Reproductive Biology

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

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Abstract

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

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

1. Introduction

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

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

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

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

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

1.1 Accelerating genetic progress

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

1.2 Movement of genetics across international borders

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

1.3 Animal health and welfare

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

1.4 Further benefits

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

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

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

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

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

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

3. Oocyte maturation

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

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

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

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

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

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

4. Fertilisation

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

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

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**

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

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

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

Conclusions and future prospects

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

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

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511

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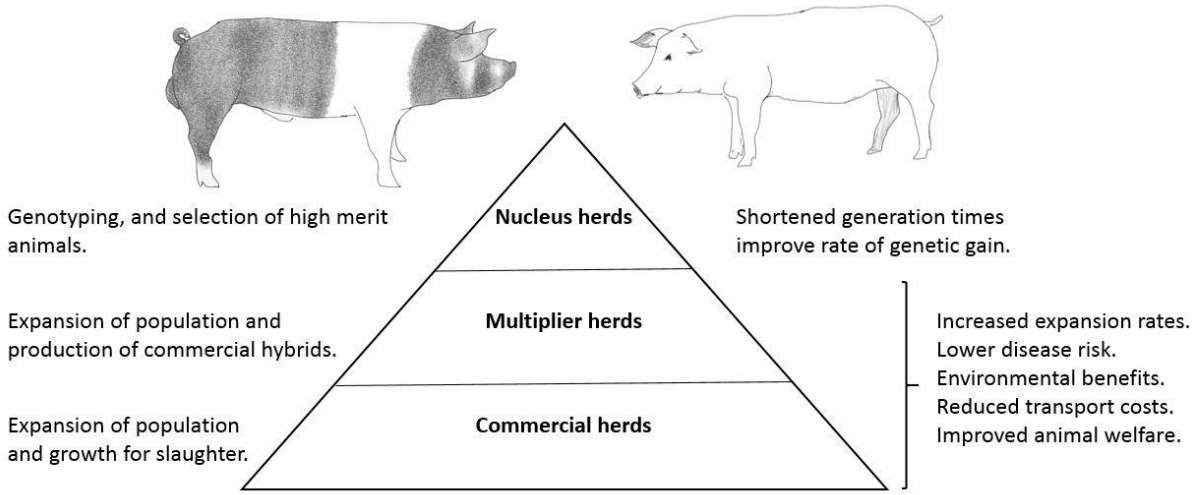
	Maturation media					Fertilisation and embryo culture media				
Concentration (mmol/L)	**TCM-199**	**NCSU-23**	**mWM**	**NCSU-37 (glucose medium)**	**NCSU-37 (pyruvate/ lactate medium)**	**BECM-7**	**PZM-3**	**PZM-4**	**PZM-5**	**NCSU-23**
Basal Medium Eagle amino acids (ml/L)	-	-	-	-	-	20.00	20.00	20.00	20.00	-
$CaCl_2$	1.80	1.70	-	-	-	-	-	-	-	-
$CaCl_2.2H_2O$	-	-	-	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_2PO_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
$MgS0_4.7H_2O$	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

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

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

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

889 **Figure 1**



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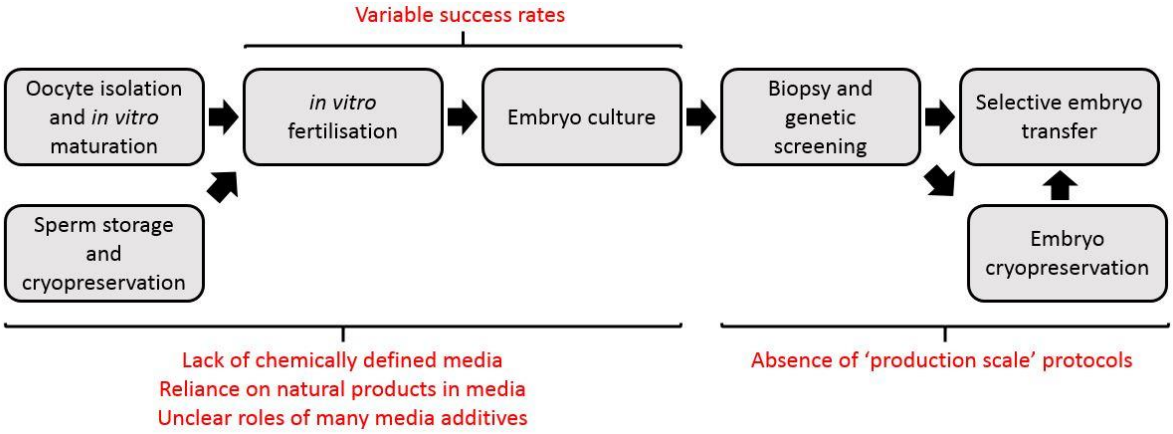
892 **Figure 2**



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