl asts	<0.05	<0.05	↔	NS
		BAL IL-4	6	Murine Bone Marrow	0	1x10 ⁶ viable MSC cells	Frozen MSCs washed 3 times	Naïve (PBS model)	AHE + PBS, Huma n Lung	<0.05	<0.05	↔	NS

						prior to use		Fibroblasts					
	BAL IL-5	10 (Fresh) and 7 (Frozen)	Human Bone Marrow	0	1x10^6 viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS	
	BAL IL-5	6	Murine Bone Marrow	0	1x10^6 viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS	
	BAL IL-6	10 (Fresh) and 7 (Frozen)	Human Bone Marrow	0	1x10^6 viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS	
	BAL IL-6	6	Murine Bone Marrow	0	1x10^6 viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS	
	BAL IL-10	10 (Fresh) and 7 (Frozen)	Human Bone Marrow	0	1x10^6 viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS	

		BAL IL-10	6	Murine Bone Marrow	0	1x10 ⁶ viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS
		BAL IL-12-p40	10 (Fresh) and 7 (Frozen)	Human Bone Marrow	0	1x10 ⁶ viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS
		BAL IL-12-p40	6	Murine Bone Marrow	0	1x10 ⁶ viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS
		BAL IL-13	10 (Fresh) and 7 (Frozen)	Human Bone Marrow	0	1x10 ⁶ viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS
		BAL IL-13	6	Murine Bone Marrow	0	1x10 ⁶ viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS
		BAL IL-17	10 (Fresh) and 7 (Frozen)	Human Bone Marrow	0	1x10 ⁶ viable MSC cells	Frozen MSCs washed 3 times	Naïve (PBS model)	AHE + PBS, Human Lung	<0.05	<0.05	Fresh better	<0.05

						prior to use		Fibroblasts				
	BAL IL-17	6	Murine Bone Marrow	0	1x10^6 viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS
	BAL KC	10 (Fresh) and 7 (Frozen)	Human Bone Marrow	0	1x10^6 viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	Fresh better	<0.05
	BAL KC	6	Murine Bone Marrow	0	1x10^6 viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	Frozen better	<0.05
	BAL RANTES	10 (Fresh) and 7 (Frozen)	Human Bone Marrow	0	1x10^6 viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS
	BAL RANTES	6	Murine Bone Marrow	0	1x10^6 viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS

		IFN- γ	10 (Fresh) and 7 (Frozen)	Human Bone Marrow	0	1x10 ⁶ viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS
		IFN- γ	6	Murine Bone Marrow	0	1x10 ⁶ viable MSC cells	Frozen MSCs washed 3 times prior to use	Naïve (PBS model)	AHE + PBS, Human Lung Fibroblasts	<0.05	<0.05	↔	NS
Curley (2017)	Acute respiratory distress syndrome by intratracheal instillation of <i>E. coli</i> in rats.	Arterial Oxygenation (FiO ₂ = 0.3)	8-10	Human Umbilical Cord (Frozen) and Bone marrow (Fresh) MSCs	NR	1 × 10 ⁷ MSCs/kg	N/A	Sham model + PBS	<i>E. coli</i> + PBS	<0.05	<0.05	↔	NS
		Arterial Oxygenation (FiO ₂ = 1)	8-10	Human Umbilical Cord (Frozen) and Bone marrow (Fresh) MSCs	NR	1 × 10 ⁷ MSCs/kg	N/A	Sham model + PBS	<i>E. coli</i> + PBS	<0.05	<0.05	↔	NS
		Lung Compliance	8-10	Human Umbilical Cord (Frozen) and Bone marrow	NR	1 × 10 ⁷ MSCs/kg	N/A	Sham model + PBS	<i>E. coli</i> + PBS	<0.05	<0.05	↔	NS

				(Fresh) MSCs									
		Wet:Dry Lung Ratio	8-10	Human Umbilical Cord (Frozen) and Bone marrow (Fresh) MSCs	NR	1×10^7 MSCs/kg	N/A	Sham model + PBS	E. coli + PBS	<0.05	<0.05	↔	NS
		BAL Neutrophils	8-10	Human Umbilical Cord (Frozen) and Bone marrow (Fresh) MSCs	NR	1×10^7 MSCs/kg	N/A	Sham model + PBS	E. coli + PBS	<0.05	<0.05	↔	NS
		BAL Bacteria	8-10	Human Umbilical Cord (Frozen) and Bone marrow (Fresh) MSCs	NR	1×10^7 MSCs/kg	N/A	Sham model + PBS	E. coli + PBS	<0.05	<0.05	↔	NS
Barcia (2017)	1) Chronic adjuvant-induced arthritis (AIA) model 2) Hindlimb ischemia	Arthritis Index	6	Human Umbilical Cord MSCs	0	1.7×10^6 MSCs	Fresh MSCs were cryopreserved and then cultured	Sham model + PBS	N/A	p<0.0001	p<0.0001	↔	NS

	model in mice						for up to 5 days						
		Left Paw Volume	6	Human Umbilical Cord MSCs	0	1.7×10^6 MSCs	Fresh MSCs were cryopreserved and then cultured for up to 5 days	Sham model + PBS	N/A	p< 0.0001	p< 0.0001	↔	NS
		Right Paw Volume	6	Human Umbilical Cord MSCs	0	1.7×10^6 MSCs	Fresh MSCs were cryopreserved and then cultured for up to 5 days	Sham model + PBS	N/A	p< 0.0001	p< 0.0001	↔	NS
		Weight	6	Human Umbilical Cord MSCs	0	1.7×10^6 MSCs	Fresh MSCs were cryopreserved and then cultured for up to 5 days	Sham model + PBS	N/A	p< 0.0001	p< 0.0001	↔	NS

							to 5 days						
		Blood Flow Ratio in Hindlimb D0	12	Human Umbilical Cord MSCs	0	2×10^5 MSCs	Fresh MSCs were cryopreserved and then cultured for up to 5 days	N/A	PBS	NS	NS	↔	NS
		Blood Flow Ratio in Hindlimb D7	12	Human Umbilical Cord MSCs	0	2×10^5 MSCs	Fresh MSCs were cryopreserved and then cultured for up to 5 days	N/A	PBS	p=0.008	p=0.019	↔	NS
		Blood Flow Ratio in Hindlimb D14	12	Human Umbilical Cord MSCs	0	2×10^5 MSCs	Fresh MSCs were cryopreserved and then cultured for up to 5 days	N/A	PBS	p=0.012	p=0.031	↔	NS

							to 5 days						
		Blood Flow Ratio in Hindlimb D21	12	Human Umbilical Cord MSCs	0	2×10^5 MSCs	Fresh MSCs were cryopreserved and then cultured for up to 5 days	N/A	PBS	p= 0.004	p= 0.002	↔	NS
Salmenkari (2019)	Acute phase and Regenerative Phase of Colitis model in mice	Macroscopic Score	9	Human Bone Marrow	NR	0.5×10^6 MSCs	N/A	Sham model with PBS	Colitis + Vehicle	PC: NS	PC: NS	↔	NS
		Colon Weight (% change)	9	Human Bone Marrow	NR	0.5×10^6 MSCs	N/A	Sham model with PBS	Colitis + Vehicle	PC: NS NC= p= 0.001	PC: NS NC: p= 0.001	↔	NS
		Colon Length	9	Human Bone Marrow	NR	0.5×10^6 MSCs	N/A	Sham model with PBS	Colitis + Vehicle	PC: NS NC= p= 0.018	PC: NS NC: p= 0.014	↔	NS
		Histopathology Score	9	Human Bone Marrow	NR	0.5×10^6 MSCs	N/A	Sham model with PBS	Colitis + Vehicle	PC: NS NC= p= 0.004	PC: NS NC: p= 0.001	↔	NS
		Regeneration	9	Human Bone Marrow	NR	0.5×10^6 MSCs	N/A	Sham model with PBS	Colitis + Vehicle	PC: NS	PC: NS	↔	NS

		IL-1b in colon tissue homogenates	9	Human Bone Marrow	NR	0.5 x 10 ⁶ MSCs	N/A	Sham model with PBS	Colitis + Vehicle	PC: NS	PC: NS	↔	NS
		TNFa in colon tissue homogenates	9	Human Bone Marrow	NR	0.5 x 10 ⁶ MSCs	N/A	Sham model with PBS	Colitis + Vehicle	PC: NS	PC: NS	↔	NS
		IL-1b mRNA in colon	9	Human Bone Marrow	NR	0.5 x 10 ⁶ MSCs	N/A	Sham model with PBS	Colitis + Vehicle	PC: NS	PC: NS	↔	NS
		Corticosterone in colon tissue homogenates	9	Human Bone Marrow	NR	0.5 x 10 ⁶ MSCs	N/A	Sham model with PBS	Colitis + Vehicle	PC: NS	PC: NS	↔	NS
		Tissue ACE levels	9	Human Bone Marrow	NR	0.5 x 10 ⁶ MSCs	N/A	Sham model with PBS	Colitis + Vehicle	PC: NS	PC: p<0.05	↔	NS
		Atgr1a mRNA expression	9	Human Bone Marrow	NR	0.5 x 10 ⁶ MSCs	N/A	Sham model with PBS	Colitis + Vehicle	PC: NS	PC: NS	↔	NS
		ACE shedding	9	Human Bone Marrow	NR	0.5 x 10 ⁶ MSCs	N/A	Sham model with PBS	Colitis + Vehicle	PC: NS	PC: p<0.001	↔	NS
Somal (2017)	Wound Healing of surgical dorsal limb wound in rats	Wound Area D0	3	Caprine Amniotic Fluid	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS
		Wound Area D7	3	Caprine Amniotic Fluid	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	P<0.05	P<0.05	↔	NS

	Wound Area D14	3	Caprine Amniotic Fluid	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS
	Wound Area D21	3	Caprine Amniotic Fluid	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS
	Wound Area D28	3	Caprine Amniotic Fluid	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS
	% Wound Contraction D7	3	Caprine Amniotic Fluid	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	P<0.05	NS	↔	NS
	% Wound Contraction D14	3	Caprine Amniotic Fluid	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS
	% Wound Contraction D21	3	Caprine Amniotic Fluid	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS
	% Wound Contraction D28	3	Caprine Amniotic Fluid	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS
	Epithelization	3	Caprine Amniotic Fluid	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	P<0.05	P<0.05	↔	NS
	Neovascularization	3	Caprine Amniotic Fluid	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	P<0.05	P<0.05	↔	NS
	Collagen Thickness	3	Caprine Amniotic Fluid	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	P<0.05	P<0.05	↔	NS
	Collagen Density	3	Caprine Amniotic Fluid	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	P<0.05	P<0.05	↔	NS

	Wound Area D0	3	Caprine Amniotic Sac	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS
	Wound Area D7	3	Caprine Amniotic Sac	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS
	Wound Area D14	3	Caprine Amniotic Sac	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS
	Wound Area D21	3	Caprine Amniotic Sac	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS
	Wound Area D28	3	Caprine Amniotic Sac	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS
	% Wound Contraction D7	3	Caprine Amniotic Sac	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS
	% Wound Contraction D14	3	Caprine Amniotic Sac	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS
	% Wound Contraction D21	3	Caprine Amniotic Sac	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS
	% Wound Contraction D28	3	Caprine Amniotic Sac	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS
	Epithelization	3	Caprine Amniotic Sac	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	P<0.05	P<0.05	↔	NS
	Neovascularization	3	Caprine Amniotic Sac	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	P<0.05	P<0.05	↔	NS

	Collagen Thickness	3	Caprine Amniotic Sac	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	P<0.05	↔	NS
	Collagen Density	3	Caprine Amniotic Sac	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	P<0.05	P<0.05	↔	NS
	Wound Area D0	3	Caprine Wharton's Jelly	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS
	Wound Area D7	3	Caprine Wharton's Jelly	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	P<0.05	NS	↔	NS
	Wound Area D14	3	Caprine Wharton's Jelly	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS
	Wound Area D21	3	Caprine Wharton's Jelly	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS
	Wound Area D28	3	Caprine Wharton's Jelly	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS
	% Wound Contraction D7	3	Caprine Wharton's Jelly	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	P<0.05	NS	↔	NS
	% Wound Contraction D14	3	Caprine Wharton's Jelly	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS
	% Wound Contraction D21	3	Caprine Wharton's Jelly	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS
	% Wound Contraction D28	3	Caprine Wharton's Jelly	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS

	Epithelization	3	Caprine Wharton's Jelly	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	P<0.05	P<0.05	↔	NS
	Neovascularization	3	Caprine Wharton's Jelly	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	P<0.05	P<0.05	↔	NS
	Collagen Thickness	3	Caprine Wharton's Jelly	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	P<0.05	P<0.05	↔	NS
	Collagen Density	3	Caprine Wharton's Jelly	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	P<0.05	P<0.05	↔	NS
	Wound Area D0	3	Caprine Cord Blood	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS
	Wound Area D7	3	Caprine Cord Blood	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	P<0.05	NS	↔	NS
	Wound Area D14	3	Caprine Cord Blood	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS
	Wound Area D21	3	Caprine Cord Blood	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS
	Wound Area D28	3	Caprine Cord Blood	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS
	% Wound Contraction D7	3	Caprine Cord Blood	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	P<0.05	NS	↔	NS
	% Wound Contraction D14	3	Caprine Cord Blood	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS

		% Wound Contraction D21	3	Caprine Cord Blood	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS
		% Wound Contraction D28	3	Caprine Cord Blood	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS
		Epithelization	3	Caprine Cord Blood	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	P<0.05	P<0.05	↔	NS
		Neovascularization	3	Caprine Cord Blood	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	P<0.05	P<0.05	↔	NS
		Collagen Thickness	3	Caprine Cord Blood	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	NS	NS	↔	NS
		Collagen Density	3	Caprine Cord Blood	NR	1x10 ⁶ MSC cells	N/A	N/A	PBS	P<0.05	NS	Frozen better	P<0.05
Lohan (2018)	Corneal Transplantation in rats	Opacity Score, measured from day 5 post-implantation to day 30	Fresh= 13, Frozen= 10	Rat Bone Marrow	0	1 × 10 ⁶ MSC	Frozen MSCs pre-treated with allogenic splenocytes, and co-intervention with MMF. No MMF	N/A	Transplantation + No treatment	NS	NS	↔	NR

							for Fresh MSCs.						
		Neovascularization Score, measured from day 5 post-implantation to day 30	Fresh= 13, Frozen= 10	Rat Bone Marrow	0	1×10^6 MSC	Frozen MSCs pre-treated with allogenic splenocytes, and co-intervention with MMF. No MMF for Fresh MSCs.	N/A	Transplantation + No treatment	P<0.001	NS	↔	NR
Gramlich (2016)	Retinal ischemia/reperfusion model in mice	Retinal ganglion cells/mm ²	Fresh= 10, Frozen = 8	Human MSCs	<1hr	3×10^4 MSC	N/A	Sham model	PBS	P=0.019	P=0.024	↔	NS
Perlee (2019)	Pneumosepsis Caused by Klebsiella pneumoniae	Lung Bacterial Load at 16 hours	8	Human Adipose Tissue	0	1×10^6 ASCs	MSCs infused at 1 or 6 hours after infection.	N/A	PBS	NS	P<0.001	↔	NS

		Lung Bacterial Load at 48 hours	8	Human Adipose Tissue	0	1×10^6 ASCs	MSCs infused at 1 or 6 hours after infection.	N/A	PBS	$P < 0.0001$	$P < 0.001$	$\leftrightarrow$	NS
		Blood Bacterial Load at 16 hours	8	Human Adipose Tissue	0	1×10^6 ASCs	MSCs infused at 1 or 6 hours after infection.	N/A	PBS	NS	NS	$\leftrightarrow$	NS
		Blood Bacterial Load at 48 hours	8	Human Adipose Tissue	0	1×10^6 ASCs	MSCs infused at 1 or 6 hours after infection.	N/A	PBS	$P < 0.001$	$P < 0.001$	$\leftrightarrow$	NS
		Liver Bacterial Load at 16 hours	8	Human Adipose Tissue	0	1×10^6 ASCs	MSCs infused at 1 or 6 hours after infection.	N/A	PBS	NS	NS	$\leftrightarrow$	NS
		Liver Bacterial Load at 48 hours	8	Human Adipose Tissue	0	1×10^6 ASCs	MSCs infused at 1 or 6 hours	N/A	PBS	$P < 0.0001$	$P < 0.001$	$\leftrightarrow$	NS

							after infectio n.						
	Spleen Bacterial Load at 16 hours	8	Human Adipose Tissue	0	1 × 10^6 ASCs	MSCs infused at 1 or 6 hours after infectio n.	N/A	PBS	NS	NS	↔	NS	
	Spleen Bacterial Load at 48 hours	8	Human Adipose Tissue	0	1 × 10^6 ASCs	MSCs infused at 1 or 6 hours after infectio n.	N/A	PBS	P<0.001	P<0.01	↔	NS	
	Lung TNFa at 16 hours	8	Human Adipose Tissue	0	1 × 10^6 ASCs	MSCs infused at 1 or 6 hours after infectio n.	N/A	PBS	P<0.000 1	P<0.05	↔	NS	
	Lung TNFa at 48 hours	8	Human Adipose Tissue	0	1 × 10^6 ASCs	MSCs infused at 1 or 6 hours after infectio n.	N/A	PBS	P<0.001	P<0.05	↔	NS	
	Lung IL-1b at 16 hours	8	Human Adipose Tissue	0	1 × 10^6 ASCs	MSCs infused	N/A	PBS	P<0.05	P<0.01	↔	NS	

						at 1 or 6 hours after infection.							
		Lung IL-1b at 48 hours	8	Human Adipose Tissue	0	1×10^6 ASCs	MSCs infused at 1 or 6 hours after infection.	N/A	PBS	P<0.001	P<0.05	↔	NS
		Lung IL-6 at 16 hours	8	Human Adipose Tissue	0	1×10^6 ASCs	MSCs infused at 1 or 6 hours after infection.	N/A	PBS	P<0.05	P<0.01	↔	NS
		Lung IL-6 at 48 hours	8	Human Adipose Tissue	0	1×10^6 ASCs	MSCs infused at 1 or 6 hours after infection.	N/A	PBS	P<0.01	NS	↔	NS
		MIP-2 at 16 hours	8	Human Adipose Tissue	0	1×10^6 ASCs	MSCs infused at 1 or 6 hours after infection.	N/A	PBS	P<0.05	P<0.01	↔	NS

		MIP-2 at 48 hours	8	Human Adipose Tissue	0	1×10^6 ASCs	MSCs infused at 1 or 6 hours after infection.	N/A	PBS	P<0.001	P<0.05	↔	NS
Horie and O'Toole (2020)	<i>E. coli</i> -induced lung injury.	Arterial Oxygenation	8	Human Umbilical Cord	0	1×10^7 MSCs/kg	Isolated CD362+ MSCs for use	N/A	PBS	P<0.05	P<0.05	↔	NS
		Lung Wet:Dry Ratio	8	Human Umbilical Cord	0	1×10^7 MSCs/kg	Isolated CD362+ MSCs for use	N/A	PBS	NS	NS	↔	NS
		Lung Compliance	8	Human Umbilical Cord	0	1×10^7 MSCs/kg	Isolated CD362+ MSCs for use	N/A	PBS	P<0.05	NS	↔	NS
		BAL E. Coli Counts	8	Human Umbilical Cord	0	1×10^7 MSCs/kg	Isolated CD362+ MSCs for use	N/A	PBS	P<0.05	P<0.05	↔	NS
		BAL WCC levels	8	Human Umbilical Cord	0	1×10^7 MSCs/kg	Isolated CD362+ MSCs for use	N/A	PBS	P<0.05	P<0.05	↔	NS
		BAL Neutrophils	8	Human Umbilical Cord	0	1×10^7 MSCs/kg	Isolated CD362+ MSCs for use	N/A	PBS	P<0.05	P<0.05	↔	NS
		BAL IL-1b	8	Human Umbilical Cord	0	1×10^7 MSCs/kg	Isolated CD362+	N/A	PBS	P<0.05	P<0.05	↔	NS

							MSCs for use						
		BAL CINC-1	8	Human Umbilical Cord	0	1 × 10 ⁷ MSCs/kg	Isolated CD362+ MSCs for use	N/A	PBS	NS	NS	↔	NS
		BAL IL-6	8	Human Umbilical Cord	0	1 × 10 ⁷ MSCs/kg	Isolated CD362+ MSCs for use	N/A	PBS	P<0.05	P<0.05	↔	NS
Hori and Laffey (2020)	Ventilator- induced Lung Injury	Arterial Oxygenation	Fresh, n = 7–8; Cryopre served, n = 5– 6	Human Bone Marrow	NR	1 × 10 ⁷ MSCs/ kg	Pre- activate d MSCs (fresh and frozen were also used)	Sham model	PBS	P<0.001	P<0.001	↔	NS
		Lung Compliance	Fresh, n = 7–8; Cryopre served, n = 5– 6	Human Bone Marrow	NR	1 × 10 ⁷ MSCs/ kg	Pre- activate d MSCs (fresh and frozen were also used)	Sham model	PBS	NS	NS	↔	NS
		Lung Wet:Dry Ratio	Fresh, n = 7–8; Cryopre served, n = 5– 6	Human Bone Marrow	NR	1 × 10 ⁷ MSCs/ kg	Pre- activate d MSCs (fresh and frozen	Sham model	PBS	P<0.05	P<0.05	↔	NS

						were also used)							
	BAL Protein	Fresh, n = 7–8; Cryopreserved, n = 5–6	Human Bone Marrow	NR	1 × 10^7 MSCs/kg	Pre-activate d MSCs (fresh and frozen were also used)	Sham model	PBS	NS	NS	↔	NS	
	Percentage of Alveolar Airspace	Fresh, n = 8; Cryopreserved, n = 6	Human Bone Marrow	NR	1 × 10^7 MSCs/kg	Pre-activate d MSCs (fresh and frozen were also used)	Sham model	PBS	P<0.001	P<0.001	↔	NS	
	BAL Neutrophils	Fresh, n = 6–8; Cryopreserved, n = 5–6	Human Bone Marrow	NR	1 × 10^7 MSCs/kg	Pre-activate d MSCs (fresh and frozen were also used)	Sham model	PBS	P<0.05	P<0.01	↔	NS	
	BAL CINC-1	Fresh, n = 6–8;	Human Bone Marrow	NR	1 × 10^7 MSCs/kg	Pre-activate d MSCs (fresh	Sham model	PBS	P<0.05	P<0.05	↔	NS	

			Cryopreserved, n = 5–6				and frozen were also used)						
		BAL IL-6	Fresh, n = 6–8; Cryopreserved, n = 5–6	Human Bone Marrow	NR	1×10^7 MSCs/kg	Pre-activated MSCs (fresh and frozen were also used)	Sham model	PBS	P<0.05	P<0.001	↔	NS
		BAL IL-10	Fresh, n = 6–8; Cryopreserved, n = 5–6	Human Bone Marrow	NR	1×10^7 MSCs/kg	Pre-activated MSCs (fresh and frozen were also used)	Sham model	PBS	NS	NS	↔	NS
		BAL KGF	Fresh, n = 6–8; Cryopreserved, n = 5–6	Human Bone Marrow	NR	1×10^7 MSCs/kg	Pre-activated MSCs (fresh and frozen were also used)	Sham model	PBS	NS	NS	↔	NS

		BAL PGE2	Fresh, n = 6–8; Cryopreserved, n = 5–6	Human Bone Marrow	NR	1×10^7 MSCs/kg	Pre-activate d MSCs (fresh and frozen were also used)	Sham model	PBS	NS	NS	↔	NS
Tan (2019)	Polymicrobial sepsis induced by cecal-ligation-and-puncture (CLP)	%CD11b+/E. coli+ cells in Peritoneal Fluid	Fresh, n = 12; Cryopreserved, n = 11	Human Bone Marrow	0	2.5×10^5 MSC cells	N/A	Sham model	PBS	P<0.0001	P<0.0001	↔	NS
		Peritoneal CFU #	Fresh, n = 12; Cryopreserved, n = 11	Human Bone Marrow	0	2.5×10^5 MSC cells	N/A	Sham model	PBS	NS	NS	↔	NS
		Plasma Lactate	Fresh, n = 12; Cryopreserved, n = 11	Human Bone Marrow	0	2.5×10^5 MSC cells	N/A	Sham model	PBS	P<0.05	P<0.05	↔	NS
		Plasma CCL5	Fresh, n = 12; Cryopreserved, n = 11	Human Bone Marrow	0	2.5×10^5 MSC cells	N/A	Sham model	PBS	NS	P<0.01	↔	NS
		Plasma JE	Fresh, n = 12; Cryopreserved, n = 11	Human Bone Marrow	0	2.5×10^5 MSC cells	N/A	Sham model	PBS	NS	NS	↔	NS

		Plasma KC	Fresh, n = 12; Cryopreserved, n = 11	Human Bone Marrow	0	2.5×10^5 MSC cells	N/A	Sham model	PBS	P<0.05	NS	↔	NS
		Plasma LIX	Fresh, n = 12; Cryopreserved, n = 11	Human Bone Marrow	0	2.5×10^5 MSC cells	N/A	Sham model	PBS	NS	NS	↔	NS
		Plasma IL-10	Fresh, n = 12; Cryopreserved, n = 11	Human Bone Marrow	0	2.5×10^5 MSC cells	N/A	Sham model	PBS	NS	NS	↔	NS
		Plasma IL-1b	Fresh, n = 12; Cryopreserved, n = 11	Human Bone Marrow	0	2.5×10^5 MSC cells	N/A	Sham model	PBS	NS	NS	↔	NS
Bharti (2020)	Wound healing model with $2 \times 2\text{cm}^2$ full-thickness excision skin wound in guinea pigs	Percent wound contraction D7	5	Dog Bone Marrow	NR	1×10^6 MSC cells	MSCs attached to polypropylene mesh of $2 \times 2\text{ cm}^2$ size	N/A	Antibiotic only, Mesh only, and MSCs only as control groups	NS	NS	↔	NS
		Percent wound contraction D14	5	Dog Bone Marrow	NR	1×10^6 MSC cells	MSCs attached to	N/A	Antibiotic only,	P<0.05	P<0.05	↔	NS

							polypropylene mesh of 2×2 cm2 size		Mesh only, and MSCs only as control groups				
		Percent wound contraction D21	5	Dog Bone Marrow	NR	1×10^6 MSC cells	MSCs attached to polypropylene mesh of 2×2 cm2 size	N/A	Antibiotic only, Mesh only, and MSCs only as control groups	P<0.05	P<0.05	↔	NS
		Percent wound contraction D28	5	Dog Bone Marrow	NR	1×10^6 MSC cells	MSCs attached to polypropylene mesh of 2×2 cm2 size	N/A	Antibiotic only, Mesh only, and MSCs only as control groups	P<0.05	P<0.05	↔	NS
		Epithelialization	5	Dog Bone Marrow	NR	1×10^6 MSC cells	MSCs attached to polypropylene mesh of 2×2 cm2 size	N/A	Antibiotic only, Mesh only, and MSCs only as control groups	P<0.05	P<0.05	↔	NS

							pylene mesh of 2×2 cm2 size		only, and MSCs only as control groups				
		Neovascularization	5	Dog Bone Marrow	NR	1×10 ⁶ MSC cells	MSCs attached to polypropylene mesh of 2×2 cm2 size	N/A	Antibiotic only, Mesh only, and MSCs only as control groups	P<0.05	P<0.05	↔	NS
		Collagen Density	5	Dog Bone Marrow	NR	1×10 ⁶ MSC cells	MSCs attached to polypropylene mesh of 2×2 cm2 size	N/A	Antibiotic only, Mesh only, and MSCs only as control groups	P<0.05	P<0.05	↔	NS
		Collagen Thickness	5	Dog Bone Marrow	NR	1×10 ⁶ MSC cells	MSCs attached to polypropylene	N/A	Antibiotic only, Mesh only,	P<0.05	P<0.05	↔	NS

							mesh of 2×2 cm2 size		and MSCs only as contro l groups				
Rogulska (2019)	Wound Healing of Full- thickness excisional skin wounds in mice	Percent Wound Closure D3	14	Human Adipose Tissue	24 hours	0.25-0.3 × 10 ⁶ cells in 50 µl	MSCs placed on 3D gel containi ng PPP, 0.2M sucrose, 1% DMSO	N/A	Sponta neous healin g, and 3D gel contai ning PPP, 0.2M sucros e, 1% DMSO alone	P<0.05	P<0.05	↔	NS
		Percent Wound Closure D7	14	Human Adipose Tissue	24 hours	0.25-0.3 × 10 ⁶ cells in 50 µl	MSCs placed on 3D gel containi ng PPP, 0.2M sucrose, 1% DMSO	N/A	Sponta neous healin g, and 3D gel contai ning PPP, 0.2M sucros e, 1% DMSO alone	P<0.05	P<0.05	↔	NS

		Percent Wound Closure D14	14	Human Adipose Tissue	24 hours	0.25-0.3 × 10 ⁶ cells in 50 µl	MSCs placed on 3D gel containing PPP, 0.2M sucrose, 1% DMSO	N/A	Spontaneous healing, and 3D gel containing PPP, 0.2M sucrose, 1% DMSO alone	P<0.05	P<0.05	↔	NS
		Percent Wound Closure D28	14	Human Adipose Tissue	24 hours	0.25-0.3 × 10 ⁶ cells in 50 µl	MSCs placed on 3D gel containing PPP, 0.2M sucrose, 1% DMSO	N/A	Spontaneous healing, and 3D gel containing PPP, 0.2M sucrose, 1% DMSO alone	P<0.05	P<0.05	↔	NS
Khan (2019)	Acute Spinal Cord Injury in dogs	Motor activity of hind limbs assessed by using the canine Basso Beattie Bresnahan (cBBB) score at Week 1	4	Dog Adipose Tissue	0	1 × 10 ⁷ MSC cells	Lentivirus Mediated HO-1 Gene Insertion into Ad-	N/A	Fresh MSCs expressing GFP only.	NS	NS	↔	NS

						MSCs.						
	cBBB score at Week 2	4	Dog Adipose Tissue	0	1 × 10^7 MSC cells	Lentivir us Mediate d HO-1 Gene Insertio n into Ad- MSCs.	N/A	Fresh MSCs expres sing GFP only.	NS	NS	↔	NS
	cBBB score at Week 3	4	Dog Adipose Tissue	0	1 × 10^7 MSC cells	Lentivir us Mediate d HO-1 Gene Insertio n into Ad- MSCs.	N/A	Fresh MSCs expres sing GFP only.	NS	NS	↔	NS
	cBBB score at Week 4	4	Dog Adipose Tissue	0	1 × 10^7 MSC cells	Lentivir us Mediate d HO-1 Gene Insertio n into Ad- MSCs.	N/A	Fresh MSCs expres sing GFP only.	P<0.05	NS	↔	NS
	% age of gross lesion area	4	Dog Adipose Tissue	0	1 × 10^7 MSC cells	Lentivir us Mediate d HO-1 Gene Insertio	N/A	Fresh MSCs expres sing GFP only.	NS	NS	↔	NS

							n into Ad- MSCs.						
		Fibrotic areas relative to normal	4	Dog Adipose Tissue	0	1×10^7 MSC cells	Lentivir us Mediate d HO-1 Gene Insertio n into Ad- MSCs.	Norma l (no SCI)	Fresh MSCs expres sing GFP only.	P<0.05	NS	↔	NS
		Myelinated areas relative to normal	4	Dog Adipose Tissue	0	1×10^7 MSC cells	Lentivir us Mediate d HO-1 Gene Insertio n into Ad- MSCs.	Norma l (no SCI)	Fresh MSCs expres sing GFP only.	P<0.05	NS	↔	NS

391

392 * ↔ indicates equal efficacy of Freshly-cultured and Cryopreserved MSCs.

393 **NS indicates Not Significant- statistical analysis did not yield significant difference between Freshly-cultured and Cryopreserved MSCs. NR=Not
394 reported.

395 ***If direct comparison of Freshly-cultured vs. Cryopreserved MSC was not presented in the same graph by a study, the results and discussion sections of
396 that study were used to judge efficacy of Freshly-cultured vs. Cryopreserved MSCs for the table above.

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399

400 Table 5. Summary of similar *in-vivo* outcomes reported across studies.

Outcome Measure	Study	Unit of Measurement	Number of samples (n)	Fresh MSC Mean	Fresh MSC Std Dev	Frozen MSC Mean	Frozen MSC Std Dev
Arterial Oxygenation	Curley (2017)	mmHg	8 to 10	217.77	77.93	242.75	84.14
	Devaney (2015)	mmHg	10	265.5	67.86	247.64	68.232
	Horie and O'Toole (2020)	mmHg	8	73.084	11.526	69.148	9.222
Lung Compliance	Curley (2017)	mL/mmHg	8 to 10	0.862	0.0816	0.81842	0.0979
	Devaney (2015)	mL/mmHg	12	0.82264	0.13208	0.76451	0.12794
	Horie and O'Toole (2020)	mL/mmHg	8	0.55939	0.08881	0.45055	4.53075
Wet:Dry Lung Ratio	Curley (2017)	Ratio	8 to 10	4.72779	0.18804	4.7693	0.1567
	Horie and O'Toole (2020)	Ratio	8	4.7643	0.0735	4.9387	0.2942
BAL IL-6 levels	Devaney (2015)	pg/ml	12	348.93	207.5	363.22	142.5
	Horie and O'Toole (2020)	pg/ml	8	224.67	119.86	181.51	126.72
% of Wound Contraction on D7	Somal (2017)	Percentage	3	60.076	16.667	55.679	12.755
	Bharti (2020)	Percentage	5	16.104	1.062	14.521	2.123
	Rogulska (2019)	Percentage	14	51.402	5.741	52.069	4.94
% of Wound Contraction on D14	Somal (2017)	Percentage	3	96.374	0.85	89.937	5.103
	Bharti (2020)	Percentage	5	67.363	1.689	71.537	2.123
	Rogulska (2019)	Percentage	14	99.065	2.8	99.866	2.804

% of Wound Contraction on D21	Somal (2017)	Percentage	3	99.85	0.681	98.515	2.89
	Bharti (2020)	Percentage	5	84.141	1.926	89.457	1.769
% of Wound Contraction on D28	Somal (2017)	Percentage	3	100.433		100.288	0.681
	Bharti (2020)	Percentage	5	99.583	0.885	99.415	0.885

414 Table 6. In-vitro outcomes where freshly-cultured vs. cryopreserved MSCs were compared directly.

Study	Outcome	Assay Used	Number (n)	Type and Source of MSCs	Time of cell preparation with out MSC (hr)	Time of outcome measurement from MSC intervention (hr)	Concentration of MSCs	Pre-Treatment of MSCs	Negative Control (NC)	Positive Control (PC)	p-value for Fresh MSCs vs. control	p-value for Frozen MSCs vs. control	Fresh or Frozen MSC more effective?	p-value for Fresh vs. Frozen comparison
Barcia (2017)	Viability	Trypan Blue	Fresh/Cultured (12); cryo <1yr(12); cryo>3yrs (5)	Human Umbilical Cord MSCs	N/A	0	NR	Fresh/Cultured MSCs were cryopreserved and then cultured for up to 5 days	N/A	N/A	N/A	N/A	↔	NS
	Apoptosis	Annexin V (and flow cytometry)	N/A	Human Umbilical Cord MSCs	N/A	2	NR	Fresh/Cultured MSCs were cryopreserved and then cultured for up to 5 days	N/A	Cultured cells incubated with H ₂ O ₂ (2mmol/L) for 2hr	NR	NR	↔	NS

	Angiogenesis: Number of master junctions (branching points)	Matrigel/ Human umbilical vein endothelial cell (HUVEC) tube formation assay	2	Human Umbilical Cord MSCs	1	16	1x10 ⁶ cells	Fresh/Cultured MSCs were cryopreserved and then cultured for up to 5 days; fresh and cryo co-cultured in basal media	N/A	HUVEC in Basal Media and HUVECs in Basal media with VEGF (100ng/mL)	NR	NR	↔	NS
	Angiogenesis: segment/tube length	Matrigel/ Human umbilical vein endothelial cell (HUVEC) tube formation assay	2	Human Umbilical Cord MSCs	1	16	1x10 ⁶ cells	Fresh/Cultured MSCs were cryopreserved and then cultured for up to 5 days; fresh and cryo co-cultured in basal media	N/A	HUVEC in Basal Media and HUVECs in Basal media with VEGF (100ng/mL)	NR	NR	↔	NS

	Angiogenesis: to tal mesh area	Matrigel/ Human umbilical vein endothelial cell (HUVEC) tube formation assay	2	Human Umbilical Cord MSCs	1	16	1x10 ⁶ cells	Fresh/Cultured MSCs were cryopreserved and then cultured for up to 5 days; fresh and cryo co-cultured in basal media	N/A	HUVEC in Basal Media and HUVECs in Basal media with VEGF (100ng/mL)	NR	NR	↔	NS
Gramlich (2016)	Viability	TUNEL staining via Apo-Direct Apoptosis Detection Kit	5	Human MSCs	N/A	24	30,000 MSCs	Both fresh and frozen cells were washed twice, resuspended in PBS and analyzed immediately or after 1h storage on wet ice	N/A	N/A	N/A	N/A	Fresh better	p<0.001
	Viability	TUNEL staining via Apo-Direct Apoptosis	5	Human MSCs	N/A	48	30,000 MSCs	Both fresh and frozen cells were washed twice,	N/A	N/A	N/A	N/A	Fresh better	p<0.001

		Detection Kit						resuspended in PBS and analyzed immediately or after 1h storage on wet ice						
	Viability	TUNEL staining via Apo-Direct Apoptosis Detection Kit	5	Human MSCs	N/A	72	30,000 MSCs	Both fresh and frozen cells were washed twice, resuspended in PBS and analyzed immediately or after 1h storage on wet ice	N/A	N/A	N/A	N/A	Fresh better	p=0.002
	Metabolic Activity (measured by XXT)	XTT Assay	6	Human MSCs	N/A	24	15,000 MSCs	N/A	N/A	N/A	N/A	N/A	↔	NS p=0.352

	Metabolic Activity (measured by XXT)	XTT Assay	6	Human MSCs	N/A	48	15,000 MSCs	N/A	N/A	N/A	N/A	N/A	↔	NS p=0.312
	Metabolic Activity (measured by XXT)	XTT Assay	6	Human MSCs	N/A	72	15,000 MSCs	N/A	N/A	N/A	N/A	N/A	↔	NS p=0.971
	IDO activity: unstimulated MSC	Concentration of kynurenine in conditioned media	6	Human MSC	N/A	48	NR	N/A	N/A	N/A	N/A	N/A	↔	NS p=0.998
	IDO activity: MSC exposed to IFN-γ	Concentration of kynurenine in conditioned media	6	Human MSC	N/A	48	NR	N/A	N/A	N/A	N/A	N/A	↔	NS p=0.099

	IDO activity: MSC exposed to IFN- γ +TNF- α	Concentration of kynurenine in conditioned media	6	Human MSC	N/A	48	NR	N/A	N/A	N/A	N/A	N/A	$\leftrightarrow$	NS p=0.951
	GDF-15: unstimulated	Human Growth Factor Array Q1	4	Human MSC	N/A	48	200,000 MSCs	N/A	N/A	Media Control	N/A	N/A	Frozen better	p=0.01
	GDF-15: stimulated with IFN- γ /TNF- α	Human Growth Factor Array Q1	4	Human MSC	N/A	48	200,000 MSCs	N/A	N/A	Media Control	N/A	N/A	$\leftrightarrow$	NS p=0.99
	IGFBP-2: unstimulated	Human Growth Factor Array Q1	4	Human MSC	N/A	48	200,000 MSCs	N/A	N/A	Media Control	N/A	N/A	$\leftrightarrow$	NS p=0.32
	IGFBP-2: stimulated with IFN- γ /TNF- α	Human Growth Factor Array Q1	4	Human MSC	N/A	48	200,000 MSCs	N/A	N/A	Media Control	N/A	N/A	$\leftrightarrow$	NS p=0.68

	IGFBP-3: unstimulated	Human Growth Factor Array Q1	4	Human MSC	N/A	48	200,000 MSCs	N/A	N/A	Media Control	N/A	N/A	↔	NS p=0.47
	IGFBP-3: stimulated with IFN- γ /TNF- α	Human Growth Factor Array Q1	4	Human MSC	N/A	48	200,000 MSCs	N/A	N/A	Media Control	N/A	N/A	↔	NS p=0.75
	IGFBP-4: unstimulated	Human Growth Factor Array Q1	4	Human MSC	N/A	48	200,000 MSCs	N/A	N/A	Media Control	N/A	N/A	↔	NS p=0.39
	IGFBP-6: unstimulated	Human Growth Factor Array Q1	4	Human MSC	N/A	48	200,000 MSCs	N/A	N/A	Media Control	N/A	N/A	↔	NS p=0.69
	IGFBP-6: stimulated with IFN- γ /TNF- α	Human Growth Factor Array Q1	4	Human MSC	N/A	48	200,000 MSCs	N/A	N/A	Media Control	N/A	N/A	Fresh better	p=0.03

	Insulin: stimulated with IFN- γ /TNF- α	Human Growth Factor Array Q1	4	Human MSC	N/A	48	200,000 MSCs	N/A	N/A	Media Control	N/A	N/A	$\leftrightarrow$	NS p=0.71
	OPG: unstimulated	Human Growth Factor Array Q1	4	Human MSC	N/A	48	200,000 MSCs	N/A	N/A	Media Control	N/A	N/A	$\leftrightarrow$	NS p=0.39
	OPG: stimulated with IFN- γ /TNF- α	Human Growth Factor Array Q1	4	Human MSC	N/A	48	200,000 MSCs	N/A	N/A	Media Control	N/A	N/A	$\leftrightarrow$	NS p=0.65
	PDGF-AA: unstimulated	Human Growth Factor Array Q1	4	Human MSC	N/A	48	200,000 MSCs	N/A	N/A	Media Control	N/A	N/A	$\leftrightarrow$	NS p=0.43
	PDGF-AA: stimulated with IFN- γ /TNF- α	Human Growth Factor Array Q1	4	Human MSC	N/A	48	200,000 MSCs	N/A	N/A	Media Control	N/A	N/A	Frozen better	p=0.04

	PIGF: unstimulated	Human Growth Factor Array Q1	4	Human MSC	N/A	48	200,000 MSCs	N/A	N/A	Media Control	N/A	N/A	↔	NS p=0.83
	SCF R: stimulated with IFN- γ /TNF- α	Human Growth Factor Array Q1	4	Human MSC	N/A	48	200,000 MSCs	N/A	N/A	Media Control	N/A	N/A	↔	NS p=0.06
	TGFb1: unstimulated	Human Growth Factor Array Q1	4	Human MSC	N/A	48	200,000 MSCs	N/A	N/A	Media Control	N/A	N/A	N/A	N/A
	TGFb1: stimulated with IFN- γ /TNF- α	Human Growth Factor Array Q1	4	Human MSC	N/A	48	200,000 MSCs	N/A	N/A	Media Control	N/A	N/A	Fresh better	p=0.05
	VEGF: unstimulated	Human Growth Factor Array Q1	4	Human MSC	N/A	48	200,000 MSCs	N/A	N/A	Media Control	N/A	N/A	↔	NS p=0.30
	VEGF: stimulated with	Human Growth Factor Array Q1	4	Human MSC	N/A	48	200,000 MSCs	N/A	N/A	Media Control	N/A	N/A	↔	NS p=0.96

	IFN- γ/TNF-α													
Tan (2019)	Viability	Trypan Blue	NR	Human BM	N/A	0	NR	N/A	N/A	N/A	N/A	N/A	↔	NS
	Viability	Trypan Blue	NR	Human BM	N/A	2	NR	N/A	N/A	N/A	N/A	N/A	Fresh better	p<0.05
	Viability	Trypan Blue	NR	Human BM	N/A	4	NR	N/A	N/A	N/A	N/A	N/A	↔	NS
	Viability	Trypan Blue	NR	Human BM	N/A	6	NR	N/A	N/A	N/A	N/A	N/A	↔	NS
	Viability (Viable Cells)	Annexin V + Propidium iodide (AV/PI)	NR	Human BM	N/A	0	NR	N/A	N/A	N/A	N/A	N/A	↔	NS
	Viability (Viable Cells)	Annexin V + Propidium	NR	Human BM	N/A	2	NR	N/A	N/A	N/A	N/A	N/A	↔	NS

		iodide (AV/PI)												
	Viability (Viable Cells)	Annexin V + Propidium iodide (AV/PI)	NR	Human BM	N/A	4	NR	N/A	N/A	N/A	N/A	N/A	↔	NS
	Viability (Viable Cells)	Annexin V + Propidium iodide (AV/PI)	NR	Human BM	N/A	6	NR	N/A	N/A	N/A	N/A	N/A	Fresh better	p<0.05
	Viability (Early apoptot ic cells)	Annexin V + Propidium iodide (AV/PI)	NR	Human BM	N/A	0	NR	N/A	N/A	N/A	N/A	N/A	↔	NS
	Viability (Early apoptot ic cells)	Annexin V + Propidium iodide (AV/PI)	NR	Human BM	N/A	2	NR	N/A	N/A	N/A	N/A	N/A	↔	NS

	Viability (Early apoptotic cells)	Annexin V + Propidium iodide (AV/PI)	NR	Human BM	N/A	4	NR	N/A	N/A	N/A	N/A	N/A	↔	NS
	Viability (Early apoptotic cells)	Annexin V + Propidium iodide (AV/PI)	NR	Human BM	N/A	6	NR	N/A	N/A	N/A	N/A	N/A	Fresh better	p<0.05
	Viability (Late apoptotic cells)	Annexin V + Propidium iodide (AV/PI)	NR	Human BM	N/A	0	NR	N/A	N/A	N/A	N/A	N/A	↔	NS
	Viability (Late apoptotic cells)	Annexin V + Propidium iodide (AV/PI)	NR	Human BM	N/A	2	NR	N/A	N/A	N/A	N/A	N/A	↔	NS
	Viability (Late apoptotic cells)	Annexin V + Propidium iodide (AV/PI)	NR	Human BM	N/A	4	NR	N/A	N/A	N/A	N/A	N/A	Fresh better	p<0.05

	Viability (Late apoptotic cells)	Annexin V + Propidium iodide (AV/PI)	NR	Human BM	N/A	6	NR	N/A	N/A	N/A	N/A	N/A	Fresh better	p<0.05
	Phagocytosis	PBMCs pre-treated with LPS the co-culture with MSC at ratio of 1:5 for 24hr	3-6	Human BM MSC: Donor 1	N/A	24	NR	N/A	Naive PBMC	LPS treated PBMC	PC: p<0.0001	PC: p<0.0001	↔	NS
	Phagocytosis	PBMCs pre-treated with LPS the co-culture with MSC at ratio of 1:5 for 24hr	3-6	Human BM MSC: Donor 2	N/A	24	NR	N/A	Naive PBMC	LPS treated PBMC	NS	NS	↔	NS

	Phagocytosis	PBMCs pre-treated with LPS the co-culture with MSC at ratio of 1:5 for 24hr	3-6	Human BM MSC: Donor 3	N/A	24	NR	N/A	Naive PBMC	LPS treated PBMC	PC: p<0.001	PC: p<0.001	↔	NS
	Permeability	Endothelial cell (EC) treated with LPS for 6hr then co-culture with MSC for 24hr at ratio of 1:2 followed by adding FITC-dextran to the transwell insert	NR	Human BM MSC: Donor 1	N/A	24	NR	N/A	Non-treated EC	LPS treated EC	PC: p<0.01	PC: p<0.01	↔	NS

	Permeability	Endothelial cell (EC) treated with LPS for 6hr then co-culture with MSC for 24hr at ratio of 1:2 followed by adding FITC-dextran to the transwell insert	NR	Human BM MSC: Donor 2	N/A	24	NR	N/A	Non-treated EC	LPS treated EC	PC: p<0.01	PC: p<0.01	↔	NS
	Permeability	Endothelial cell (EC) treated with LPS for 6hr then co-culture with MSC for 24hr at ratio of	NR	Human BM MSC: Donor 3	N/A	24	NR	N/A	Non-treated EC	LPS treated EC	PC: p<0.001	PC: p<0.001	↔	NS

		1:2 followed by adding FITC-dextran to the transwell insert												
Bharti (2020)	Growth Curve	Countess automated cell counter	NR	Canine BM	N/A	24	1x10 ⁴ cells/ml	Frozen cells were thawed in distilled water at 36C for 45-60s then enzymatically detached from mesh and added in re-warmed media with 15% FBS and washed twice at 1200rpm for 5min	N/A	N/A	N/A	N/A	↔	NS
	Growth Curve	Countess automated cell counter	NR	Canine BM	N/A	48	1x10 ⁴ cells/ml	Frozen cells were thawed in distilled water at 36C	N/A	N/A	N/A	N/A	↔	NS

								for 45-60s then enzymatically detached from mesh and added in re-warmed media with 15% FBS and washed twice at 1200rpm for 5min						
	Growth Curve	Countess automated cell counter	NR	Canine BM	N/A	72	1x10 ⁴ cells/ml	Frozen cells were thawed in distilled water at 36C for 45-60s then enzymatically detached from mesh and added in re-warmed media with 15% FBS and washed twice at 1200rpm for 5min	N/A	N/A	N/A	N/A	↔	NS

	Growth Curve	Countess automated cell counter	NR	Canine BM	N/A	96	1x10 ⁴ cells/ml	Frozen cells were thawed in distilled water at 36C for 45-60s then enzymatically detached from mesh and added in re-warmed media with 15% FBS and washed twice at 1200rpm for 5min	N/A	N/A	N/A	N/A	↔	NS
	Growth Curve	Countess automated cell counter	NR	Canine BM	N/A	120	1x10 ⁴ cells/ml	Frozen cells were thawed in distilled water at 36C for 45-60s then enzymatically detached from mesh and added in re-warmed media with	N/A	N/A	N/A	N/A	↔	NS

								15% FBS and washed twice at 1200rpm for 5min						
	Growth Curve	Countess automated cell counter	NR	Canine BM	N/A	144	1x10 ⁴ cells/ml	Frozen cells were thawed in distilled water at 36C for 45-60s then enzymatically detached from mesh and added in re-warmed media with 15% FBS and washed twice at 1200rpm for 5min	N/A	N/A	N/A	N/A	↔	NS
	Growth Curve	Countess automated cell counter	NR	Canine BM	N/A	168	1x10 ⁴ cells/ml	Frozen cells were thawed in distilled water at 36C for 45-60s then enzymatically detached	N/A	N/A	N/A	N/A	↔	NS

								from mesh and added in re-warmed media with 15% FBS and washed twice at 1200rpm for 5min						
	Growth Curve	Countess automated cell counter	NR	Canine BM	N/A	192	1x10 ⁴ cells/ml	Frozen cells were thawed in distilled water at 36C for 45-60s then enzymatically detached from mesh and added in re-warmed media with 15% FBS and washed twice at 1200rpm for 5min	N/A	N/A	N/A	N/A	↔	NS
	Growth Curve	Countess automated cell counter	NR	Canine BM	N/A	216	1x10 ⁴ cells/ml	Frozen cells were thawed in distilled water at 36C	N/A	N/A	N/A	N/A	↔	NS

								for 45-60s then enzymatically detached from mesh and added in re-warmed media with 15% FBS and washed twice at 1200rpm for 5min						
	Growth Curve	Countess automated cell counter	NR	Canine BM	N/A	240	1x10 ⁴ cells/ml	Frozen cells were thawed in distilled water at 36C for 45-60s then enzymatically detached from mesh and added in re-warmed media with 15% FBS and washed twice at 1200rpm for 5min	N/A	N/A	N/A	N/A	↔	NS

	Growth Curve	Countess automated cell counter	NR	Canine BM	N/A	264	1x10 ⁴ cells/ml	Frozen cells were thawed in distilled water at 36C for 45-60s then enzymatically detached from mesh and added in re-warmed media with 15% FBS and washed twice at 1200rpm for 5min	N/A	N/A	N/A	N/A	↔	NS
	Growth Curve	Countess automated cell counter	NR	Canine BM	N/A	288	1x10 ⁴ cells/ml	Frozen cells were thawed in distilled water at 36C for 45-60s then enzymatically detached from mesh and added in re-warmed media with	N/A	N/A	N/A	N/A	↔	NS

								15% FBS and washed twice at 1200rpm for 5min						
	Growth Curve	Countess automated cell counter	NR	Canine BM	N/A	312	1x10 ⁴ cells/ml	Frozen cells were thawed in distilled water at 36C for 45-60s then enzymatically detached from mesh and added in re-warmed media with 15% FBS and washed twice at 1200rpm for 5min	N/A	N/A	N/A	N/A	↔	NS
	CD 105 expression	Antibody assay	NR	Canine BM	N/A	Overnight	NR	Primary antibodies (1:100 dilutions) were used for localizing different	N/A	N/A	N/A	N/A	↔	NS

								markers (CD73, CD90, CD105, CD34) with an overnight incubation period at 4 °C.						
	CD 90 expression	Antibody assay	NR	Canine BM	N/A	Overnight	NR	Primary antibodies (1:100 dilutions) were used for localizing different markers (CD73, CD90, CD105, CD34) with an overnight incubation period at 4 °C.	N/A	N/A	N/A	N/A	↔	NS
	CD 73 expression	Antibody assay	NR	Canine BM	N/A	Overnight	NR	Primary antibodies (1:100	N/A	N/A	N/A	N/A	↔	NS

								dilutions) were used for localizing different markers (CD73, CD90, CD105, CD34) with an overnight incubation period at 4 °C.						
	Population Doubling Time	N/A	NR	Canine BM	N/A	N/A	1x10 ⁴ cells/ml	N/A	N/A	N/A	N/A	N/A	↔	NS
Rogulka (2019)	Metabolic Activity/Proliferation rate	Alamar Blue	3	Human Adipose	N/A	48	NR	MSCs culture in PS1D-based gel	N/A	N/A	N/A	N/A	Fresh better	p<0.05
	Metabolic Activity/Proliferation	Alamar Blue	3	Human Adipose	N/A	96	NR	MSCs culture in PS1D-based gel	N/A	N/A	N/A	N/A	Fresh better	p<0.05

	ation rate													
	Metabol ic Activity/ Prolifer ation rate	Alamar Blue	3	Human Adipose	N/A	144	NR	MSCs culture in PS1D- based gel	N/A	N/A	N/A	N/A	↔	NS
	Viability	Alamar Blue	3	Human Adipose	N/A	24	NR	N/A	N/A	N/A	N/A	N/A	Fresh better	p<0.05
Khan (2019)	Antioxid ant Concent ration (2 fresh groups: GFP- MSC and HO- 1 MSC)	Antioxida nt Assay	6	Canine adipose	NR	NR	NR	Lentivirus- mediated GFP and HO- 1 gene insertion into Ad-MSCs	N/A	N/A	N/A	N/A	Fresh better	p<0.05

415 *N/A= Not applicable (e.g. if the experiment set up did not include a particular variable). NR= Not reported (e.g. if a particular variable was part of the
 416 experiment set up but not explicitly reported on in results section or graph).

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419 **Table 7. Summary of all in-vitro PBMC Proliferation assays from included studies.**

Study	MSCs Used	Solution	Addition to solution	Responder Cells	Fresh vs. Frozen Comparison	Duration of Culture	Proliferation Measurement	Ratio (MSC:Responder Cells)					
								1:1	1:3	1:6	1:10	1:12	1:50
Barcia (2017)	Cultured and Freshly-thawed MSCs were irradiated with 50 Gy prior to use	RPMI	5% HEPES, 5% Pen-Strep, 5% NaPyr and 5% human serum	PBMC stimulated with anti-CD3, anti-CD28, and IL-2.	Yes	16 hours	Percentage of T cells proliferation/suppression	Yes			Yes		Yes
Gramlich (2016)	Cultured and Freshly-thawed MSCs	RPMI	10% (v/v) FBS, 1% (v/v) Penicillin/Streptomycin, and 1% (v/v) L-glutamine	PBMC stimulated with 250,000 Human T-activator CD3+/D28+ Dynabeads	Yes	144 hours	CFSE Cell Proliferation Kit		Yes	Yes		Yes	
Tan (2019)	Cultured and Freshly-thawed MSCs	NR	NR	PBMC stimulated with Dynabeads Human T-Activator CD3/CD28	Yes	120 hours			Yes				

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623 **Supporting Information**

624 **S1 Fig. Search Strategy.**

625 **S2 Table. PRISMA Checklist.**

626 **S3 Table. Data Collection Items.**

Figures

Figure 1

Literature search and study inclusion.

Figure 2

Primary in-vivo outcomes. All the outcomes related to function and composition of tissues are presented below. Number of experiments represent the number of separate comparisons between fresh and cryopreserved MSCs on surrogate measures of in-vivo efficacy.

Figure 3

Primary in-vivo outcomes. All the outcomes related to protein (cytokine) expression and secretion are presented below. Number of experiments represent the number of separate comparisons between fresh and cryopreserved MSCs on surrogate measures of in-vivo efficacy

Figure 4

In-vitro potency outcomes. All the in-vitro reported outcomes are displayed below. Number of experiments represent the number of separate comparisons between fresh and cryopreserved MSCs on surrogate measures of in-vivo efficacy.

Viability

Total Experiments= 22

Figure 5

Comparison of Viability. Experiments where viability at varying time points of freshly-cultured and cryopreserved MSCs were compared directly are presented below.

Supplementary Files

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- [S3Table.pdf](#)
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