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1   **Cryopreservation of animal oocytes and embryos: current progress and**

2   **future prospects**

3

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21

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

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

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

Embryology and vitrification progress in agricultural animals

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

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

In comparison to mice, numerous studies have revealed that freezing oocytes and embryos of agricultural species (especially the pig) is difficult. It has been established for example, that both porcine oocytes and embryos are particularly susceptible to cellular damage by freezing and some trials have reported subsequent blastocyst development rates as low as 5% [51]. Difficulties associated with oocyte and embryo vitrification in the porcine model are primarily due to their high intracellular 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

Conflict of interest

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

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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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575

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.

582

Initial cell
Before cooling

SLOW-FREEZING

VITRIFICATION

Cell inserted into
freezing medium
5°C

Cell inserted into
vitrification medium
-5°C

Water flows out of cell
due to extracellular ice
crystal formation

Damage to cell,
extracellular ice crystals form
-196°C

Cell remains intact, ''glassy''
layer forms, no ice crystals
-196°C