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1 **Reproductive Biology**

2 **The production of pig preimplantation embryos *in vitro*: current progress and future**
3 **prospects**

4
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22 **Abstract**

23 Human assisted reproductive technology procedures are routinely performed in clinics
24 globally, and some of these approaches are now common in other mammals such as cattle.
25 This is currently not the case in pigs. Given that the global population is expected to increase
26 by over two billion people between now and 2050, the demand for meat will also
27 undoubtedly increase. With this in mind, a more sustainable way to produce livestock;
28 increasing productivity and implementing methods that will lead to faster genetic selection,
29 is imperative. The establishment of routine and production scale pig embryo *in vitro*
30 production could be a solution to this problem. Producers would be able to increase the
31 overall number of offspring born, animal transportation would be more straightforward and
32 *in vitro* produced embryos could be produced from the gametes of selected elite. Here we
33 review the most recent developments in pig embryology, outline the current barriers and key
34 challenges that exist, and outline research priorities to surmount these difficulties.

35

36 **Key words:** pig; embryology; *in vitro* fertilisation; *in vitro* production; embryo culture

37 **1. Introduction**

38 Human assisted reproductive technology (ART) procedures such as *in vitro* fertilisation (IVF),
39 preimplantation genetic diagnosis (PGD) and gamete and embryo cryopreservation are well
40 established and implemented in clinics worldwide; in fact in 2016 in the UK alone over 68,000
41 IVF treatment cycles were performed, resulting in 20,028 births [1]. Similarly, the mouse is
42 widely used as a model for human ART procedures. In domestic farm animals, the motivation
43 for performing IVF, and possibly PGD, is quite different. By 2050 the world population is
44 predicted to increase from 7.6 to 9.8 billion [2], and the *per capita* increase in consumption
45 of meat and milk is expected to increase by 20% [2]. Livestock production is also a significant
46 contributor to global warming [3]. Solving these problems means that more meat needs to
47 be produced from fewer animals in less time. This could potentially place an untenable
48 demand, both on the environment and on food producers without sufficient innovation. This
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50 producers without sufficient innovation.

51

52 IVP in pigs is an attractive option for research fields such as reproductive biotechnology,
53 transgenesis and biomedicine. Moreover, taking into account the genetic, anatomical and
54 physiological similarities between pigs and humans, transgenic pigs may represent suitable
55 donors of tissues and organs for xenotransplantation, regenerative medicine, as animal
56 models of human hereditary diseases, or as animal bioreactors of recombinant human
57 proteins/biopharmaceuticals [4–14].

58

59 The strategies of IVP that are commonly applied to generate porcine embryos encompass
60 three crucial steps: 1) *in vitro* maturation (IVM); 2) IVF or somatic cell nuclear transfer (SCNT);

61 and 3) *in vitro* culture (IVC) of fertilised or cloned embryos [15–26]. Although multiple
62 methods have been used to create *in vitro* fertilised or nuclear transferred pig embryos, their
63 developmental potential and quality are low in comparison both to their *in vivo* produced
64 counterparts and to IVP embryos from other livestock species [27–36]. Therefore, more work
65 is needed to achieve the efficient generation of high quality IVP derived pig embryos for the
66 purposes of biotechnological and biomedical research [37–46].

67

68 As pigs account for c.40% of global meat consumption [4] a sustainable supply of pork to both
69 developed and developing countries also requires increased productivity through rapid
70 selection for greater feed conversion efficiency, improved disease resistance and enhanced
71 fertility. With this in mind, IVF, or more specifically, IVP could be greatly beneficial in the
72 following ways.

73

74 **1.1 Accelerating genetic progress**

75 IVP embryos produced from the gametes of selected elite parents represent an excellent
76 resource for improving food production. In recent years, food producers have made use of
77 high-throughput genomic platforms, primarily single nucleotide polymorphism (SNP) chips
78 [5], to determine genetic merit in new-borns. The speed and efficiency at which genetic
79 improvement for such traits can be introduced is however constrained by the delay between
80 conception and birth. Use of IVP embryos would theoretically have the potential to increase
81 selection intensity as the first selection step would occur before the embryo is implanted,
82 thereby immediately removing the requirement to gestate lower genetic merit animals and
83 hence ensuring uterine resource is focused only on the genetically superior candidates.

84

85 **1.2 Movement of genetics across international borders**

86 As artificial insemination (AI) is widely used in animals of agricultural importance, semen
87 samples (male genetics) are routinely distributed both nationally and internationally. For
88 female genetics however, currently the only option in pigs is to transport live animals for
89 establishing nucleus farms overseas. Use of vitrified IVP embryos on the other hand would
90 facilitate the global transport of genetically superior stock in way that delivered higher
91 animal-welfare, a lower-cost and increased bio-security. Moreover, if the embryos are sexed
92 beforehand, the drawbacks of the waste from genetically unwanted males that have to be
93 reared to market weight, including ammonia, methane and nitrous oxide [7], is eliminated.

94

95 **1.3 Animal health and welfare**

96 Farm animals carry a considerable number of endemic diseases and often it is necessary to
97 move infected, and potentially infected, pigs into a “clean” farm. Current practice involves a
98 pregnant female receiving a hysterectomy with foetuses *in utero*, followed by sacrificing the
99 mother. In contrast, embryos produced in a lab have the potential to be “clean” and could be
100 implanted into recipients on the farm significantly reduced disease risk (explored later).
101 Similarly, when re-stocking a farm, it is imperative to have one supply at a time as mixing
102 multiple populations risks transmission of disease. Embryos on the other hand, could be
103 implanted into existing sows (following improvements in transfer techniques) which means
104 that subsequent live births would receive the endemic immunological challenge of the farm
105 at birth, and thereby would not introduce new disease to the existing population.

106

107 **1.4 Further benefits**

108 By producing IVP pig embryos, a resource for future work on genome editing, which could be
109 used to improve livestock, is created. IVP embryos are also a useful resource for bio-banking,
110 in particular, maintaining biodiversity by preserving rare breeds or lines. Finally, both pig and
111 cattle embryos are an excellent model system for fundamental research into human IVF.
112 Being large mammals, like ourselves, pigs and cattle (and, by extension, aspects of the cell
113 biology of their embryos) have much closer similarity to humans than the classical mouse
114 model for fundamental biological studies. As such, and in addition to other sources of
115 embryos, these could be used to improve media, culture conditions and standard operating
116 procedures when ethical issues preclude direct experimentation on human embryos.

117

118 Cattle IVP is now comparatively well established, thus enabling vast improvements to both
119 beef and dairy production [8,15]; for example, the first use of Karyomapping, (a universal
120 means of detecting chromosome disorders) for non-human purposes has recently been
121 reported in cattle [47]. In pigs however, much work is still to be done and the received wisdom
122 is that pig IVP is notoriously difficult to achieve. Given that challenges previously faced in
123 human embryology have now been overcome, it seems that, with sufficient time and
124 resources, a re-invention of the pig IVP process could be accomplished. Furthermore, with
125 working protocols for embryo biopsy, genetic screening, sexing and possibly genome
126 sequencing there is great potential for success. These benefits are summarised in Figure 1,
127 there however remain a number of significant challenges to the implementation of pig IVP.
128 The purpose of this review is to summarise the state of the art in pig IVP, to outline the key
129 challenges and to provide a road map for research priorities to surmount these.

130

131 **2. The challenge of pig embryology and the importance of using chemically defined culture**
132 **medium**

133 The strived for, but not yet accomplished, 100% success rate in human IVF procedures is less
134 important in agricultural animal embryological procedures where the key drivers are embryo
135 quantity and cost. Given the comparatively high number of embryos required, the processes
136 of IVM, IVF and IVC in these species is generally referred to as *in vitro* production (IVP). Whilst
137 the first successful pig IVP was reported in 1986, IVP still has a relatively low success rate
138 [16,19,20,48]. More recently, several pig IVP approaches have been developed that
139 successfully generate embryos [34,43,49], however upscaling the process to the levels
140 required for production and commercial implementation remains challenging due to a high
141 incidence of polyspermy, the notorious four cell block (associated with genome activation in
142 mammalian species), and low blastulation rates.

143
144 A significant complication in the production of pig embryos is the high endogenous lipid
145 content. This lipid excess makes the oocytes and embryos look far darker and less transparent
146 under the microscope than mouse or human cells [28], thus hindering the observation of
147 initial indicators of successful fertilisation such as pronuclear development and assessment
148 of morphology, usually the first port of call in human embryology. Studies indicate that pig
149 oocytes contain more than double the amount of lipid (135-156ng) [29,30] when compared
150 to bovine oocytes (58-59ng) [31]. The role of this lipid is not well understood, but it has been
151 hypothesised that pig oocytes use intracellular triglyceride as a source of energy for
152 maturation [29]. Interestingly, however high lipid content has been correlated with impaired
153 oocyte developmental competence and low cryo-survival due to temperature sensitivity
154 [28,37].

155

156 When considering embryological procedures in any species, it is important to reflect on the
157 entire process, from oocyte collection and subsequent maturation, to fertilisation, embryo
158 culture and finally cryopreservation of material and/or embryo transfer (where applicable).
159 Ideally, both the maturation and embryo culture medium used are chemically defined, and of
160 a consistently high quality. It is also often the case that media need to be specific to each
161 developmental stage. The use of chemically defined media permits analysis of the impact of
162 the various essential components required for successful embryonic development.
163 Additionally, the composition of chemically undefined supplements, such as human serum
164 albumin (HSA) or fetal bovine serum (FBS), may vary between batches and result in the
165 possibility of media contamination. The following sections reflect sequentially on each stage
166 of the process and these issues are shown in Figure 2.

167

168 **3. Oocyte maturation**

169 It is essential that oocytes are matured to the correct stage (either *in vivo* or *in vitro*) prior to
170 fertilisation. Generally, in order to generate a sufficient volume of oocytes for research,
171 oocytes are harvested from slaughterhouse derived gilt or sow pig ovaries [38], rather
172 embryos being recovered via superovulation and uterine flushing. In part, this is due to
173 complications in the anatomy of the female pig reproductive tract, including, the fact that the
174 uterine horns are coiled with cervical folds [39]. In some cases, ovaries are obtained from
175 non-synchronised animals of unknown age and breed which can make sample control
176 difficult. Further to this, there are also inherent complications in sample acquisition; examples
177 include ovary collection procedures (only a trained person can collect ovaries, and there are
178 associated issues with biosecurity), the distance from the point of collection to the laboratory

179 and how the ovaries are stored in the laboratory before until and during oocyte retrieval.
180 Interestingly, there is some evidence in the literature that when oocytes derived from sows
181 as opposed to gilts are used for IVF, a higher proportion develop to the blastocyst stage, and
182 that susceptibility to polyspermy may be also be reduced [50–53]. In addition, a primary
183 consideration is ensuring that the mode of oocyte retrieval does not disrupt or damage the
184 cumulus-oocyte-complex (COC), pivotal to oocyte maturation [40]. Cumulus cells provide a
185 range of functions including supporting oocyte maturation (predominantly cytoplasmic
186 maturation) by allowing metabolite transfer via gap junctions, and by raising intracellular
187 cyclic adenosine monophosphate (cAMP) levels to maintain the oocyte under meiotic arrest
188 [41].

189

190 Collected pig oocytes are immature (germinal vesicle stage) and hence, need to be matured
191 (nuclear maturation stage) *in vitro* prior to fertilisation. As mentioned previously, the
192 development of oocyte maturation culture medium is a vital initial step in the process to
193 ensure that both nuclear and cytoplasmic oocyte maturation are achieved and that these
194 events are co-ordinated [54]. This is particularly important given that there is considerable
195 variation in germinal vesicle morphology at the time of oocyte collection [50]. Nuclear
196 maturation involves the processes that reverse meiotic arrest at prophase I and thereby allow
197 resumption of meiosis. In contrast, cytoplasmic maturation describes the vital processes
198 which prepare the oocyte at the germinal vesicle stage to undergo oocyte activation and
199 development following fertilisation, for example, the co-ordinated arrangement of proteins
200 and organelles [51]. Three main types of IVM media are now commonly used; Tissue Culture
201 Medium (TCM)-199, North Carolina State University (NCSU)-23 medium and modified
202 Whitten’s Medium (mWM) as those that offer the best oocyte developmental competence.

203 Whilst the main constituents of these media remain the same, some differences exist (Table
204 1) and there is clear evidence that even quite small changes in the concentration of the
205 individual components can alter success rates. For example, Funahashi and colleagues found
206 that the concentration of organic osmolytes in mWMM affected cytoplasmic maturation [52].
207 In this case, the presence of the organic osmolytes taurine and sorbitol (6mM and 12mM in
208 maturation media that contained 68.49 or 92.40 mM of sodium chloride) had a positive effect
209 on the concentration of oocyte glutathione content, but a higher concentration of sodium
210 chloride (92.40mM) disrupted the organisation of microfilaments in the oocytes [52].
211 Luteinising hormone (LH) has also been shown to improve cytoplasmic maturation, whilst the
212 presence of both follicle stimulating hormone (FSH) and LH in maturation media has been
213 shown to accelerate meiotic maturation [19]. Glucose and pyruvate have been shown to
214 support meiosis resumption through the pentose phosphate pathway (PPP), consequently
215 leading to improved rates of cytoplasmic maturation [55]. The obvious objective here is to
216 develop a suitable single medium that combines all of these factors and components; a
217 chemically defined media that supports both cytoplasmic and nuclear maturation. Numerous
218 other media supplementations have been investigated, including epidermal growth factor
219 (EGF) [38,56], insulin-like growth factor I (IGF-I) [57,58] and PG600, an approved drug used
220 for the stimulation of the oestrous cycle in gilts. This is a combination of pregnant mare serum
221 gonadotropin (PMSG) and human chorionic gonadotropin (hCG) and has a similar function to
222 FSH and LH [38]. The use of other agents to aid with meiotic resumption (such as forskolin
223 and hypoxanthine) has also been investigated in various different species, but such studies
224 are limited in the pig [59,60].
225

226 As shown in Table 1, IVM media is traditionally supplemented with porcine follicular fluid
227 (pFF) as provides oxidative stress protection and theoretically has the potential to act as a
228 non-invasive biochemical predictor of oocyte quality [61]. In theory, pFF should provide the
229 ideal microenvironment for oocyte development and currently, the supplementation of
230 media with pFF is common. There are however significant complications around this
231 component of media. Routine preparation of pFF requires aspiration from ovarian follicles,
232 centrifugation, filter sterilisation and subsequently storage at -20°C until supplementation of
233 maturation media [62,63]. This means that pFF varies between batches, and will be derived
234 from follicles at, potentially very, different stages of development. To date, proteomic
235 analysis of pFF is limited [64] and characterisation of the metabolomic profile is yet to be
236 achieved. Such analyses have been undertaken in other species and show that follicular fluid
237 is highly complex. For example, analysis of human follicular fluid (hFF) has successfully
238 identified critical roles for a large number of acute-phase proteins and antioxidant enzymes
239 including glutathione transferase, catalase and heat shock protein 27, providing evidence that
240 the human follicle is protected from oxidative stress induced toxic injury during maturation
241 [65]. Additionally, and unsurprisingly, it has been shown that many steroid and pituitary
242 hormones are present in hFF including FSH, LH, prolactin, oestradiol and progesterone, and
243 that the concentrations of these have been correlated with successful follicle growth, oocyte
244 maturation and the secretory activity of the granulosa cells both prior and subsequent to
245 ovulation [66]. Interestingly, a handful of studies have used solely follicular fluid for pig oocyte
246 maturation and subsequent fertilisation *in vitro*. Here, both static (petri dish culture) and non-
247 static (rotating, test tube based culture) systems were trialled, with positive results for the
248 non-static, solo pFF culture [67]. There are however, obvious drawbacks to the use of pFF as
249 a solo culture media, the most critical being the chemically undefined nature of the pFF and

250 size dependent difference in the composition of follicle contents [68]. In fact, the inefficiency
251 of pig IVP has been correlated with follicle size; the smaller the follicle, the less
252 developmentally competent the oocyte [69].

253

254 **4. Fertilisation**

255 Fertilisation results from the successful penetration of an oocyte by a single spermatozoon,
256 which when performed *in vitro*, is achieved by co-culturing oocytes that have been matured
257 to the correct stage with either frozen-thawed or fresh spermatozoa in a fertilisation medium.
258 Generally, due to the difficulties associated with cryopreserving boar semen [70,71], many
259 laboratories opt to use fresh, extended ejaculates as the source of spermatozoa for IVF. The
260 establishment of a block to polyspermic fertilisation is necessary for embryo survival in
261 mammals and it has been shown that polyspermic events are more common during IVF
262 procedures than *in vivo*. As such, the occurrence of polyspermy in pig IVP remains one of the
263 biggest and unsolved challenges in the field [70,72–75]. An obvious solution to minimising the
264 incidence of polyspermy would be to reduce the concentration of spermatozoa during *in vitro*
265 culture, however reduction in the spermatozoon concentration has been shown to
266 significantly reduce IVF success rates [38,76]. During natural (*in vivo*) mammalian fertilisation,
267 two mechanisms reduce the incidence of polyspermy: fast block and slow block. The fast block
268 depolarises the oocyte plasma membrane by causing an instantaneous change in sodium ion
269 permeability. In sea urchins, this has been shown to occur immediately after sperm first bind
270 with the oocyte, thereby preventing additional spermatozoa-oocyte fusion [77], but
271 remarkably, this phenomenon is yet to be established in pigs. The phenomenon of pre-
272 fertilisation zona pellucida hardening, first discovered in pigs and described by [78]
273 highlighted that the presence of an oviduct-specific glycoprotein–heparin protein complex is

274 necessary for the correct regulation of polyspermy in pigs. This again has further implications
275 for the eradication of using biological fluids in pig IVP, which must be taken into consideration.
276 Interestingly, it has also been shown that the addition of snap-frozen pFF (rapid freezing using
277 dry ice or liquid nitrogen) to fertilisation medium reduces the incidence of polyspermy [79].

278

279 Whilst *in vivo* rates of polyspermy are not known in pigs, it is clear that elements of ART can
280 increase polyspermy. For example, in comparison to naturally ovulated oocytes obtained via
281 surgical flushing of the oviduct, the incidence of polyspermy was 38% higher in oocytes
282 matured *in vitro* and subsequently fertilised under the same culture conditions [56]. Given
283 that the function of the pig ZP is not well understood, Tanihara and colleagues attempted ZP
284 removal to ascertain the function in pigs. This showed that removal of the zona can actually
285 decrease polyspermic penetration, meaning that the ZP may not be a competent factor for
286 polyspermy prevention in pigs [72]. Interestingly, studies have revealed that similarly to
287 maturation media, alterations in the constituents of fertilisation media can impact associated
288 success rates, especially when considering polyspermy [38,80,81]. Various different
289 compounds such as heparin, bovine serum albumin, ethanol, pentoxifylline and caffeine have
290 been used *in vitro* to induce the acrosome reaction in mammalian sperm [82]. Caffeine, for
291 example, has been shown to improve sperm motility by increasing levels of cyclic adenosine
292 monophosphate (cAMP) and to have an effect on the induction of capacitation, the
293 penultimate step in mammalian spermatozoa maturation [82,83]. Caffeine may however
294 induce spontaneous acrosome reactions resulting in a higher number of matured sperm cells
295 that are incapable of oocyte penetration [81]. As previously mentioned, polyspermic
296 fertilisation is common in pig IVP, and whilst washing of presumptive zygotes following co-
297 culture has been shown to decrease the incidence of polyspermy somewhat, and that the

298 sperm preparation method and co-culture time has an effect on monospermic penetration
299 [84], various compounds have been added to fertilisation media to assist with this.
300 Supplementation with adenosine, caffeine, adenosine or pyroglutamylglutamylproline
301 amide, a fertilisation promoting peptide, all increased fertilisation rates, but supplementation
302 with caffeine increased the incidence of polyspermy [85], whereas supplementation with
303 exogenous hyaluronan reduced polyspermic events [86]. The effect of calcium on oocyte
304 penetration has also been discussed in the literature, with fertilisation media
305 supplementation between 7.5 and 10mM successfully increasing the penetration rate [80].
306 Conversely, and highlighting the importance of media constituent accuracy, pig oocytes can
307 be parthenogenetically activated by supplementation with calcium ionophore A23187; the
308 calcium increase and associated cortical reaction preventing sperm penetration in intact
309 oocytes [56]. As mentioned previously, the concentration of sodium chloride is an important
310 factor to consider in pig IVP media and it has been found that a lower concentration in
311 fertilisation media led to less polyspermic events, an increase in the incidence of male
312 pronuclear formation and elevated oocyte glutathione levels, which is thought to be the main
313 non-enzymatic defence against oxygen radicals in oocytes and enzymes [87]. Another way in
314 which the incidence of polyspermy can be reduced is by the use of intracytoplasmic sperm
315 injection (ICSI), which has been successful in pigs [88–90]. Given the high lipid content in pig
316 oocytes and the associated difficulties in injecting a whole spermatozoon, the process is less
317 successful than in other species; not only is the rate of blastocyst formation lower, but the
318 quality of the embryos is inferior to IVF embryos [91]. This is also not a process that can be
319 easily scaled for IVP.

320

321 **5. Embryo culture**

322 For pigs, the literature suggests that *in vitro* fertilisation rates of approximately 45% and
323 subsequent progression to the blastocyst stage of c.30% can be achieved [53,92]. These levels
324 of success have been achieved in a variety of media, with work over the last twenty-six years
325 leading to the development of numerous types of pig embryo culture media (outlined in Table
326 1). The majority of pig embryo culture media used today is based on NCSU-23 [21], but
327 Beltsville Embryo Culture Medium (BECM) [22], Whitten's Medium (WM) and Porcine Zygote
328 Medium (PZM) [27] have also been shown to support embryogenesis. Unfortunately, and
329 similarly to the case for maturation media, none of these represent chemically defined media
330 capable of supporting embryo development from the point of fertilisation, to the hatched
331 blastocyst stage. When comparing ingredients, one key issue is that embryo culture media
332 can be very different to the *in vivo* environment. For example, NCSU-23 contains glucose,
333 which is used as an energy source for embryo development, but at a concentration
334 approximately 32 times higher than that found *in vivo* [29]. Such high levels are surprising,
335 given that this concentration is inhibitory in hamster and mouse embryology, but necessary
336 in pigs. Before the embryo's genome is activated, the metabolism of glucose occurs via the
337 pentose phosphate pathway (PPP), rather than by glycolysis [93]. Given that there is evidence
338 that suggests that glucose metabolism via the PPP has been correlated with an increase in
339 reactive oxygen species, this high concentration of glucose in NCSU-23 has successfully been
340 replaced with pyruvate and lactate as alternative energy sources [27,93]. Interestingly, it has
341 been discovered that supplementation of embryo culture media with pyruvate and lactate
342 for the first two days, followed by glucose supplementation for the subsequent four days
343 achieved the highest blastocyst formation rate [94].

344

345 Whilst embryo development to the morula and blastocyst stage is successful over 70% of the
346 time, for *in vivo* derived embryos, studies demonstrate a far lower success rate for embryo
347 development using oocytes matured *in vitro*. The “four-cell block” in pig embryo development
348 is a well-known phenomenon; there is conjecture that in pigs the transition from maternal to
349 zygotic control of development occurs at the four cell stage. The mechanism behind this is
350 however not fully understood [92]. Embryo development rates *in vitro* from the 1- or 2- cell
351 embryo to the four-cell stage are lower than rates seen with *in vivo* produced 4- cell embryos
352 that are then cultured *in vitro* [95]. Research has shown that this developmental block can be
353 overcome in a number of ways; by co-culture with oviductal or granulosa cells, the
354 supplementation of culture media with fluid from oviducts or ovarian follicles, as well as
355 modifications to culture media [96]. While these approaches have been useful during the
356 early stages of embryonic development, consistent progression to the blastocyst stage
357 remains a challenge and this again raises an issue for scaling the process to commercial
358 production. Glucose and glutamine are largely used as energy sources in pig embryo culture
359 media; a successful alternative is bovine serum albumin (BSA) which contains amino acids,
360 osmoregulators and pH stabilisers. Similarly, FBS has been shown to be beneficial for
361 continuing embryo development; in fact, it has been shown in one case that blastocyst
362 hatching only occurred in the presence of serum [97]. Similarly, Dobrinsky and colleagues
363 found that the addition of FBS to a defined medium, BECM) supported 80% of the embryos
364 cultured in the study to develop into hatched blastocysts [22]. As discussed previously
365 however, the undefined nature of the serum poses a challenge when attempting to stream-
366 lining pig embryo culture protocols; the potential variation in serum constituents may both
367 impact success rates, and make it difficult to ascertain the source of the problem. It has also

368 been shown that the stimulation of developmental progression from early cleavage to the
369 blastocyst stage can also be achieved by the presence of taurine or hypotaurine [21].

370

371 PZM is another option for embryo culture, with various iterations of this media existing, all
372 based on the same constituents. PZM-5 for example contains twice the concentration of L-
373 glutamine when compared to PZM-4 [27,98]; glutamine has been shown to supports cell
374 growth and is particularly useful for cells that have a high metabolic activity [96]. It has
375 however been shown that whilst a higher concentration of L-glutamine results in a reduction
376 in the production of reactive oxygen species [99], it can also lead to an increased
377 concentration of ammonium due to its instability. Lane and Gardner suggest that whilst a
378 build-up of ammonium may not impact blastulation rates, lower implantation rates may
379 result [100]. PZM-3 is supplemented with BSA, fatty acid free (FAF), to provide the required
380 amino acids to support the metabolic needs of the embryo, whereas PZM-4 is supplemented
381 with polyvinyl alcohol (PVA) rather than BSA. Naturally occurring chemical variations in BSA
382 have been shown to impact embryonic development; this is avoided by the use of PVA, an
383 appealing option due to its chemically defined nature. The effect of oxygen tension on embryo
384 development has been investigated in many species including pigs; while there is no definite
385 conclusion as to the effectiveness of a low oxygen environment on embryo development,
386 evidence suggests that embryo quality can be improved, but blastocyst quality is not affected
387 [101].

388

389 The osmolality of the culture media used is a key factor that influences success in this regard;
390 it has been shown that osmotic stress can have an effect on DNA replication, transcription
391 and mRNA translation, causing cellular damage [102]. There is also some debate in the

392 literature pertaining to the use, or not, of mineral oil as an overlay during both IVM and
393 embryo culture [103] to prevent evaporation, thereby maintaining the osmotic pressure and
394 the pH of the culture medium being used. Some studies have shown that oocyte nuclear
395 maturation is delayed when using mineral oil [104], and it has been suggested that toxic waste
396 products may accumulate in the media. Conversely, other studies have shown that the use of
397 mineral oil does not affect the time taken for oocyte maturation, or oocyte developmental
398 competence [103]. Oxygen tension, temperature and pH levels *in vivo* have been explored
399 extensively in humans (reviewed in [105]), and have shown that for both successful
400 embryogenesis and subsequent implantation, avoidance of oxidative stress by controlling
401 cyclic variation in oxygen, temperature and pH are important. For example, temperature and
402 pH *in vivo* has been shown to affect sperm motility and overall embryonic development. A
403 similar systematic review is however, yet to be conducted in pigs.

404

405 The exclusive use of chemically defined media does nonetheless come with some drawbacks
406 that have only recently been elucidated. The absence of proteins, growth factors and other
407 naturally occurring components has been shown to have an epigenetic impact on both
408 embryos and the resulting offspring [106–109]. Notably, [110] found that use of chemically
409 defined media can cause alterations in DNA methylation and gene expression patterns in *in*
410 *vitro* produced pig blastocysts, and that these changes can be decreased by the addition of
411 reproductive fluids in the culture media. This epigenetic impact is not to be dismissed, and
412 certainly warrants further investigation.

413

414 **6. Verification methods**

415 The efficiency of IVM and hence, subsequent embryo production can be deduced by
416 investigating nuclear maturation in oocytes [111] using oocyte staining methods. For
417 example, aceto-orcein staining enables confirmation of successful IVM of oocytes by the
418 observation of an intact germinal vesicle or germinal vesicle breakdown [112,113]. The
419 method involves fixing oocytes to slides with methanol and acetic acid (3:1) followed by
420 staining with 1% natural orcein in 45% acetic acid [112]. Whilst aceto-orcein staining allows
421 observation of morphological changes within the nuclei of cells using phase-contrast
422 microscopy [112], others have shown that this technique can result in a significant loss of
423 oocytes during the fixation step of the protocol and that results can be inconclusive for a large
424 proportion of oocytes studied due to ambiguous observations of oocyte morphology and
425 unclear results following staining [114]. Thus, alternative methods involve staining with
426 fluorescent dyes such as 4',6-diamidino-2-phenylindole (DAPI) [115,116] and Hoechst 33342
427 [117,118], however, a key limitation of the use of fluorescent dyes is the inability to accurately
428 differentiate between the germinal vesicle and germinal vesicle breakdown stages of oocytes.
429 As a solution to this, Prentice-Biensch and colleagues developed a combination staining
430 method using DAPI and anti-lamin A/C antibody (a protein present in the germinal vesicle
431 stage of bovine oocytes [111,119]). This protocol enabled identification of specific stages
432 (germinal vesicle, germinal vesicle breakdown, metaphase I and metaphase II) of nuclear
433 maturation in bovine oocytes [114]. Whilst there is no evidence to date that demonstrates
434 the successful use of the anti-lamin A/C – DAPI stain in establishing successful nuclear
435 maturation in porcine oocytes, the presence of lamin A/C in the nuclear envelope of porcine
436 oocytes in the germinal vesicle stage [111,119] suggests that the method could be also be
437 used to verify IVM of oocytes in pigs [114]. Methods for the observation of nuclei within
438 embryos include the use of a rapid fluorescent staining method which included

439 counterstaining embryos with trypan blue, followed by staining with Hoechst 33342. This
440 technique was applied in various mammals including animals of agricultural importance such
441 as pigs, cows and sheep [120]. Such methods have however now been superseded by non-
442 invasive approaches, including the development of time-lapse devices, incubators with
443 integrated time-lapse functionality. Here, culture conditions are less disturbed and various
444 morphokinetic parameters can be analysed, such timings of cleavage timings and how these
445 parameters may be indicative of ongoing embryonic development [121]. Such studies in the
446 pig are limited [122], and therefore the routine integration of such technology in pig
447 embryology is currently not feasible, but this is inevitable in the near future.

448

449 **7. Embryo transfer**

450 Subsequent to the processes involved in embryo culture is either embryo storage, or embryo
451 transfer (ET). The first successful ET in a mammal was in 1890, and since then, in cattle much
452 progress has been made; in fact, ET in this species is now relatively commonplace, and has
453 been for over 40 years [123]. This is not the case in pigs. Until relatively recently, the only
454 option for ET in pigs was surgical implantation; this is costly and high risk when compared to
455 routine AI. More recently, non-surgical deep intrauterine (NsDU) ET of non-sedated gilts has
456 become an option [39,124]. This is a far more attractive option for the industry to consider,
457 particularly given that recent studies have demonstrated that transfer of vitrified, *in vivo*
458 produced embryos morulae or blastocysts is successful [125]. Given that consistent
459 progression to these stages is challenging in pig embryology, there is a school of thought that
460 suggests performing early NsDU ETs to avoid this common developmental block. One of the
461 putative major problems in ET is asynchrony between the embryos transferred and the uterus
462 of the recipient; this means that usually, a large number of embryos (over 30 in most cases)

463 [38] are transferred to the recipients to increase the likelihood of pregnancy. Given that pig
464 IVP is not particularly robust, this adds to the problem; over double the number of embryos
465 that have the chance of implantation need to be produced for every transfer.

466

467 Whilst vitrification and subsequent shipping of cattle embryos is now relatively
468 commonplace, this is not the case in pigs. The improvement of such downstream processes
469 would assist in making pig embryo transfer procedures more achievable and cost efficient
470 [124]. The current process in pigs is not well described and has many limitations, as
471 comprehensively reviewed in Mandawala *et al.*, 2016 [126]. Additionally, there are also
472 implications of vitrification and thawing in an agricultural environment – particularly the
473 increased contamination risk and issue of upscaling protocols to facilitate larger sample
474 numbers.

475

476 **Conclusions and future prospects**

477 It is clear from the success achieved in cattle [8,15] that ART and IVP have the potential to be
478 transformative techniques in pigs. It is however also clear that, despite recent progress,
479 significant challenges remain. The ultimate aim of a successful pig IVP system would therefore
480 involve: 1) generating pig embryos from mothers as young as possible, to reduce generation
481 times; 2) genetic profiling of embryos, including sexing, use of SNP chips and sequencing; 3)
482 transport and selective implantation of embryos on farm. As discussed above, in pig IVP,
483 problems usually arise with the number of embryos that develop to the later stages of
484 development and therefore the need for chemically defined media for oocyte maturation and
485 embryo development is critical. Given that IVM currently requires media supplementation
486 with pFF, determining the critical component(s) of pFF is therefore a priority. Other

487 complications include oocyte and embryo freezing, incidence of polyspermy, and the fact that
488 many have gross genetic abnormalities (e.g. extra or missing chromosomes). There is great
489 potential to integrate PGD in pig IVP procedures, given that it is commonly used in both
490 humans and cattle [127], and that the technique is transferrable. In humans, the interrogation
491 of biopsied cells is already performed for screening for chromosome disorders and monogenic
492 traits simultaneously (Karyomapping) [128]. Karyomapping makes use of SNP chips, the like
493 of which are already used for determining estimated breeding values in pigs and cattle. If pig
494 IVP be made to work effectively, it should be possible to incorporate PGD with SNP chips to
495 reduce generation intervals and increase selection intensity. Other future novel protocols
496 may include improving IVM procedures, application of state-of-the-art morphokinetic tools
497 to monitor embryos, reducing the lipid content in embryos and screening for chromosome
498 abnormalities. This would ultimately reduce levels of chromosome abnormality, metabolic
499 problems and stress in embryos and would make on-farm trials of embryo transfer more
500 successful. More productive sows would reduce the sow overhead costs per piglet, lead to a
501 lower food conversion ratio thus reducing animal feed usage, increase selection intensity and
502 thus result in less animals required to meet market demands. Moreover, through pig IVP,
503 disease management and animal welfare concerns have the potential to be significantly
504 reduced. Pig IVP is an issue of great global significance; one that requires considerable new
505 research and development.

506

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511

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517

518 **References**

- 519 [1] Human Fertility and Embryology Authority. Fertility treatment 2014-2016 trends and
520 figures, [https://www.hfea.gov.uk/media/2563/hfea-fertility-trends-and-figures-2017-](https://www.hfea.gov.uk/media/2563/hfea-fertility-trends-and-figures-2017-v2.pdf?platform=hootsuite)
521 [v2.pdf?platform=hootsuite](https://www.hfea.gov.uk/media/2563/hfea-fertility-trends-and-figures-2017-v2.pdf?platform=hootsuite); 2017 [accessed 04 January 2018].
- 522 [2] Alexandratos N, Bruinsma J. World agriculture: towards 2015/2030: an FAO
523 perspective. *Land Use Policy* 2003;20:375.
- 524 [3] FAO. The State of Food and Agriculture 2012, <http://www.fao.org/3/a-i3028e.pdf>;
525 2013 [accessed 04 January 2018].
- 526 [4] Tilman D, Cassman KG, Matson PA, Naylor R, Polasky S. Agricultural sustainability and
527 intensive production practices. *Nature* 2002;418:671–7.
- 528 [5] Jonas E, de Koning DJ. Genomic selection needs to be carefully assessed to meet
529 specific requirements in livestock breeding programs. *Front Genet* 2015;5:1–8.
- 530 [6] Jakobsen JE, Johansen MG, Schmidt M, Liu Y, Li R, Callesen H, et al. Expression of the
531 Alzheimer’s disease mutations A β PP695sw and PSEN1M146I in double-transgenic
532 göttingen minipigs. *J Alzheimer’s Dis* 2016;53:1617–30.
- 533 [7] Eilerman SJ, Peischl J, Neuman JA, Ryerson TB, Aikin KC, Holloway MW, et al.
534 Characterization of ammonia, methane, and nitrous oxide emissions from
535 concentrated animal feeding operations in Northeastern Colorado. *Environ Sci Technol*
536 2016;50:10885–93.
- 537 [8] Perkel KJ, Tscherner A, Merrill C, Lamarre J, Madan P. The ART of selecting the best
538 embryo: A review of early embryonic mortality and bovine embryo viability assessment
539 methods. *Mol Reprod Dev* 2015;82:822–38.
- 540 [9] Samiec M, Skrzyszowska M. The possibilities of practical application of transgenic
541 mammalian species generated by somatic cell cloning in pharmacology, veterinary
542 medicine and xenotransplantation. *Pol J Vet Sci* 2011;14:329–40.
- 543 [10] Opiela J, Samiec M. Characterization of mesenchymal stem cells and their application
544 in experimental embryology. *Pol J Vet Sci* 2013;16:593–9.
- 545 [11] Hryhorowicz M, Zeyland J, Słomski R, Lipiński D. Genetically modified pigs as organ
546 donors for xenotransplantation. *Mol Biotechnol* 2017;59:435–44.
- 547 [12] Shim J, Poulsen CB, Hagensen MK, Larsen T, Heegaard PMH, Christoffersen C, et al.
548 Apolipoprotein E deficiency increases remnant lipoproteins and accelerates
549 progressive atherosclerosis, but not xanthoma formation, in gene-modified minipigs.
550 *JACC Basic to Transl Sci* 2017;2:591–600.
- 551 [13] Callesen MM, Árnadóttir SS, Lyskjær I, Ørntoft MW, Høyer S, Dagnæs-Hansen F, et al.
552 A genetically inducible porcine model of intestinal cancer. *Mol Oncol* 2017;11:1616–
553 29.
- 554 [14] Staunstrup NH, Stenderup K, Mortensen S, Primo MN, Rosada C, Steiniche T, et al.
555 Psoriasiform skin disease in transgenic pigs with high-copy ectopic expression of
556 human integrins α 2 and β 1. *Dis Model Mech* 2017;10:869–80.
- 557 [15] Van Eetvelde M, Heras S, Leroy JLMR, Van Soom A, Opsomer G. The importance of the
558 periconception period: immediate effects in cattle breeding and in assisted
559 reproduction such as artificial insemination and embryo transfer periconception in
560 physiology and medicine. In: Fazeli A, Holt W V, editors., Cham: Springer International
561 Publishing; 2017, p. 41–68.
- 562 [16] Cheng WTK, Moor RM, Polge C. In vitro fertilization of pig and sheep oocytes matured
563 in vivo and in vitro. *Theriogenology* 1986;25:146. doi:10.1016/0093-691X(86)90200-1.

- 564 [17] Samiec M, Skrzyszowska M, Opiela J. Creation of cloned pig embryos using contact-
565 inhibited or serum-starved fibroblast cells analysed in TRA VITAM for apoptosis
566 occurrence. *Ann Anim Sci* 2013;13:275–93.
- 567 [18] Opiela J, Samiec M, Romanek J. In vitro development and cytological quality of inter-
568 species (porcine→bovine) cloned embryos are affected by trichostatin A-dependent
569 epigenomic modulation of adult mesenchymal stem cells. *Theriogenology* 2017;97:27–
570 33.
- 571 [19] Mattioli M, Bacci ML, Galeati G, Seren E. Developmental competence of pig oocytes
572 matured and fertilized in vitro. *Theriogenology* 1989;31:1201–7.
- 573 [20] Somfai T, Ozawa M, Noguchi J, Kaneko H, Nakai M, Maedomari N, et al. Live piglets
574 derived from in vitro-produced zygotes vitrified at the pronuclear stage1. *Biol Reprod*
575 2009;80:42–9.
- 576 [21] Petters RM, Wells KD. Culture of pig embryos. *J Reprod Fertil Suppl* 1993;48:61–73.
- 577 [22] Dobrinsky JR, Johnson L a, Rath D. Development of a culture medium (BECM-3) for
578 porcine embryos: effects of bovine serum albumin and fetal bovine serum on embryo
579 development. *Biol Reprod* 1996;55:1069–74.
- 580 [23] Yoshioka K, Suzuki C, Itoh S, Kikuchi K, Iwamura S, Rodriguez-Martinez H. Production of
581 piglets derived from in vitro-produced blastocysts fertilized and cultured in chemically
582 defined media: effects of theophylline, adenosine, and cysteine during in vitro
583 fertilization. *Biol Reprod* 2003;69:2092–9.
- 584 [24] Samiec M. The effect of mitochondrial genome on architectural remodeling and
585 epigenetic reprogramming of donor cell nuclei in mammalian nuclear transfer-derived
586 embryos. *J Anim Feed Sci* 2005;14:393–422.
- 587 [25] Gil MA, Cuello C, Parrilla I, Vazquez JM, Roca J, Martinez EA. Advances in swine in vitro
588 embryo production technologies. *Reprod Domest Anim* 2010;45:40–8.
- 589 [26] Lee S-E, Moon JJ-M, Kim E-Y, Park S-P. Stem cell-derived bioactive materials accelerate
590 development of porcine in vitro-fertilized embryos. *Cell Reprogram* 2015;17:181–90.
- 591 [27] Yoshioka K, Suzuki C, Tanaka A, Anas IM-K, Iwamura S. Birth of piglets derived from
592 porcine zygotes cultured in a chemically defined medium1. *Biol Reprod* 2002;66:112–
593 9.
- 594 [28] Genicot G, Leroy JLMR, Van Soom A, Donnay I. The use of a fluorescent dye, Nile red,
595 to evaluate the lipid content of single mammalian oocytes. *Theriogenology*
596 2005;63:1181–94.
- 597 [29] Sturmev RG, Leese HJ. Energy metabolism in pig oocytes and early embryos.
598 *Reproduction* 2003;126:197–204.
- 599 [30] McEvoy TG, Coull GD, Broadbent PJ, Hutchinson JSM, Speake BK. Fatty acid
600 composition of lipids in immature cattle, pig and sheep oocytes with intact zona
601 pellucida. *J Reprod Fertil* 2000;118:163–70.
- 602 [31] Ferguson EM, Leese HJ. Triglyceride content of bovine oocytes and early embryos. *J*
603 *Reprod Fertil* 1999;116:373–8.
- 604 [32] Gil MA, Almiñana C, Cuello C, Parrilla I, Roca J, Vazquez JM, et al. Brief coincubation of
605 gametes in porcine in vitro fertilization: Role of sperm:oocyte ratio and post-
606 coincubation medium. *Theriogenology* 2007;67:620–6.
- 607 [33] Samiec M, Skrzyszowska M. High developmental capability of porcine cloned embryos
608 following trichostatin A-dependent epigenomic transformation during in vitro
609 maturation of oocytes pre-exposed to R -roscovitine*. *Animal Science Papers and*
610 *Reports* 2012;30:383–93.

- 611 [34] Samiec M, Skrzyszowska M. Biological transcomplementary activation as a novel and
612 effective strategy applied to the generation of porcine somatic cell cloned embryos.
613 *Reprod Biol* 2014;14:128–39.
- 614 [35] Samiec M, Opiela J, Lipiński D, Romanek J. Trichostatin A-mediated epigenetic
615 transformation of adult bone marrow-derived mesenchymal stem cells biases the in
616 vitro developmental capability, quality, and pluripotency extent of porcine cloned
617 embryos. *Biomed Res Int* 2015;2015.
- 618 [36] Glanzner WG, Rissi VB, de Macedo MP, Mujica LKS, Gutierrez K, Bridi A, et al. Histone
619 3 lysine 4, 9, and 27 demethylases expression profile in fertilized and cloned bovine
620 and porcine embryos†. *Biol Reprod* 2018;98:742–51.
- 621 [37] Prates EG, Nunes JT, Pereira RM. A role of lipid metabolism during cumulus-oocyte
622 complex maturation: Impact of lipid modulators to improve embryo production.
623 *Mediators Inflamm* 2014;2014.
- 624 [38] Abeydeera LR. In vitro production of embryos in swine. *Theriogenology* 2002;57:257–
625 73.
- 626 [39] Martinez EA, Caamaño JN, Gil MA, Rieke A, McCauley TC, Cantley TC, et al. Successful
627 nonsurgical deep uterine embryo transfer in pigs. *Theriogenology* 2004;61:137–46.
- 628 [40] Tanghe S, Van Soom A, Nauwynck H, Coryn M, De Kruif A. Minireview: Functions of the
629 cumulus oophorus during oocyte maturation, ovulation, and fertilization. *Mol Reprod*
630 *Dev* 2002;61:414–24.
- 631 [41] Coticchio G, Dal Canto M, Renzini MM, Guglielmo MC, Brambillasca F, Turchi D, et al.
632 Oocyte maturation: Gamete-somatic cells interactions, meiotic resumption,
633 cytoskeletal dynamics and cytoplasmic reorganization. *Hum Reprod Update*
634 2014;21:427–54.
- 635 [42] Kamiya C, Kobayashi M, Fukui Y. In vitro culture conditions using chemically defined
636 media for in vitro matured and intracytoplasmically inseminated porcine oocytes. *J*
637 *Reprod Dev* 2006;52:625–32.
- 638 [43] Deshmukh RS, Østrup O, Østrup E, Vejlsted M, Niemann H, Lucas-Hahn A, et al. DNA
639 methylation in porcine preimplantation embryos developed in vivo and produced by in
640 vitro fertilization, parthenogenetic activation and somatic cell nuclear transfer.
641 *Epigenetics* 2011;6:177–87.
- 642 [44] Diao YF, Lin T, Li X, Oqani RK, Lee JE, Kim SY, et al. Dynamic changes of SETD2, a histone
643 H3K36 methyltransferase, in porcine oocytes, IVF and SCNT embryos. *PLoS One*
644 2018;13:1–13.
- 645 [45] Samiec M, Skrzyszowska M. Molecular conditions of the cell nucleus
646 remodelling/reprogramming process and nuclear transferred embryo development in
647 the intraooplasmic karyoplast injection technique: A review. *Czech J Anim Sci*
648 2005;50:185–95.
- 649 [46] Samiec M, Skrzyszowska M. Intrinsic and extrinsic molecular determinants or
650 modulators for epigenetic remodeling and reprogramming of somatic cell-derived
651 genome in mammalian nuclear-transferred oocytes and resultant embryos. *Pol J Vet*
652 *Sci* 2018;21:217–27.
- 653 [47] Turner K, Silvestri G, Smith C, Dobson G, Black D, Handyside A, et al. Cattle
654 karyomapping to optimise food production and delivery of superior genetics: the first
655 liveborn calves. *Reprod Biomed Online* 2018;36:e20.
- 656 [48] Grupen CG. The evolution of porcine embryo invitro production. *Theriogenology*
657 2014;81:24–37.

- 658 [49] Zhang W, Yi K, Yan H, Zhou X. Advances on in vitro production and cryopreservation of
659 porcine embryos. *Anim Reprod Sci* 2012;132:115–22..
- 660 [50] Funahashi H, Cantley TC, Day BN. Synchronization of meiosis in porcine oocytes by
661 exposure to dibutyryl cyclic adenosine monophosphate improves developmental
662 competence following in vitro fertilization. *Biol Reprod* 1997;57:49–53.
- 663 [51] Sirard MA, First NL. In vitro inhibition of oocyte nuclear maturation in the bovine. *Biol*
664 *Reprod* 1988;39:229–34.
- 665 [52] Funahashi H, Kim NH, Stumpf TT, Cantley TC, Day BN. Presence of organic osmolytes in
666 maturation medium enhances cytoplasmic maturation of porcine oocytes. *Biol Reprod*
667 1996;54:1412–9.
- 668 [53] Li R, Hs YL, Callesen PH. Effect of cumulus cells and sperm concentration on fertilization
669 and development of pig oocytes 2018:4–7.
- 670 [54] Combelles CMH, Cekleniak NA, Racowsky C, Albertini DF. Assessment of nuclear and
671 cytoplasmic maturation in in-vitro matured human oocytes. *Hum Reprod*
672 2002;17:1006–16.
- 673 [55] Sutton-McDowall ML, Gilchrist RB, Thompson JG. The pivotal role of glucose
674 metabolism in determining oocyte developmental competence. *Reproduction*
675 2010;139:685–95.
- 676 [56] Abeydeera LR, Wang W, Prather RS, Day BN. Maturation in vitro of pig oocytes in
677 protein-free culture media: fertilization and subsequent embryo development in vitro.
678 *Biol Reprod* 1998;1320:1316–20.
- 679 [57] Xia P, Tekpetey FR, Armstrong DT. Effect of IGF-I on pig oocyte maturation, fertilization,
680 and early embryonic development in vitro, and on granulosa and cumulus cell
681 biosynthetic activity. *Mol Reprod Dev* 1994;38:373–9.
- 682 [58] Grupen CG, Nagashima H, Nottle MB. Role of epidermal growth factor and insulin-like
683 growth factor-I on porcine oocyte maturation and embryonic development in vitro.
684 *Reprod Fertil Dev* 1998;9:571–6.
- 685 [59] Shu YM, Zeng HT, Ren Z, Zhuang GL, Liang XY, Shen HW, et al. Effects of cilostamide
686 and forskolin on the meiotic resumption and embryonic development of immature
687 human oocytes. *Hum Reprod* 2008;23:504–13.
- 688 [60] Hegele-Hartung C. Nuclear and cytoplasmic maturation of mouse oocytes after
689 treatment with synthetic meiosis-activating sterol in vitro. *Biol Reprod* 1999;61:1362–
690 72.
- 691 [61] Revelli A, Piane LD, Casano S, Molinari E, Massobrio M, Rinaudo P. Follicular fluid
692 content and oocyte quality: From single biochemical markers to metabolomics. *Reprod*
693 *Biol Endocrinol* 2009;7:1–13.
- 694 [62] Funahashi H, Day BN. Effects of the duration of exposure to hormone supplements on
695 cytoplasmic maturation of pig oocytes in vitro. *J Reprod Fertil* 1993;98:179–85.
- 696 [63] Yoshida M, Ishizaki Y, Kawagishi H, Bamba K, Kojima Y. Effects of pig follicular fluid on
697 maturation of pig oocytes in vitro and on their subsequent fertilizing and
698 developmental capacity in vitro. *J Reprod Fertil* 1992;95:481–8.
- 699 [64] Bijttebier J, Tilleman K, Dhaenens M, Deforce D, Van Soom A, Maes D. Comparative
700 proteome analysis of porcine follicular fluid and serum reveals that excessive α 2-
701 macroglobulin in serum hampers successful expansion of cumulus-oocyte complexes.
702 *Proteomics* 2009;9:4554–65.
- 703 [65] Angelucci S, Ciavardelli D, Di Giuseppe F, Eleuterio E, Sulpizio M, Tiboni GM, et al.
704 Proteome analysis of human follicular fluid. *Biochim Biophys Acta - Proteins*

- 705 Proteomics 2006;1764:1775–85.
- 706 [66] McNatty KP, Hunter WM, Mcneilly AS, Sawers RS, Biology R, Street C. Changes in the
707 concentration of pituitary and steroid hormones in the follicular fluid of human
708 graafian follicles throughout the menstrual cycle. *J Endocrinol* 1975;64:555–71
- 709 [67] Agung B, Otoi T, Fuchimoto D, Senbon S, Onishi A, Nagai T. In vitro fertilization and
710 development of porcine oocytes matured in follicular fluid. *J Reprod Dev* 2013;59:103–
711 6.
- 712 [68] Algriany O, Bevers M, Schoevers E, Colenbrander B, Dieleman S. Follicle size-dependent
713 effects of sow follicular fluid on in vitro cumulus expansion, nuclear maturation and
714 blastocyst formation of sow cumulus oocytes complexes. *Theriogenology*
715 2004;62:1483–97.
- 716 [69] Bagg MA, Nottle MB, Armstrong DT, Grupen CG. Relationship between follicle size and
717 oocyte developmental competence in prepubertal and adult pigs. *Reprod Fertil Dev*
718 2007;19:797–803.
- 719 [70] Coy P, Aviles M. What controls polyspermy in mammals, the oviduct or the oocyte?
720 *Biol Rev* 2010;85:593–605.
- 721 [71] Knox R V. The fertility of frozen boar sperm when used for artificial insemination.
722 *Reprod Domest Anim* 2015;50:90–7.
- 723 [72] Tanihara F, Nakai M, Kaneko H, Noguchi J, Otoi T, Kikuchi K. Evaluation of zona pellucida
724 function for sperm penetration during in vitro fertilization in pigs. *J Reprod Dev*
725 2013;59:385–92.
- 726 [73] Kosman ET, Levitan DR. Sperm competition and the evolution of gametic compatibility
727 in externally fertilizing taxa. *Mol Hum Reprod* 2014;20:1190–7.
- 728 [74] Romar R, Funahashi H, Coy P. In vitro fertilization in pigs: New molecules and protocols
729 to consider in the forthcoming years. *Theriogenology* 2016;85:125–34.
- 730 [75] Saavedra MD, Mondéjar I, Coy P, Betancourt M, González-Márquez H, Jiménez-Movilla
731 M, et al. Calreticulin from subolemmal vesicles affects membrane regulation of
732 polyspermy. *Reproduction* 2014;147:369–78.
- 733 [76] Rath D. Experiments to improve in vitro fertilization techniques for in vivo-matured
734 porcine oocytes. *Theriogenology* 1992;37:885–96.
- 735 [77] Jaffe LA. Fast block to polyspermy in sea urchin eggs is electrically mediated. *Nature*
736 1976;261:68–71.
- 737 [78] Coy P, Canovas S, Mondejar I, Saavedra MD, Romar R, Grullon L, et al. Oviduct-specific
738 glycoprotein and heparin modulate sperm-zona pellucida interaction during
739 fertilization and contribute to the control of polyspermy. *Proc Natl Acad Sci*
740 2008;105:15809–14.
- 741 [79] Vatzias G, Hagen DR. Effects of porcine follicular fluid and oviduct-conditioned media
742 on maturation and fertilization of porcine oocytes in vitro. *Biol Reprod* 1999;60:42–8.
- 743 [80] Abeydeera LR, Day BN. In vitro penetration of pig oocytes in a modified Tris-buffered
744 medium: Effect of BSA, caffeine and calcium. *Theriogenology* 1997;48:537–44.
- 745 [81] Gil MA, Almiñana C, Roca J, Vázquez JM, Martínez EA. Boar semen variability and its
746 effects on IVF efficiency. *Theriogenology* 2008;70:1260–8.
- 747 [82] Nabavi N, Todehdeghan F, Shiravi A. Effect of caffeine on motility and vitality of sperm
748 and in vitro fertilization of outbreed mouse in T6 and M16 media. *Iran J Reprod Med*
749 2013;11:741–6.
- 750 [83] Yamaguchi S, Funahashi H. Effect of the addition of beta-mercaptoethanol to a thawing
751 solution supplemented with caffeine on the function of frozen-thawed boar sperm and

- 752 on the fertility of sows after artificial insemination. *Theriogenology* 2012;77:926–32.
- 753 [84] Matás C, Coy P, Romar R, Marco M, Gadea J, Ruiz S. Effect of sperm preparation method
754 on in vitro fertilization in pigs. *Reproduction* 2003;125:133–41.
- 755 [85] Funahashi H, Fujiwara T, Nagai T. Modulation of the function of boar spermatozoa via
756 adenosine and fertilization promoting peptide receptors reduce the incidence of
757 polyspermic penetration into porcine oocytes. *Biol Reprod* 2000;63:1157–63.
- 758 [86] Suzuki K, Eriksson B, Shimizu H, Nagai T, Rodriguez-Martinez H. Effect of hyaluronan on
759 monospermic penetration of porcine oocytes fertilized in vitro. *Int J Androl*
760 2000;23:13–21.
- 761 [87] Funahashi H, Cantley TC, Stumpf TT, Terlouw SL, Day BN. Use of low-salt culture
762 medium for in vitro maturation of porcine oocytes is associated with elevated oocyte
763 glutathione levels and enhanced male pronuclear formation after in vitro fertilization.
764 *Biol Reprod* 1994;51:633–9.
- 765 [88] Kolbe T, Holtz W. Birth of a piglet derived from an oocyte fertilized by intracytoplasmic
766 sperm injection (ICSI). *Anim Reprod Sci* 2000;64:97–101.
- 767 [89] Nakai M, Kashiwazaki N, Takizawa A, Hayashi Y, Nakatsukasa E, Fuchimoto D-I, et al.
768 Viable Piglets Generated from Porcine Oocytes Matured In Vitro and Fertilized by
769 Intracytoplasmic Sperm Head Injection. *Biol Reprod* 2002;68:1003–8.
- 770 [90] Herrero L, Martínez M, Garcia-Velasco JA. Current status of human oocyte and embryo
771 cryopreservation. *Curr Opin Obstet Gynecol* 2011;23:245–50.
- 772 [91] Nakai M, Ozawa M, Maedomari N, Noguchi J, Kaneko H, Ito J, et al. Delay in cleavage
773 of porcine embryos after Intracytoplasmic Sperm Injection (ICSI) shows poorer
774 embryonic development. *J Reprod Dev* 2014;60:256–9.
- 775 [92] Arrell VL, Day BN, Prather RS. The Transition from maternal to zygotic control of
776 development occurs during the 4-cell stage in the domestic pig, *sus scrofa*: quantitative
777 and qualitative aspects of protein synthesis. *Biol Reprod* 1991;44:62–8.
- 778 [93] Karja NWK, Kikuchi K, Fahrudin M, Ozawa M, Somfai T, Ohnuma K, et al. Development
779 to the blastocyst stage, the oxidative state, and the quality of early development stage
780 of porcine embryos cultured in alteration of glucose concentrations in vitro under
781 different oxygen tensions. *Reprod Biol Endocrinol* 2006;4:1–12.
- 782 [94] Kikuchi K, Onishi A, Kashiwazaki N, Iwamoto M, Noguchi J, Kaneko H, et al. Successful
783 piglet production after transfer of blastocysts produced by a modified in vitro system.
784 *Biol Reprod* 2002;66:1033–41.
- 785 [95] Davis DL. Culture and storage of pig embryos. *J Reprod Fertil Suppl* 1985;33:115–24.
- 786 [96] Petters R, Johnson B, Reed M, Archibong A. Glucose , glutamine and inorganic
787 phosphate in early development of the pig embryo in vitro. *Reproduction*
788 1990;89:269–75.
- 789 [97] Robl JM, Davis DL. Effects of serum on swine morulae and blastocysts in vitro. *J Anim*
790 *Sci* 1981;52:1450–6.
- 791 [98] Yoshioka K, Suzuki C, Onishi A. Defined system for in vitro production of porcine
792 embryos using a single basic medium. *J Reprod Dev* 2008;54:208–13.
- 793 [99] Suzuki C, Yoshioka K, Sakatani M, Takahashi M. Glutamine and hypotaurine improves
794 intracellular oxidative status and in vitro development of porcine preimplantation
795 embryos. *Zygote* 2007;15:317–24.
- 796 [100] Lane M, Gardner DK. Vitrification of mouse oocytes using a nylon loop. *Mol Reprod Dev*
797 2001;58:342–7.
- 798 [101] Kang JT, Atikuzzaman M, Kwon DK, Park SJ, Kim SJ, Moon JH, et al. Developmental

- 799 competence of porcine oocytes after in vitro maturation and in vitro culture under
800 different oxygen concentrations. *Zygote* 2012;20:1–8.
- 801 [102] Burg MB, Ferraris JD, Dmitrieva NI. Cellular response to hyperosmotic stresses. *Physiol*
802 *Rev* 2007;87:1441–74.
- 803 [103] Martinez CA, Nohalez A, Cuello C, Vazquez JM, Roca J, Martinez EA, et al. The use of
804 mineral oil during in vitro maturation, fertilization, and embryo culture does not impair
805 the developmental competence of pig oocytes. *Theriogenology* 2015;83:693–702.
- 806 [104] Shimada M, Kawano N, Terada T. Delay of nuclear maturation and reduction in
807 developmental competence of pig oocytes after mineral overlay of in vitro maturation
808 media. *Reproduction* 2002;124:557–64.
- 809 [105] Ng KYB, Mingels R, Morgan H, Macklon N, Cheong Y. In vivo oxygen, temperature and
810 pH dynamics in the female reproductive tract and their importance in human
811 conception: a systematic review. *Hum Reprod Update* 2017:1–20.
- 812 [106] Ventura-Juncá P, Irarrázaval I, Rolle AJ, Gutiérrez JI, Moreno RD, Santos MJ. In vitro
813 fertilization (IVF) in mammals: Epigenetic and developmental alterations. *Scientific and*
814 *bioethical implications for IVF in humans. Biol Res* 2015;48:1–13.
- 815 [107] Calle A, Fernandez-Gonzalez R, Ramos-Ibeas P, Laguna-Barraza R, Perez-Cerezales S,
816 Bermejo-Alvarez P, et al. Long-term and transgenerational effects of in vitro culture on
817 mouse embryos. *Theriogenology* 2012;77:785–93.
- 818 [108] Khosla S, Dean W, Brown D, Reik W, Feil R. Culture of preimplantation mouse embryos
819 affects fetal development and the expression of imprinted genes. *Biol Reprod*
820 2001;64:918–26.
- 821 [109] Fernández-Gonzalez R, Ramirez MA, Bilbao A, De Fonseca FR, Gutiérrez-Adán A.
822 Suboptimal in vitro culture conditions: an epigenetic origin of long-term health effects.
823 *Mol Reprod Dev* 2007;74:1149–56.
- 824 [110] Canovas S, Ivanova E, Romar R, García-Martínez S, Soriano-Úbeda C, García-Vázquez
825 FA, et al. DNA methylation and gene expression changes derived from assisted
826 reproductive technologies can be decreased by reproductive fluids. *Elife* 2017;6:1–24.
- 827 [111] Wang Q, Sun Q-Y. Evaluation of oocyte quality: morphological, cellular and molecular
828 predictors. *Reprod Fertil Dev* 2006;19:1–12.
- 829 [112] Hunter RH, Polge C. Maturation of follicular oocytes in the pig after injection of human
830 chorionic gonadotrophin. *J Reprod Fertil* 1966;12:525–31.
- 831 [113] McGaughey R, Polge C. Cytogenetic Analysis of Pig Oocytes Matured In Vitro. *Therio*
832 1971.
- 833 [114] Prentice-Biensch JR, Singh J, Alfoteisy B, Anzar M. A simple and high-throughput
834 method to assess maturation status of bovine oocytes: Comparison of anti-lamin A/C-
835 DAPI with an aceto-orcein staining technique. *Theriogenology* 2012;78:1633–8.
- 836 [115] Chohan KR, Hunter AG. Meiotic competence of bovine fetal oocytes following in vitro
837 maturation. *Anim Reprod Sci* 2003;76:43–51.
- 838 [116] Izadyar F, Colenbrander B, Bevers MM. In vitro maturation of bovine oocytes in the
839 presence of growth hormone accelerates nuclear maturation and promotes
840 subsequent embryonic development. *Mol Reprod Dev* 1996;45:372–7.
- 841 [117] Critser ES, First NL. Use of A Fluorescent Stain for Visualization of Nuclear Material in
842 Living Oocytes and Early Embryos. *Stain Technol* 1986;61:1–5.
- 843 [118] Lodde V, Modina S, Galbusera C, Franciosi F, Luciano AM. Large-scale chromatin
844 remodeling in germinal vesicle bovine oocytes: Interplay with gap junction
845 functionality and developmental competence. *Mol Reprod Dev* 2007;74:740–9.

- 846 [119] Nagai T. Parthenogenetic activation of cattle follicular oocytes in vitro with ethanol.
847 Gamete Res 1987;16:243–9.
- 848 [120] Pursel VG, Wall RJ, Rexroad CE, Hammer RE, Brinster RL. A rapid whole-mount staining
849 procedure for nuclei of mammalian embryos. Theriogenology 1985;24:687–91.
- 850 [121] Mandawala AA, Harvey SC, Roy TK, Fowler KE. Time-lapse embryo imaging and
851 morphokinetic profiling: Towards a general characterisation of embryogenesis. Anim
852 Reprod Sci 2016;174:2–10.
- 853 [122] Callesen H, Holm P. Developmental characteristics of later-stage porcine embryos
854 produced in vivo or in vitro. Reprod Fertil Dev 2016;28:158–9.
- 855 [123] Hasler JF. Forty years of embryo transfer in cattle: A review focusing on the journal
856 Theriogenology, the growth of the industry in North America, and personal reminiscences.
857 Theriogenology 2014;81:152–69.
- 858 [124] Martinez EA, Cuello C, Parrilla I, Martinez CA, Nohalez A, Vazquez JL, et al. Recent
859 advances toward the practical application of embryo transfer in pigs. Theriogenology
860 2016;85:152–61.
- 861 [125] Martinez EA, Martinez CA, Nohalez A, Sanchez-Osorio J, Vazquez JM, Roca J, et al.
862 Nonsurgical deep uterine transfer of vitrified, in vivo-derived, porcine embryos is as
863 effective as the default surgical approach. Sci Rep 2015;5:1–9.
- 864 [126] Mandawala AA, Harvey SC, Roy TK, Fowler KE. Cryopreservation of animal oocytes and
865 embryos: Current progress and future prospects. Theriogenology 2016;86:1637–44.
- 866 [127] Ponsart C, Le Bourhis D, Knijn H, Fritz S, Guyader-Joly C, Otter T, et al. Reproductive
867 technologies and genomic selection in dairy cattle. Reprod Fertil Dev 2013;26:12–21.
- 868 [128] Handyside AH, Harton GL, Mariani B, Thornhill AR, Affara N, Shaw MA, et al.
869 Karyomapping: A universal method for genome wide analysis of genetic disease based
870 on mapping crossovers between parental haplotypes. J Med Genet 2010;47:651–8.
- 871 [129] Wang WH, Abeydeera LR, Cantley TC, Day BN. Effects of oocyte maturation media on
872 development of pig embryos produced by in vitro fertilization. J Reprod Fertil
873 1997;111:101–8.
- 874 [130] Long, C R; Dobrinsky J. In vitro production of pig embryos comparison of culture and
875 boars. Theriogenology 1999;51:1375-90.
876

| Concentration (mmol/L) | Maturation media | | | | | Fertilisation and embryo culture media | | | | |
|----------------------------------------------------------|------------------|---------|--------|--------------------------|-----------------------------------|----------------------------------------|--------|--------|-------|---------|
| | TCM-199 | NCSU-23 | mWM | NCSU-37 (glucose medium) | NCSU-37 (pyruvate/lactate medium) | BECM-7 | PZM-3 | PZM-4 | PZM-5 | NCSU-23 |
| Basal Medium Eagle amino acids (ml/L) | - | - | - | - | - | 20.00 | 20.00 | 20.00 | 20.00 | - |
| CaCl ₂ | 1.80 | 1.70 | - | - | - | - | - | - | - | - |
| CaCl ₂ .2H ₂ O | - | - | - | 1.70 | 1.70 | 1.71 | - | - | - | 1.70 |
| Calcium lactate | - | - | 1.71 | - | - | - | 2.00 | 2.00 | 2.00 | - |
| Cysteine | 0.57 | 0.57 | 0.57 | - | - | - | - | - | - | - |
| Fatty acid-free BSA (mg/ml) | - | - | - | 4.00 | 4.00 | 4.00 | 3.00 | - | - | 4.00 |
| Gentamycin (mg/ml) | - | - | - | - | - | - | 0.05 | 0.05 | 0.01 | 0.05 |
| Glucose | 5.55 | 5.55 | 5.56 | 5.55 | - | 0.33 | - | - | - | 5.55 |
| Hypotaurine | - | 5.00 | - | - | - | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 |
| KCl | 5.36 | 4.78 | 4.78 | 4.78 | 4.78 | 6.00 | 10.00 | 10.00 | 10.00 | 4.78 |
| KH ₂ PO ₄ | - | 1.19 | 1.19 | 1.19 | 1.19 | - | 0.35 | 0.35 | 0.35 | 1.19 |
| L-Glutamine | 0.68 | 1.00 | - | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 2.00 | 1.00 |
| MgSO ₄ .7H ₂ O | 0.81 | 1.19 | 1.19 | 1.19 | 1.19 | 1.19 | 0.40 | 0.40 | 0.40 | 1.19 |
| Minimum Essential Medium nonessential amino acids (ml/L) | - | - | - | - | - | 10.00 | 10.00 | 10.00 | 10.00 | - |
| NaCl | 116.36 | 108.73 | 68.49 | 108.73 | 108.73 | 94.59 | 108.00 | 108.00 | 108.0 | 108.73 |
| NaHCO ₃ | 26.19 | 25.07 | 25.07 | 25.07 | 25.07 | 25.07 | 25.07 | 25.07 | 25.00 | 25.07 |
| Penicillin G | 100.00 | 100.00 | 100.00 | - | - | - | - | - | - | - |
| Phenol Red (g/L) | - | - | - | - | - | 0.001 | - | - | - | - |
| Polyvinyl alcohol (mg/ml) | - | - | - | - | - | - | - | 3.00 | 3.00 | - |
| Porcine follicular fluid (% v/v) | 10.00 | 10.00 | 10.00 | - | - | - | - | - | - | - |
| Sodium lactate | - | - | 25.20 | - | 2.73 | - | - | - | - | - |
| Sodium pyruvate | - | - | 0.33 | - | 0.17 | 23.00 | 0.20 | 0.20 | 0.20 | - |
| Sorbitol | - | - | - | - | 12.00 | - | - | - | - | - |
| Streptomycin (µg/ml) | 50.00 | 50.00 | 50.00 | - | - | - | - | - | - | - |
| Taurine | - | 7.00 | - | - | - | 7.00 | - | - | - | 7.00 |

877 **Table 1:** Composition of existing media used for *in vitro* maturation of oocytes, *in vitro* fertilisation and subsequent embryo culture. The table
878 demonstrates the constituents present in Tissue Culture Medium (TCM)-199, North Carolina State University (NCSU)-23 medium, modified
879 Whitten's Medium (mWM), North Carolina State University (NCSU)-37 medium (with glucose), North Carolina State University (NCSU)-37
880 medium (with pyruvate and lactate), Beltsville Embryo Culture Medium (BECM)-7, three iterations of Porcine Zygote Medium (PZM) and North
881 Carolina State University (NCSU)-23 medium specific to embryo culture. Values given are in mmol/L (unless otherwise stated) [21,27,98,129,130].

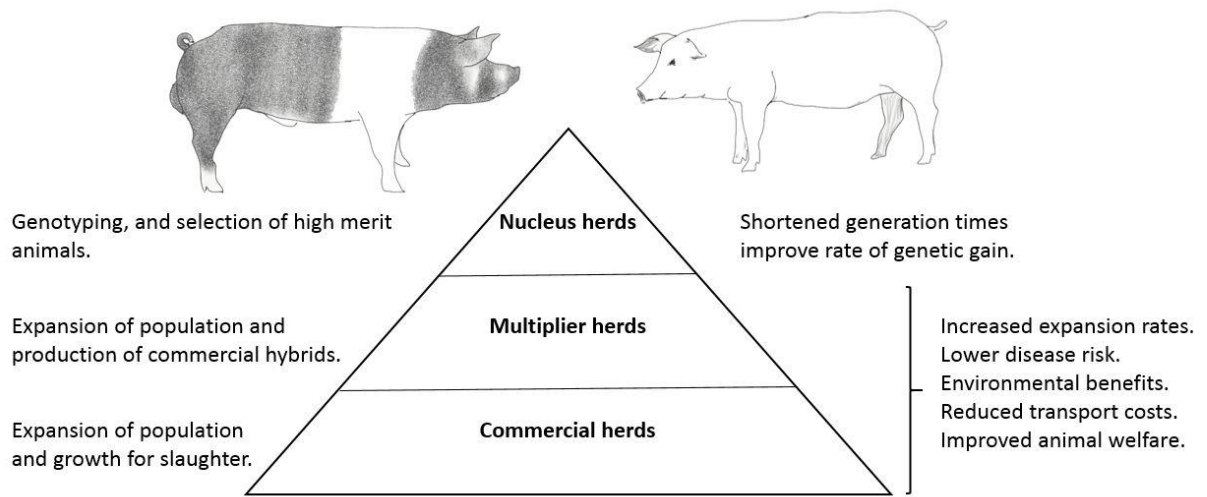
882 **Figure 1:** Schematic representation of selection and production herds in pig production
883 indicating where *in vitro* production can achieve production gains.

884

885 **Figure 2:** Flowchart indicating the pig *in vitro* production process. The main challenges for
886 commercial implementation are noted in red, these define the current research priorities in
887 the field.

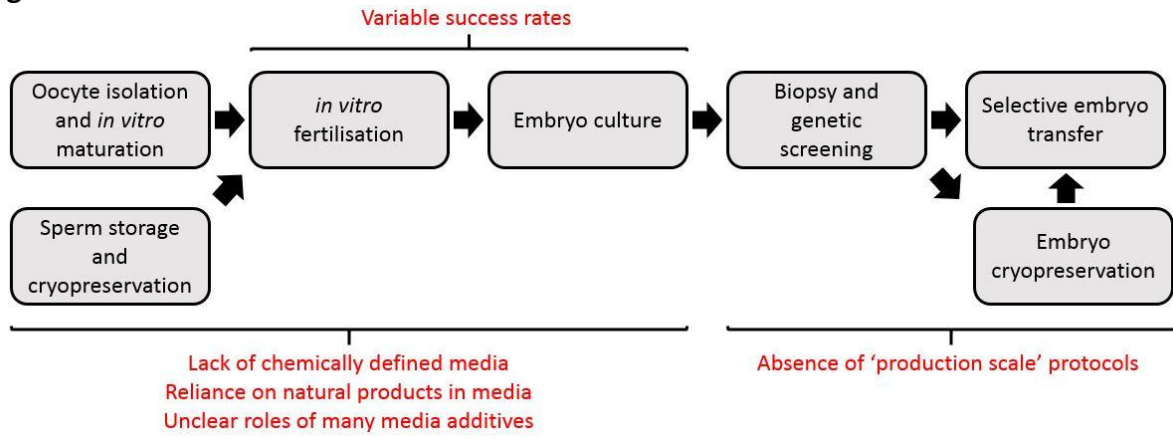
888

889 **Figure 1**



890
891

892 **Figure 2**



893