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22 **Abstract**

23

24 Cryopreservation describes techniques that permit freezing and subsequent warming of biological
25 samples without loss of viability. The application of cryopreservation in assisted reproductive
26 technology encompasses the freezing of gametes, embryos and primordial germ cells. Whilst some
27 protocols still rely on slow-freezing techniques, most now use vitrification, or ultra-rapid freezing, for
28 both oocytes and embryos due to an associated decreased risk of damage caused by the lack of ice
29 crystal formation, unlike in slow-freezing techniques. Vitrification has demonstrated its use in many
30 applications, not only following *in vitro* fertilisation (IVF) procedures in human embryology clinics, but
31 also following *in vitro* production (IVP) of embryos in agriculturally important, or endangered animal
32 species, prior to embryo transfer. Here we review the various cryopreservation and vitrification
33 technologies that are used in both humans and other animals and discuss the most recent innovations
34 in vitrification with a particular emphasis on their applicability to animal embryology.

35

36 Keywords – bovine, cryopreservation, embryo, oocyte, porcine, vitrification

37

38 **Introduction**

39

40 Over the last few decades, cryopreservation techniques have progressed rapidly. This progress has
41 made a significant impact in many fields, with reproductive medicine possibly the most significant.
42 From initial success in cryopreservation of sperm [1], it is now routinely used for the preservation of
43 oocytes, sperm and embryos within both agricultural systems and in assisted reproductive technology
44 (ART) in humans. Cryopreservation is a process by which biological cells or tissues are preserved at
45 sub-zero temperatures resulting in a radical decrease in the rate of metabolic processes and the ability
46 to store samples for extended periods [2]. However, as would be expected, freezing cells causes

47 damage and this must be circumvented. The two major causes of cellular damage are the physical
48 damage caused by the formation of ice crystals and the chemical damage that results from changes in
49 intracellular solute concentrations. Both of these damage types can be avoided, or at least
50 ameliorated, by controlling how the temperature is reduced and by modifying the cellular conditions.
51 For instance, the mechanical damage that results from the piercing action of ice crystals can be
52 avoided by making the freezing process very rapid and the significant rise in intracellular solute
53 concentration as the formation of ice crystals increases can be avoided by use of cryoprotectants [3].
54 Permeating cryoprotectants replace intracellular liquid and decrease ice formation [4]; as such they
55 need to have low toxicity, be capable of penetrating cells and be able to withstand extremely low
56 temperatures. Examples of commonly used cryoprotectants include glycerol, ethanediol, dimethyl
57 sulphoxide (DMSO), ethylene glycol (EG) and propanediol [5]. Whilst most cells cannot survive the
58 freezing process without use of a cryoprotectant, it is also important to note that simply using such
59 solutions alone is insufficient for cell survival after freezing (and thawing); survival also depends on
60 the cell type and its ability to withstand various stresses caused by physical and physiochemical
61 changes during the process, as well as rates of cooling and warming [5].

62

63 **The promise of vitrification**

64 Vitrification eradicates damage caused due to ice crystal formation during the cooling process. The
65 method involves rapid cooling and liquid solidification due to a substantial rise in viscosity and results
66 in the formation of a solid glass-like form [6]. This solid 'glassy' layer is amorphous; meaning that it
67 can readjust and take the shape of the cell, hence enabling the cell to maintain its structure and remain
68 intact, unlike in slow-freezing, where the formation of ice crystals during cooling prevents the cell from
69 maintaining its structure. See Figure 1 for a schematic comparison of slow-freezing and vitrification.
70 There have been a number of studies that have compared slow-freezing techniques and vitrification
71 in human embryology [7][8]; whilst it seems that there is a gradual move towards more widespread
72 use of vitrification clinically, the literature to date describes no consensus as to which method is the

73 best. For example, Herrero and colleagues established that cryopreservation of both human oocytes
74 and blastocysts demonstrate competitive pregnancy rates when compared to those obtained through
75 implantation of fresh samples and that vitrification was preferable in terms of minimised cellular
76 damage and higher post-warming survival rates when compared to traditional freezing processes
77 [9][10]. Whilst some still opt for a traditional slow-cooling method via the use of insemination straws,
78 others now use fast-cooling vitrification techniques, employing an array of different vitrification
79 systems including; thin capillaries or straws, Cryotop, Cryoloop, Cryolock, CryoTip, nylon mesh, plastic
80 blades, Vitri-ingá, electron microscopic grid, Gavi and the minimum drop size technique [11]. For
81 example, Kuwayama (2007) discusses the efficiency of the Cryotop method, wherein he states that
82 cryopreservation of blastocysts using the Cryotop method resulted in more live births when compared
83 to any other vitrification system [12]. Moreover, Mukaida and colleagues used clinical results from
84 725 human blastocysts (of which 80.4% survived vitrification and warming) to establish that the
85 Cryoloop system can be used as an effective method for vitrification of human blastocysts [13].
86 Additionally, Sugiyama *et al.* (2010) tested the effect of a plastic blade as a cryopreservation device
87 on survival rates of human embryos and blastocysts following vitrification and warming. Interestingly,
88 this demonstrated that whilst 98.4% of cleavage stage embryos survived vitrification and subsequent
89 warming, all of the blastocysts survived [14]. In a comparative study, Desai and colleagues
90 comparatively assessed three cryo-devices on the impact of vitrification (nylon mesh, micro-capillary
91 tips and an electron microscopy grid), of murine pre-antral follicles and found no significant
92 differences for subsequent *in vitro* development following vitrification. However, a low survival rate
93 was observed for follicles vitrified using micro-capillary tips, and it was revealed that when a large
94 number of follicles required vitrification, a nylon-mesh was most successful [15]. In 2008, Vitri-ingá,
95 was developed and tested on bovine oocytes; the device showed promising results with an 86%
96 survival rate post warming [16]. The method was subsequently adapted for use with human oocytes
97 and in 2010, Almodin and colleagues evaluated the device's success, by comparing gestational results
98 achieved via use of frozen-warmed human oocytes vitrified using Vitri-ingá and by those that did not

99 undergo vitrification. The technology was tested clinically on 125 human patients, of which 79 patients
100 received embryos that were derived from fresh oocytes, while 46 patients were implanted with
101 embryos that were developed using frozen-warmed oocytes vitrified by Vitri-ingá technology; a high
102 survival rate of 84.9% was demonstrated by oocytes that underwent vitrification. Moreover, no
103 significant differences were reported for fertilisation, implantation or pregnancy rates between the
104 patients of the two groups [17]. Successes of vitrification methods have resulted in IVF clinics around
105 the world progressively shifting away from traditional slow-freezing methods for routine use in ART
106 [18][19]. As is evident here, new vitrification techniques are constantly being developed and these can
107 be broadly classified as open or closed – the distinction depending on the degree to which there is, or
108 is not, direct contact between the media and the liquid nitrogen used during the cooling process.

109

110 **Open and closed vitrification systems: a comparison**

111 In an open system, the oocytes or embryos come into direct contact with liquid nitrogen, whereas in
112 a closed system, they do not. Direct comparisons between these types of systems have been limited;
113 however, the available evidence suggests that the viability of oocytes and embryos post-warming can
114 be similar. For instance, Papatheodorou and colleagues compared open and closed systems by
115 conducting a randomised trial using human sibling oocytes. Whilst survival rates following vitrification
116 using the closed system (82.9%) were slightly lower than that of the open system (91.0%), there was
117 no significant effect on observed, clinical, or ongoing pregnancies between the two groups. Moreover,
118 the closed system group produced higher live birth rates as well as a higher number of healthy babies
119 (27 versus 18) [20]. Researchers in Tokyo demonstrated similar findings, showing no significant
120 difference between blastocyst survival rates using the CryoTip™ (closed system) and the Cryotop
121 (available as either an open or closed carrier system) [21]. Comparisons were also made between
122 slow-freezing and ultra-rapid vitrification of human embryos, which indicated that vitrification was
123 the most reliable; these results are summarised in Table 1. Similarly, comparisons between Rapid-i®

124 and Cryotop [22], and between Vit Kit Freeze/Thaw (Irvine Scientific, CA) Global Fast Freeze/Thaw Kits
125 (LifeGlobal, Canada) [23] indicate that these systems can produce comparable results.

126

127 Interestingly, conflicting evidence by Paffoni *et al.* (2011) revealed considerably lower pregnancy rates
128 and a higher ratio of cancelled cycles for vitrification of mature human oocytes using a closed system,
129 as opposed to an open system [24]. Moreover, when embryological parameters were compared for
130 sibling oocytes that were either fresh or vitrified using the closed system, fertilisation rates and
131 cleavage rates were considerably lower for those that underwent vitrification in an open system. The
132 closed system vitrified oocytes also produced embryos of a lower standard, when compared to their
133 fresh sibling oocytes, in terms of both quantity and quality. However, when the same parameters were
134 compared between oocytes that were vitrified using the open system and their fresh sibling
135 counterparts, no significant differences were observed. Though these observations could, most likely,
136 be due to variation in sensitivities between oocytes and blastocysts, this study suggests that an open
137 vitrification system is more reliable than a closed system [24]. As this indicates, the evidence in the
138 literature when comparing open and closed vitrification systems is conflicting. Another important
139 consideration is the potential contamination of samples during the cooling process. Whilst there is
140 currently no clinical evidence of pathogenic contamination during cryopreservation of oocytes and
141 embryos specifically [25], there has been some evidence to show contamination of other types of
142 human tissue, such as bone marrow, through liquid nitrogen during cryopreservation [26]. It has also
143 been noted that slow cooling by the use of static vapour freezers reduces the risk of pathogenic
144 contamination, unlike with use of open vitrification methods in which samples come into direct
145 contact with liquid nitrogen [27]. Closed vitrification however is considered to be aseptic due to the
146 elimination of the potential for pathogen contamination from liquid nitrogen [28] and for this reason
147 closed systems could be regarded as preferable for use in vitrification. Bielanski and Vajta (2009)
148 discuss concerns regarding sterility of liquid nitrogen in ARTs, and it seems that to date, even though
149 methods exist to produce sterile liquid nitrogen, no commercial provider exists [27]. Another,

150 relatively new vitrification device, Gavi (Genea Biomedx) permits the process to be further
151 standardised and automated. The device comprises of a thin walled pod that allows for rapid
152 vitrification and warming rates; the pod also incorporates a microfluidic design in order to maintain
153 the embryo's location thereby permitting automated exchange of fluids. Finally, the instrument
154 automatically seals the pods, which, unlike the manual Cryotop method or in other existing open
155 vitrification technologies, eradicates the risk of pathogenic contamination from liquid nitrogen by
156 confirming that the pod is completely closed [10]. Results to date demonstrate that mouse (zygotes,
157 cleavage stage embryos and blastocysts) and human (vitrified-warmed blastocysts) vitrified samples
158 using the Gavi technology produced similar results to those of the control samples which were vitrified
159 using the manual Cryotop method that is currently considered to be the gold standard for vitrification
160 [10].

161

162 As is evident from the literature, both open and closed vitrification systems are now used clinically for
163 cryopreservation of human oocytes and embryos; further to this, a number of studies (discussed later)
164 have also been executed in other species.

165

166 **The mouse as a model**

167 In some cases, the vitrification of non-human oocytes and embryos can be particularly challenging,
168 such as is the case in the pig model; these challenges are discussed later, in addition to a discussion
169 pertaining to oocyte and embryo vitrification of other important agricultural species. The mouse (*Mus*
170 *musculus*) is a particularly powerful model for studying mammalian embryo development due to
171 broad morphological similarities [29]. The extensive genome similarities between mouse and human
172 coupled with the experimental tractability of the mouse also provide significant benefits to using this
173 species. Additionally, mouse embryos are also more readily available than those of many
174 agriculturally-important animal species [30]. As such, it is unsurprising that numerous experiments
175 have been conducted on cryopreservation of murine oocytes, embryos and ovaries. Similarly to the

176 case for humans, comparative studies have demonstrated that vitrification of murine oocytes
177 [15][31][32][33][34][35] and embryos at blastocyst stage [36][37] results in higher post-warming
178 survival rates, fertilisation, and better subsequent *in vitro* embryonic development than conventional
179 slow-freezing techniques. Similar findings based on vitrification of murine ovarian tissue indicates that
180 cryopreservation of mouse ovaries can be used to preserve fertility, as well as endocrine functions of
181 ovaries, an approach that has been implemented in human models [38][39][40]. Mice have also been
182 used to develop (and test) novel vitrification devices. For example, using murine blastocysts, Kong and
183 colleagues demonstrated that an open pulled straw and a glass micropipette could be used
184 independently as vitrification vessels to obtain high embryo survival rates [41]. Similarly, in a more
185 recent study, a new, simplified technique, ‘needle immersed vitrification’, was developed as a
186 vitrification approach for preservation of ovarian tissue in mice [42]. One of the key benefits of this
187 method is that the technique, which can also be used to vitrify human ovarian tissue, uses a minimal
188 volume of cryoprotectants at a low concentration, thereby resulting in lower toxicity of vitrification
189 solutions, hence resulting in less cellular-damage [42].

190

191 **Embryology and vitrification progress in agricultural animals**

192 Human ART procedures are now used clinically worldwide and such methods give families the chance
193 to have healthy offspring, which in many cases would not have been possible before such advances.
194 The ‘one child at a time’ report, published by the Human Fertilisation and Embryology Authority
195 (HFEA) in 2006 [43], aimed to reduce the incidence of multiple births following ART; however, the
196 challenges associated with agricultural animal IVF are somewhat different; procedures developed for
197 humans need to be adapted and scalable for a far larger number of viable embryos to be produced.
198 Additionally, the 100% success rate that is strived for in human IVF is perhaps not such an important
199 consideration in other animal IVF, especially considering the quantity of embryos that are required in
200 comparison. There is increasing interest within agricultural breeding communities to cryopreserve
201 oocytes and embryos of agriculturally important animals such as pig, cow and sheep [44][30]. Both

202 the domestic pig (*Sus scrofa domestica*) and cattle (*Bos taurus*) are of significant importance for meat
203 (and milk) production. The United Nations suggests that the world population is predicted to grow to
204 a projected 9.15 billion by 2050 [45]; and it has been indicated that the consumption of meat is
205 expected to rise in order to supply the 20% increase in *per capita* calorific intake. Not only this, but it
206 is current practice to transport live animals that are either genetically 'superior' or that are better to
207 matched to particular markets between countries. The transportation of live animals is both expensive
208 and logistically demanding, and hence the prospect of transporting frozen embryos as opposed to live
209 animals is an attractive one [46][47]. Not only would the ability to transport vitrified (and viable)
210 embryos be an approach that would be more practical and cost effective, but it would also minimise
211 the risk of disease transmission both within and between species [48]. In addition, routinely used
212 techniques in human embryology, such as preimplantation genetic diagnosis (PGD) and sex
213 determination techniques, could be usefully adapted for both pig and cow species. This could facilitate
214 in vitro production (IVP) of viable embryos that are predominantly female, which again, would be
215 economically favourable to agricultural breeding companies [49]. Moreover, it is important to note
216 that the challenges faced in animal embryo vitrification and warming (particularly in livestock species)
217 are somewhat different when compared to those faced in human IVF clinics; warming protocols may
218 need to be performed on farm which would inherently increase the risk of potential pathogenic
219 contamination [50]. Furthermore, existing vitrification protocols would need to be adapted for a large
220 number of viable embryos.

221

222 In comparison to mice, numerous studies have revealed that freezing oocytes and embryos of
223 agricultural species (especially the pig) is difficult. It has been established for example, that both
224 porcine oocytes and embryos are particularly susceptible to cellular damage by freezing and some
225 trials have reported subsequent blastocyst development rates as low as 5% [51]. Difficulties associated
226 with oocyte and embryo vitrification in the porcine model are primarily due to their high intracellular
227 lipid content [30]; the relative abundance of lipid droplets, particularly in 1-8 cell stage porcine

228 embryos, makes them exceptionally challenging to work with under a microscope as the lipid bilayers
229 darken the embryos, thus hindering observation of signs of fertilisation success (such as pronuclei)
230 during *in vitro* development. Additionally, studies have indicated that separation of membrane lipids
231 when freezing impacts post-thaw viability [52]. Hazel (1995) describes a threshold level, defined by
232 the intracellular lipid composition, below which cell membrane function is weakened due to a phase
233 transition that occurs in membrane fats [53]. The temperature at which this lipid phase transition
234 takes place, is inversely proportional to the amount of unsaturated fatty acids within the membrane,
235 and hence, by altering the composition of their lipid membranes, different organisms have the ability
236 to adjust this threshold temperature [54]. As such, Hazel & Williams (1990) explain that, the ability of
237 cells to survive at low temperatures is partly due to the increase in the ratio of unsaturated fatty acids
238 within the cell membrane [55].

239

240 As such, obtaining viable 8- to 16- cell embryos that survive freezing is extremely challenging [30].
241 However, many studies have been conducted which aim to improve prospects of porcine embryo
242 cryopreservation and, fortunately, some have had considerable success. For example, the application
243 of hydrostatic pressure, prior to vitrification has been shown to improve blastocyst survival rates post
244 warming, to over 10% [51]. Similarly, another study by Li and colleagues showed that embryo
245 treatment with trypsin or embryo exposure to a solution of high osmolality, could eradicate the lipid
246 layer in porcine embryos that are produced *in vitro* [56]. This was significant as the implementation of
247 this method meant that embryos could be centrifuged without prior micromanipulation (to deplete
248 the lipid bilayer) with only minimal damage to the zona pellucida [56]. Further studies have
249 demonstrated that the critical temperature at which damage caused to cells is no longer reversible is
250 at 15°C [30]. Furthermore, Men *et al.* (2011) demonstrated that disrupting the lipid bilayers via
251 micromanipulation and then centrifuging embryos prior to vitrification had a positive outcome on
252 survival post warm [57]. They also demonstrated that, using this approach, vitrification in a closed-
253 system was as successful as using open pulled straws, which was a major step forward in porcine

254 embryo cryopreservation [57]. Furthermore, Berthelot *et al.*, (2000) revealed that an ultra-rapid open
255 pulled straw can be used as a vitrification device for fast cooling of unhatched porcine blastocysts [58],
256 while Galeati *et al.*, (2011) noted low survival rates post thaw for porcine oocytes vitrified using the
257 open Cryotop system [59].

258

259 Whilst not as sensitive as pig embryos, cow embryos are also sensitive to cooling injury at very low
260 temperatures and initially only a few studies revealed that 8- and 16-cell embryos survived vitrification
261 at 0°C [30]. Polge and Willadsen also demonstrated that blastocysts were better adapted to withstand
262 cooling when compared to 8- and 16- cell embryos or even morulae, which has led scientists to focus
263 most of their attention to vitrifying embryos at only the blastocyst stage [30]. It is important to note
264 that there has been some evidence in the literature that flushed cattle embryos produced *in vivo*
265 exhibit differences in their cryopreservation properties when compared to their *in vitro* fertilised
266 counterparts (oocytes aspirated either from ovaries obtained from a slaughter house or by ovum pick
267 up following super stimulation). Specifically, Stachecki and Wiemer (2007) note that, in comparison to
268 their *in vivo* produced counterparts, bovine embryos that are developed *in vitro* are more sensitive to
269 cooling [60]. The definitive cause for this is still unclear, however one reason for may be due to the
270 metabolic differences prevalent during preimplantation development (from oocyte to hatched
271 blastocyst) of bovine embryos based on how they were developed (*in vivo* or *in vitro*) [61].

272

273 Methods of vitrification of bovine embryos have significantly progressed in recent years; specifically,
274 a study by Park and colleagues showed that an electron microscopy grid could be used as an effective
275 vitrification container (instead of traditional straws), to achieve high embryo survival rates when
276 vitrifying bovine blastocysts [62]. In another study, Mucci and colleagues compared embryo survival
277 (defined by blastocyst hatching rate) for bovine embryos that were either vitrified or slow-frozen, and
278 found a significant positive skew towards vitrification (43% survival rate for vitrified embryos,
279 compared to 12% for slow-frozen embryos) [63]. Similarly, another comparative study noted that,

280 whilst there might be slight differences between using fresh and vitrified oocytes for IVF, for nuclear
281 transfer experiments with cultured fibroblast cells, use of vitrified oocytes resulted in better
282 embryonic development [64]. Additionally, a review by Dalvit and colleagues indicated that
283 vitrification of *in vitro* matured bovine oocytes and embryos was successful, with no significant
284 differences being observed between vitrified-warmed and fresh oocytes, with respect to both survival
285 and embryonic development [65]. Additionally, no significant oocyte morphological differences were
286 found following oocyte vitrification-warming using the Cryoptop method and open-pulled straws [66].

287

288 In comparison to bovine embryos, studies suggest that cryopreservation of ovine (sheep) embryos is
289 relatively easier, primarily because the stage of development at which embryos are frozen is not
290 detrimental to post thaw success rates; embryos at the 1-cell, 2-cell and blastocyst stages have all
291 been viable after cooling at temperatures as low as 0°C [30]. However, findings demonstrated that
292 comparatively, slow-freezing with ethylene glycol was still more successful than vitrification [67]. In
293 the hope of developing vitrification techniques for ovine oocytes, Mullen and Fahy addressed some
294 issues associated with the approach, which included changes in levels of messenger RNA and injury to
295 the cytoskeleton of cells [51]. Whilst these are significant issues, some scientists have been successful
296 in obtaining blastocyst development rates of at least 10% post-warm [51] and better success rates
297 with using open pulled straws, for vitrification of sheep embryos, has been demonstrated more
298 recently [68][69]. Additionally, the first successful vitrification (using a cryoloop) of sheep oocytes at
299 the germinal vesicle stage was reported in 2013 by Moawad and colleagues, and since then,
300 techniques for ovine oocyte vitrification have been evolving [70]. One particular challenge pertaining
301 to mammalian oocyte cryopreservation is their extremely high cellular volume when compared to
302 other cell types; this makes them extremely sensitive and even more susceptible to intracellular ice
303 formation during the process of cryopreservation due to a lower surface-to-volume ratio [71]. Other
304 factors that make oocyte cryopreservation more challenging include the presence of the zona
305 pellucida and the decreased permeability of oocyte plasma membranes, both of which can hinder the

306 movement of water and cryoprotectants in and out of the oocyte [72]. Fertilisation however, alters
307 many of these parameters and hence, embryos are generally less challenging to cryopreserve [73].

308

309 As discussed previously, apart from the use of vitrification in achieving successful IVP in breeding
310 livestock, cryopreservation also has potential to save the fate of certain endangered species and
311 ingenious animal breeds [74]. In order to achieve this, it is important to be able to successfully
312 preserve primordial follicles (the main source of female gametes) of these species by slow-freezing or
313 fast-cooling. Gathering sufficient ovarian tissue (whole ovaries, ovarian cortical tissue or isolated
314 follicles) from endangered species can however be a significantly limiting factor and hence, many farm
315 and domestic animals are used as models to optimise cryopreservation techniques [75]. Specifically,
316 domestic livestock, cats, dogs and capuchin monkeys are commonly used as models to enhance our
317 current understanding of reproductive physiology and species-specific differences in non-domestic
318 ungulates, wild felids, rare canid breeds, and new world primates respectively [74]. Thus, Leibo and
319 Songsasen discuss how using these species as models to study cryopreservation and hence preserve
320 their genetic material, could pave the way to potentially eliminate the risk of extinction in endangered
321 species [76].

322

323 **Embryology and vitrification progress in aquaculture**

324 Many of the same challenges around the need to increase levels of production and to improve stock
325 that are found in agriculture also exist in both fish and invertebrate aquaculture. For many aquaculture
326 systems, there is also the additional problem that production depends on the harvest of broodstock
327 or seed from wild populations [77]. Cryopreservation of sperm is relatively common within
328 aquaculture, but widespread implementation of cryopreservation within the industry has however
329 only occurred in a limited number of species, particularly salmon and turbot [77]. Work that has been
330 done indicates that vitrification also works well in fish as an alternative to conventional

331 cryopreservation (e.g. [78]), but this approach has not been well explored in invertebrate systems,
332 which represents a major opportunity for future research [79].

333

334 Unfortunately, successful cryopreservation of intact fish embryos has yet to be achieved. Some
335 success has been reported (e.g. [80]), but protocols have proved difficult to replicate (e.g. see [81]).
336 The limiting factors for fish embryo cryopreservation include their large size, their multi-
337 compartmental nature, their high chilling sensitivity and their low membrane permeability [82]. Given
338 these challenges, without a significant breakthrough, the successful cryopreservation of fish embryos
339 at a commercial scale looks to be unlikely. The cryopreservation of primordial germ cells is however
340 possible (e.g. [83][84]) and this represents a viable approach for the conservation of genetic resources
341 and research purposes, but not for large-scale production.

342

343 **Future prospects**

344 Whilst there has been considerable success with vitrification of oocytes, embryos and even ovaries or
345 ovarian tissue (particularly in humans and the mouse model), advantages of using closed systems
346 instead of open systems, is a topic that is still widely debated in the literature; additionally, more
347 research is required to produce more data, and importantly, more reliable results especially in
348 agricultural animals. The development of automated devices for vitrification is potentially a huge leap
349 forward in this regard; however, adaptation of such technology for use in agriculturally important
350 animals such as pig and cow, whilst taking into consideration the scale of production that would be
351 required for use in the agricultural industry, is yet to be attempted. The successful implementation of
352 a closed, automated vitrification method could potentially revolutionise the field and would in no
353 doubt be in the interest of agricultural breeding companies worldwide.

354

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357

358 **Conflict of interest**

359 Tammie Roy is an employee of Genea Biomedx, the company that manufactures the vitrification
360 device Gavi.

361

362 **References**

363 [1] Polge C, Smith AU, Parkes A. Revival of spermatozoa after vitrification and dehydration at low
364 temperatures. *Nature* 1949;164.

365 [2] Armitage W. Cryopreservation of animal cells. *Symp Soc Exp Biol* 1986;41.

366 [3] Pegg DE. Cryopreservation and Freeze-Drying Protocols. In: Day JG, Stacey GN, editors.,
367 Totowa, NJ: Humana Press; 2007, p. 39–57.

368 [4] Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at– 196 C by vitrification.
369 *Nature* 1985;313:573–5.

370 [5] Pegg D. The history and principles of cryopreservation. *Semin Reprod Med* 2002;20:5–13.

371 [6] Fahy GM, MacFarlane DR, Angell CA, Meryman HT. Vitrification as an approach to
372 cryopreservation. *Cryobiology* 1984;21:407–26.

373 [7] Cao Y-X, Xing Q, Li L, Cong L, Zhang Z-G, Wei Z-L, et al. Comparison of survival and embryonic
374 development in human oocytes cryopreserved by slow-freezing and vitrification. *Fertil Steril*
375 2009;92:1306–11.

376 [8] Fadini R, Brambillasca F, Renzini MM, Merola M, Comi R, De Ponti E, et al. Human oocyte
377 cryopreservation: comparison between slow and ultrarapid methods. *Reprod Biomed Online*
378 2009;19:171–80.

379 [9] Herrero L, Martínez M, Garcia-Velasco JA. Current status of human oocyte and embryo

- 380 cryopreservation. *Curr Opin Obstet Gynecol* 2011;23:245–50.
- 381 [10] Roy TK, Brandi S, Tappe NM, Bradley CK, Vom E, Henderson C, et al. Embryo vitrification using
382 a novel semi-automated closed system yields in vitro outcomes equivalent to the manual
383 Cryotop method. *Hum Reprod* 2014;29:2431–8.
- 384 [11] Marco-Jiménez F, Jiménez-Trigos E, Almela-Miralles V, Vicente JS. Development of Cheaper
385 Embryo Vitrification Device Using the Minimum Volume Method. *PLoS One*
386 2016;11:e0148661.
- 387 [12] Kuwayama M. Highly efficient vitrification for cryopreservation of human oocytes and
388 embryos: the Cryotop method. *Theriogenology* 2007;67:73–80.
- 389 [13] Mukaida T, Nakamura S, Tomiyama T, Wada S, Oka C, Kasai M, et al. Vitrification of human
390 blastocysts using cryoloops: Clinical outcome of 223 cycles. *Hum Reprod* 2003;18:384–91.
- 391 [14] Sugiyama R, Nakagawa K, Shirai A, Sugiyama R, Nishi Y, Kuribayashi Y, et al. Clinical outcomes
392 resulting from the transfer of vitrified human embryos using a new device for
393 cryopreservation (plastic blade). *J Assist Reprod Genet* 2010;27:161–7.
- 394 [15] Desai N, AbdelHafez F, Ali MY, Sayed EH, Abu-Alhassan AM, Falcone T, et al. Mouse ovarian
395 follicle cryopreservation using vitrification or slow programmed cooling: Assessment of in
396 vitro development, maturation, ultra-structure and meiotic spindle organization. *J Obstet*
397 *Gynaecol Res* 2011;37:1–12.
- 398 [16] Almodin C, Câmara V, Oliveira L, Picada I, Seko M, Moron A. Vitrification in cattle models:
399 training model. *J Bras Reprod Assist* 2008;12:20–3.
- 400 [17] Almodin CG, Minguetti-Camara VC, Paixao CL, Pereira PC. Embryo development and gestation
401 using fresh and vitrified oocytes. *Hum Reprod* 2010;25:1192–8.
- 402 [18] Roy TK, Bradley CK, Bowman MC, McArthur SJ. Single-embryo transfer of vitrified-warmed
403 blastocysts yields equivalent live-birth rates and improved neonatal outcomes compared with
404 fresh transfers. *Fertil Steril* 2014;101:1294–301.
- 405 [19] Vajta G, Rienzi L, Ubaldi FM. Open versus closed systems for vitrification of human oocytes

406 and embryos. *Reprod Biomed Online* 2015;30:325–33.

407 [20] Papatheodorou A, Vanderzwalmen P, Panagiotidis Y, Prapas N, Zikopoulos K, Georgiou I, et al.

408 Open versus closed oocyte vitrification system: a prospective randomized sibling-oocyte

409 study. *Reprod Biomed Online* 2013;26:595–602.

410 [21] Kuwayama M, Vajta G, Ieda S, Kato O. Comparison of open and closed methods for

411 vitrification of human embryos and the elimination of potential contamination. *Reprod*

412 *Biomed Online* 2005;11:608–14.

413 [22] Hashimoto S, Amo A, Hama S, Ohsumi K, Nakaoka Y, Morimoto Y. A closed system supports

414 the developmental competence of human embryos after vitrification. *J Assist Reprod Genet*

415 2013;30:371–6.

416 [23] Lopes AS, Frederickx V, Kerkhoven G Van, Campo R, Puttemans P, Gordts S. Survival, re-

417 expansion and cell survival of human blastocysts following vitrification and warming using

418 two vitrification systems. *J Assist Reprod Genet* 2015;32:83–90.

419 [24] Paffoni A, Guarneri C, Ferrari S, Restelli L, Nicolosi AE, Scarduelli C, et al. Effects of two

420 vitrification protocols on the developmental potential of human mature oocytes. *Reprod*

421 *Biomed Online* 2011;22:292–8.

422 [25] Bielanski A. A review of the risk of contamination of semen and embryos during

423 cryopreservation and measures to limit cross-contamination during banking to prevent

424 disease transmission in ET practices. *Theriogenology* 2012;77:467–82.

425 [26] Tedder R, Zuckerman M, Goldstone A, Hawkins A, Fielding A, Briggs E, et al. Hepatitis B

426 transmission from contaminated cryopreservation tank. *Lancet* 1995;346:137–40.

427 [27] Bielanski A, Vajta G. Risk of contamination of germplasm during cryopreservation and

428 cryobanking in IVF units. *Hum Reprod* 2009;24:2457–67.

429 [28] Parmegiani L, Cognigni GE, Bernardi S, Cuomo S, Ciampaglia W, Infante FE, et al. Efficiency of

430 aseptic open vitrification and hermetical cryostorage of human oocytes. *Reprod Biomed*

431 *Online* 2011;23:505–12.

- 432 [29] Mukaida T, Wada S, Takahashi K, Pedro PB, An TZ, Kasai M. Vitrification of human embryos
433 based on the assessment of suitable conditions for 8-cell mouse embryos. *Hum Reprod*
434 1998;13:2874–9.
- 435 [30] Polge C, Willadsen SM. Freezing eggs and embryos of farm animals. *Cryobiology*
436 1978;15:370–3.
- 437 [31] Chen SU, Lien YR, Cheng YY, Chen HF, Ho HN, Yang YS. Vitrification of mouse oocytes using
438 closed pulled straws (CPS) achieves a high survival and preserves good patterns of meiotic
439 spindles, compared with conventional straws, open pulled straws (OPS) and grids. *Hum*
440 *Reprod* 2001;16:2350–6.
- 441 [32] Huang JYJ, Chen H-Y, Tan S-L, Chian R-C. Effect of choline-supplemented sodium-depleted
442 slow freezing versus vitrification on mouse oocyte meiotic spindles and chromosome
443 abnormalities. *Fertil Steril* 2007;88:1093–100.
- 444 [33] Valojerdi MR, Salehnia M. Developmental potential and ultrastructural injuries of metaphase
445 II (MII) mouse oocytes after slow freezing or vitrification. *J Assist Reprod Genet* 2005;22:119–
446 27.
- 447 [34] Lane M, Gardner DK. Vitrification of mouse oocytes using a nylon loop. *Mol Reprod Dev*
448 2001;58:342–7.
- 449 [35] Chen SU, Lien YR, Chao KH, Ho HN, Yang YS, Lee TY. Effects of cryopreservation on meiotic
450 spindles of oocytes and its dynamics after thawing: clinical implications in oocyte freezing—a
451 review article. *Mol Cell Endocrinol* 2003;202:101–7.
- 452 [36] Huang CC, Lee TH, Chen SU, Chen HH, Cheng TC, Liu CH, et al. Successful pregnancy following
453 blastocyst cryopreservation using super-cooling ultra-rapid vitrification. *Hum Reprod*
454 2005;20:122–8.
- 455 [37] Li L, Zhang X, Zhao L, Xia X, Wang W. Comparison of DNA apoptosis in mouse and human
456 blastocysts after vitrification and slow freezing. *Mol Reprod Dev* 2012;79:229–36.
- 457 [38] Sugimoto M, Maeda S, Manabe N, Miyamoto H. Development of infantile rat ovaries

- 458 autotransplanted after cryopreservation by vitrification. *Theriogenology* 2000;53:1093–103.
- 459 [39] Chen SU, Chien CL, Wu MY, Chen TH, Lai SM, Lin CW, et al. Novel direct cover vitrification for
460 cryopreservation of ovarian tissues increases follicle viability and pregnancy capability in
461 mice. *Hum Reprod* 2006;21:2794–800.
- 462 [40] Migishima F, Suzuki-Migishima R, Song S-Y, Kuramochi T, Azuma S, Nishijima M, et al.
463 Successful cryopreservation of mouse ovaries by vitrification. *Biol Reprod* 2003;68:881–7.
- 464 [41] Kong IK, Lee SI, Cho SG, Cho SK, Park CS. Comparison of open pulled straw (ops) vs glass
465 micropipette (gmp) vitrification in mouse blastocysts. *Theriogenology* 2000;53:1817–26.
- 466 [42] Wang Y, Xiao Z, Li L, Fan W, Li SW. Novel needle immersed vitrification: A practical and
467 convenient method with potential advantages in mouse and human ovarian tissue
468 cryopreservation. *Hum Reprod* 2008;23:2256–65.
- 469 [43] Braude P. One child at a time: reducing multiple births after IVF. *Expert Gr Mult Births after*
470 *IVF*, http://www.hfea.gov.uk/docs/MBSET_report.pdf; 2006 [accessed 10.04.16].
- 471 [44] Vajta G. Vitrification of the oocytes and embryos of domestic animals. *Anim Reprod Sci*
472 2000;60-61:357–64.
- 473 [45] Alexandratos N, Bruinsma J. World agriculture: towards 2015/2030: an FAO perspective. *Land*
474 *Use Policy* 2003;20:375.
- 475 [46] Liebermann J, Nawroth F, Isachenko V, Isachenko E, Rahimi G, Tucker MJ. Potential
476 importance of vitrification in reproductive medicine. *Biol Reprod* 2002;67:1671–80.
- 477 [47] Dobrinsky JR. Advancements in cryopreservation of domestic animal embryos.
478 *Theriogenology* 2002;57:285–302.
- 479 [48] Martinez EA, Cuello C, Parrilla I, Martinez CA, Nohalez A, Vazquez JL, et al. Recent advances
480 toward the practical application of embryo transfer in pigs. *Theriogenology* 2016;85:152–61.
- 481 [49] Massip A. Cryopreservation of embryos of farm animals. *Reprod Domest Anim* 2001;36:49–
482 55.
- 483 [50] Dochi O, Yamamoto Y, Saga H, Yoshida N, Kano N, Maeda J, et al. Direct transfer of bovine

- 484 embryos frozen-thawed in the presence of propylene glycol or ethylene glycol under on-farm
485 conditions in an integrated embryo transfer program. *Theriogenology* 1998;49:1051–8.
- 486 [51] Mullen SF, Fahy GM. A chronologic review of mature oocyte vitrification research in cattle,
487 pigs, and sheep. *Theriogenology* 2012;78:1709–19.
- 488 [52] Nagashima H, Kashiwazaki N, Ashman R, Grupen C, Seamark R, Nottle M. Removal of
489 cytoplasmic lipid enhances the tolerance of porcine embryos to chilling. *Biol Reprod*
490 1994;51:618–22.
- 491 [53] Hazel J. Thermal Adaptation in Biological Membranes: Is Homeoviscous Adaptation the
492 Explanation? *Physiology* 1995;57:19–42.
- 493 [54] Cossins A. Homeoviscous adaptation of biological membranes and its functional significance.
494 *Temp Adapt Biol Membr* 1994:63–76.
- 495 [55] Hazel JR, Eugene Williams E. The role of alterations in membrane lipid composition in
496 enabling physiological adaptation of organisms to their physical environment. *Prog Lipid Res*
497 1990;29:167–227.
- 498 [56] Li R, Murphy CN, Spate L, Wax D, Isom C, Rieke A, et al. Production of piglets after
499 cryopreservation of embryos using a centrifugation-based method for delipitation without
500 micromanipulation. *Biol Reprod* 2009;80:563–71.
- 501 [57] Men H, Zhao C, Si W, Murphy CN, Spate L, Liu Y, et al. Birth of piglets from in vitro-produced,
502 zona-intact porcine embryos vitrified in a closed system. *Theriogenology* 2011;76:280–9.
- 503 [58] Berthelot F, Martinat-Botté F, Locatelli A, Perreau C, Terqui M. Piglets born after vitrification
504 of embryos using the open pulled straw method. *Cryobiology* 2000;41:116–24.
- 505 [59] Galeati G, Spinaci M, Vallorani C, Bucci D, Porcu E, Tamanini C. Pig oocyte vitrification by
506 cryotop method: effects on viability, spindle and chromosome configuration and in vitro
507 fertilization. *Anim Reprod Sci* 2011;127:43–9.
- 508 [60] Stachecki JJ, Wiemer K. Human embryo cryopreservation and its effects on embryo
509 morphology. *Hum Preimplantation Embryo Sel* 2007:123–34.

- 510 [61] Khurana NK, Niemann H. Energy metabolism in preimplantation bovine embryos derived in
511 vitro or in vivo. *Biol Reprod* 2000;62:847–56.
- 512 [62] Park SP, Kim EY, Kim DI, Park NH, Won YS, Yoon SH, et al. Simple, efficient and successful
513 vitrification of bovine blastocysts using electron microscope grids. *Hum Reprod*
514 1999;14:2838–43.
- 515 [63] Mucci N, Aller J, Kaiser GG, Hozbor F, Cabodevila J, Alberio RH. Effect of estrous cow serum
516 during bovine embryo culture on blastocyst development and cryotolerance after slow
517 freezing or vitrification. *Theriogenology* 2006;65:1551–62.
- 518 [64] Dinnyés a, Dai Y, Jiang S, Yang X. High developmental rates of vitrified bovine oocytes
519 following parthenogenetic activation, in vitro fertilization, and somatic cell nuclear transfer.
520 *Biol Reprod* 2000;63:513–8.
- 521 [65] Dalvit G, Gutnisky C, Alvarez G, Cetica P. 11. Vitrification of bovine oocytes and embryos.
522 *Cryobiology* 2012;65:341–2.
- 523 [66] Morato R, Izquierdo D, Paramio M, Mogas T. Cryotops versus open-pulled straws (OPS) as
524 carriers for the cryopreservation of bovine oocytes: effects on spindle and chromosome
525 configuration and embryo development. *Cryobiology* 2008;57:137–41.
- 526 [67] Varago FC, Moutacas VS, Carvalho BC, Serapi??o R V., Vieira F, Chiarini-Garcia H, et al.
527 Comparison of conventional freezing and vitrification with dimethylformamide and ethylene
528 glycol for cryopreservation of ovine embryos. *Reprod Domest Anim* 2014;49:839–44.
- 529 [68] Green R, Santos B, Sicherle C, Landim-Alvarenga F, Bicudo S. Viability of OPS vitrified sheep
530 embryos after direct transfer. *Reprod Domest Anim* 2009;44:406–10.
- 531 [69] Bhat M, Sharma V, Khan F, Naykoo N, Yaqoob S, Vajta G, et al. Open pulled straw vitrification
532 and slow freezing of sheep IVF embryos using different cryoprotectants. *Reprod Fertil Dev*
533 2015;27:1175–80.
- 534 [70] Moawad AR, Tan SL, Xu B, Chen HY, Taketo T. L-carnitine supplementation during vitrification
535 of mouse oocytes at the germinal vesicle stage improves preimplantation development

536 following maturation and fertilization in vitro. *Biol Reprod* 2013;88:104.

537 [71] Toner M, Cravalho EG, Karel M. Thermodynamics and kinetics of intracellular ice formation
538 during freezing of biological cells. *J Appl Phys* 1990;67:1582.

539 [72] Mazur P. Kinetics of Water Loss From Cells At Subzero Temperatures and the Likelihood of
540 Intracellular Freezing. *J Gen Physiol* 1963;47:347–69.

541 [73] Ghetler Y, Yavin S, Shalgi R, Arav A. The effect of chilling on membrane lipid phase transition
542 in human oocytes and zygotes. *Hum Reprod* 2005;20:3385–9.

543 [74] Santos RR, Amorim C, Cecconi S, Fassbender M, Imhof M, Lornage J, et al. Cryopreservation of
544 ovarian tissue: an emerging technology for female germline preservation of endangered
545 species and breeds. *Anim Reprod Sci* 2010;122:151–63.

546 [75] Wildt DE. Genome resource banking for wildlife research, management, and conservation.
547 *ILAR J* 2000;41:228–34.

548 [76] Leibo SP, Songsasen N. Cryopreservation of gametes and embryos of non-domestic species.
549 *Theriogenology* 2002;57:303–26.

550 [77] Migaud H, Bell G, Cabrita E, McAndrew B, Davie A, Bobe J, et al. Gamete quality and
551 broodstock management in temperate fish. *Rev Aquac* 2013;5.

552 [78] Cuevas-Urbe R, Chesney EJ, Daly J, Tiersch TR. Vitrification of sperm from marine fish: effect
553 on motility and membrane integrity. *Aquac Res* 2015;46:1770–84.

554 [79] Paredes E. Exploring the evolution of marine invertebrate cryopreservation - Landmarks,
555 state of the art and future lines of research. *Cryobiology* 2015;71:198–209.

556 [80] Chen SL, Tian YS. Cryopreservation of flounder (*Paralichthys olivaceus*) embryos by
557 vitrification. *Theriogenology* 2005;63:1207–19.

558 [81] Edashige K, Valdez DM, Hara T, Saida N, Seki S, Kasai M. Japanese flounder (*Paralichthys*
559 *olivaceus*) embryos are difficult to cryopreserve by vitrification. *Cryobiology* 2006;53:96–106.

560 [82] Zhang T, Rawson DM. Studies on Chilling Sensitivity of Zebrafish (*Brachydanio rerio*) Embryos.
561 *Cryobiology* 1995;32:239–46.

- 562 [83] Riesco MF, Martínez-Pastor F, Chereguini O, Robles V. Evaluation of zebrafish (*Danio rerio*)
563 PGCs viability and DNA damage using different cryopreservation protocols. *Theriogenology*
564 2012;77:122–30, 130.e1–2.
- 565 [84] Marques LS, Bos-Mikich A, Godoy LC, Silva LA, Maschio D, Zhang T, et al. Viability of zebrafish
566 (*Danio rerio*) ovarian follicles after vitrification in a metal container. *Cryobiology*
567 2015;71:367–73.
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Sample (all human)	Parameter	Method of cryopreservation	
		Slow-freezing (%)	Vitrification (%)
Pronuclear stage embryos	Survival rate	89	100
	Development into blastocysts	41	52
4-cell embryos	Survival rate	91	98
	Development into blastocysts	84	90
	Pregnancies	51	53
Sample	Parameter	Method of vitrification	
		CryoTip™ (closed) (%)	Cryotop (open) (%)
Blastocysts	Survival rate	93	97
	Pregnancies	51	59
	Deliveries	48	51

570

571 Table 1: Summary of results obtained from [21]; comparison of slow-freezing versus vitrification, and
572 the CryoTip™ (closed) versus Cryotop (open) systems.

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576 Figure 1: Comparison of rates of ice crystal formation between slow-freezing (left) and
577 vitrification (right): figure illustrates that during slow-freezing, water flows out of cells due to
578 extracellular ice crystal formation, thus causing mechanical damage to the cell structure. In
579 contrast, during vitrification, cells are inserted into vitrification medium of high viscosity,
580 which prevents extracellular ice crystallisation and hence, cells remain intact. In both
581 instances, cryoprotectants prevent intracellular ice crystallisation.
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