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Polyspermic fertilisation of porcine oocytes *in vitro* – a current review

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Abstract: Polyspermic fertilisation has been a continual problem in porcine *in vitro* fertilisation. Over the years, researchers have tried to resolve this persistent problem, but have not been entirely successful in their endeavour. This can be attributed to several factors. This review discusses the role of sperm capacitation in the *in vitro* fertilisation and the most recent studies on the capacitation media. This includes the results obtained by testing the effect of various compounds on sperm capacitation. Additionally, the importance of the oviductal fluid on the porcine oocytes prior to fertilisation is also discussed. This is of significance, as the experiments show that oviductal fluid is crucial for the pre-fertilisation zona pellucida hardening. Also, the significance of using high quality oocytes for the *in vitro* fertilisation is highlighted. Lastly, the different and latest techniques to prevent or reduce polyspermic fertilisation have been reviewed.

Keywords: oviductal fluid; capacitation media; zona pellucida; hardening; oocyte maturation; supplementary hormones

Contents

1. Introduction

Studies on swine breeding has developed and advanced over the years due to its immense economic importance. Pork is the most consumed meat in the world followed by poultry and beef. Moreover, swine biotechnology has great significance to the scientific community as well, since the physiology of pigs has been found to be exceedingly similar to human physiology. After the advent of *in vitro* production (IVP) of swine embryos by Cheng et al. (1986), research is now being undertaken to create genetically modified animals to act as potential organ donors for humans in the future, by xenotransplantation (Gil et al. 2010).

Porcine IVF is usually carried out by the following outlined steps: (1) Oocyte and spermatozoa collection from the pigs, (2) *In vitro* maturation of the

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oocytes (IVM), (3) *In vitro* fertilisation (IVF) process of the matured oocytes with the spermatozoa, (4) culturing the embryos (Motta et al. 2018).

In vitro oocyte maturation usually takes place with the help of various supplementary hormones added to the IVF medium including foetal bovine serum (FBS), follicle stimulating hormone (FSH), pregnant mare serum gonadotrophin (PMSG), human chorionic gonadotropin (hCG), estradiol-17β, leptin and relaxin (Zhang et al. 2012). This is followed by the actual process of IVF, which has numerous variables like the media components, the quality of the oocytes and the spermatozoa that influence its success rates. Successful fertilisation then leads to the cleavage and division of the embryo starting with the one-celled stage on the 0^{th} day to the 8-cell stage after 4.5 days, before proceeding towards the formation of the morula on the 5^{th} day and the blastocyst on the 6^{th} day (Hunter 1974).

Despite the advancements made in the field of *in vitro* fertilisation in pigs, the process still has various unresolved problems such as imbalanced nuclear and cytoplasmic maturation and the low quality of the oocytes and spermatozoa leading to polyspermic penetration. Most of this can also be attributed to various other factors of the IVF environment. Since the development of assisted reproductive technology for pigs, several IVF techniques have been realised and have attempted to overcome these problems. This review discusses the novel studies made and techniques developed to overcome the persisting problem of polyspermic fertilisation in the *in vitro* fertilised porcine oocytes, which is found to be far greater than in most other species.

2. Important factors involved in sperm capacitation

The activation of the spermatozoa shortly prior to the fertilisation is known as sperm capacitation. Understanding sperm capacitation is vital for knowing the importance of its role in IVF. Most of this knowledge has been obtained from *in vitro* studies as it is quite difficult to study this process *in vivo*. In general, IVF processes attempt to mimic the oviductal environment for the sperm capacitation.

In the past, a few important compounds such as caffeine, theophylline, cysteine, adenosine, pGlu-Glu-ProNH_2 (FPP) have been found to contribute to the sperm capacitation (Funahashi et al. 2000; Yoshioka et al. 2003). Recently, a few other compounds and methods have been experimented with. Caffeine has been found to promote capacitation as well as a spontaneous acrosome reaction. However, this does tend to increase the possibility of polyspermic fertilisation. When compared to caffeine, adenosine and FPP treatments lead to significantly reduced polyspermic penetration (Funahashi et al. 2000). Theophylline has also been found to decrease polyspermy while maintaining sperm penetrability (Yoshioka et al. 2003).

The compound hyaluronan is known to be present in the epithelial folds of the sperm reservoir at the uterine tubal junction (UTJ) during the pre-ovulatory stages of the oestrous cycle. Furthermore, the hyaluronan receptor CD44 is also present which possibly indicates the importance of its signalling pathway in the sperm storage. Hyaluronan plays a key role in sperm capacitation as it helps preserve the sperm in its non-capacitated form during the pre-ovulatory stages and then induces capacitation post ovulation (Tienthai 2015). This function combined with the UTJ and sperm reservoir controlling the entry of the spermatozoa into the oviducts, reduces the occurrence of polyspermy.

Another compound, α-L-fucosidase, promotes sperm capacitation and prevents a premature acrosome reaction by increasing the sperm intracellular calcium concentration and tyrosine phosphorylation. It was also reported that α-L-fucosidase increased the ZP (zona pellucida) binding and penetration by the spermatozoa (Romero-Aguirregomezcorta et al. 2015).

A recent study shows that spermatozoa in the epididymal cauda is exposed to far less HCO_3^- concentrations than that post ejaculation in the uterine environment. This is an important factor in the female reproductive tract which promotes the sperm capacitation for fertilisation. In IVF environments, it was found that optimising the concentration of HCO_3^- is one method which could allow for the better efficiency and higher rates of monospermy (Soriano-Ubeda et al. 2019). They found that an HCO_3^- concentration of 15 mmol/l is adequate for the capacitation of the sperm *in vitro* and for the improved fertilisation efficiency.

Another study conducted by Loki et al. (2016) indicates the effects of a phosphodiesterase (PDE) type-5 inhibitor: sildenafil, on the capacitation of the spermatozoa. It was found that the oocytes

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and spermatozoa cultured together in sildenafil did not show any significant difference in the penetration as compared to the culture using caffeine benzoate, but the rate of monospermic penetration in particular, was far greater using sildenafil than caffeine benzoate. Through an immunocytochemical analysis, PDE Type-5 was found to be concentrated in the acrosome region of the sperm which helps improve the sperm penetrability.

In the study conducted on mice using creatine as a key compound of the IVF medium, Umehara et al. (2018) analysed the motility of the sperm, the capacitation levels and the state of the acrosome. Sperm motility levels were found to be much higher in the experimental group with creatine due to an increase in the ATP (adenosine triphosphate) content in the sperm. It was also found that the inclusion of creatine resulted in a significant improvement of the sperm capacitation by reducing the number of sperm required for achieving the desired rate of fertilisation which, in turn, could reduce the polyspermy levels and it was then observed that the resultant fertilised oocytes were also far greater in the experimental group than in the control group where the creatine was not utilised.

Previous studies have also indicated that the porcine oviductal fluid (OF) brings about several physiological, biochemical and physical changes in the spermatozoa when extracted from the follicular phase and cultured together. This OF from the follicular phase of the oestrous cycle, preserves the sperm viability and acrosomal conformity which, in turn, prevents premature sperm capacitation. Although this did not significantly improve the fertilisation rates, it increased the rates of polyspermy and zona binding, thereby indicating improved the physiological function of the spermatozoa. This is in contrast to the study where oocytes were cultured in OF which resulted in decreased polyspermy and improved ZP hardening, Coy et al. (2010).

3. Oviductal fluid and zonae pellucidae hardening

It was reported by Wang et al. (1998) that the zonae pellucidae was considerably thicker in the ovulated porcine oocytes than in the IVM oocytes which probably indicates that an abnormal zona reaction could be the cause for promoting polyspermic fertilisation. Additionally, it was also found that the cytoplasm of the IVM oocytes was dense and even while the ovulated oocyte had clear regions in the cortex of the cytoplasm and was uneven in nature. This uneven cytoplasm has been found to help improve the probability of the development of porcine oocytes (Nagashima et al. 1996).

It has been known that zona pellucida hardening takes place due to the release of cortical granules post entry of a sperm into the oocyte (Barros and Yanagimachi 1971). OF is highly complex in nature and is difficult to replicate during the process of media preparation. However, oviduct-specific glycoprotein (OVGP1) and heparin-like glycosaminoglycans (GAGs) in the OF have been identified by Coy et al. (2008) to promote ZP hardening to some extent prior to the entry of the spermatozoa into the oocyte and this method known as pre fertilisation zona pellucida hardening can now be included in the preparation of IVF media.

It was also observed that porcine OF induced high values of ZP enzymatic resistance to pronase solutions only around the short time of ovulation during the oestrous cycle (Coy et al. 2008) (ZP enzymatic resistance or ZP digestion time in pronase are common tests for the polyspermy rates). In the study by Mondejar et al. (2013), a relationship between the monospermy and ZP hardening was established. Under normal circumstances, the utero-tubal junction present in the pigs act as a reservoir for the sperm and, hence, only controlled amounts of sperm are allowed to enter the oviducts. This allows for the sperm to be preserved in the uterine epithelium prior to ovulation (Flechon et al. 1981). The oestrogen and progesterone hormonal changes that occur during ovulation then allow the sperm to be released from the epithelial cells in order to make their way towards the oocytes. It is at this optimal window that the monospermy levels were found to be the highest. During this window of ovulation, it is also when the OVGP1 levels are found to be high, thereby directly promoting the ZP hardening (Buhi et al. 1996). Hence, it can be said that the porcine oocytes cultured in the pre-ovulatory OF has far greater ZP hardening than the oocytes cultured in the post ovulatory OF due to the presence of higher amounts of OVGP1 combined with the heparin-like GAGs (Coy et al. 2008; Ballester et al. 2014).

In another study by Canovas et al. (2009), it was found that the compound bis(sulfosuccinimidyl) suberate (BS3) promoted ZP hardening with de-

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creased penetration and increased monospermy. However, the overall IVF productivity only improved when the oocytes were exposed to Di-(*N*-succinimidyl)-3,3'-dithiodipropionate (DSP). This was further proved when the porcine zygotes cultured *in vitro* indicated improved rates of cleavage and blastocyst formation.

The importance of understanding the composition of the oviductal fluid was further highlighted when a recent study conducted by Batista et al. (2016) on the effects of oviductal fluid (OF) on porcine oocytes indicated that there was a high rate of monospermic fertilisation at any concentration of spermatozoa introduced into the OF environment and this, in turn, improved the rate of blastocyst formation.

In mice, Tokuhiro and Dean (2018) observed that after the first spermatozoon crosses the zona pellucida, a healthy oocyte performs the exocytosis of the zinc ions which participate in the post fertilisation ZP hardening and thereby prevent polyspermy. During this period of ZP hardening, cleavage of the ZP2 glycoprotein is also observed. This has been found to prevent sperm binding to the zona pellucida and thereby forms an additional block to the polyspermic fertilisation. However, such a study has yet to be conducted on pig models to get a more holistic understanding and comparison of these processes in different species.

Moreover, oocytes, on ZP hardening by exposure to the oviductal fluid, have been found to be penetrated only by those spermatozoa which have physical durability (Bedford 2004) to surpass this layer with or without the help of acrosomal enzymes, in order to succeed at fertilisation. This acts as an additional barrier for the prevention of polyspermy (Aviles et al. 2010).

In their review, Romar et al. (2019) have also concluded that a reasonable protocol to follow for successful *in vitro* fertilisation of porcine oocytes is to begin the culturing of the oocytes in the oviductal fluid in order to allow for the ZP hardening to take place. They also emphasised the importance of 3D culture systems (Francipane and Lagasse 2016) which have been developed with the help of advanced tissue engineering to replicate the environment of the reproductive system in detail in order to achieve better results with IVF.

Fowler et al. (2018) highlighted the importance of the oocyte maturation media in their review paper which summarises some of the additional important compounds that can be added to the media like, epidermal growth factor (EGF), insulin growth factor I (IGF-I) and PG600. Apart from that, the paper also refers to the importance of the porcine follicular fluid (PFF), as an excellent supplement to the oocyte maturation media. However, the known complications to using PFF that have been described is that the preparation of PFF requires it to be aspirated from ovarian follicles, then centrifuged and filter sterilised before storing at –20 °C. Unfortunately, the components of PFF have not been extensively researched and characterised in the manner that human follicular fluid (HFF) has been and, hence, it is not recommended to use PFF as the primary medium, but only as a supplement instead.

4. Novel techniques to reduce the incidence of polyspermy

The most commonly used procedure for IVF is culturing the spermatozoa and IVM oocytes together in a droplet of media, but this usually leads to very high rates of polyspermy and polyploidy. To overcome this, Park et al. (2009) designed a modified swim-up method where the spermatozoa placed

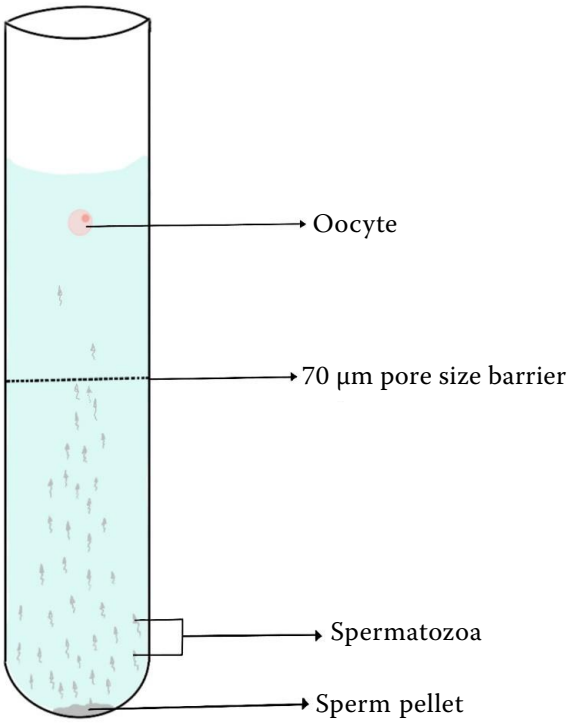

Figure 1. A modified swim up method where the spermatozoa swim across a 70 µm pore size barrier (developed by Park et al. (2009); drawing made by P. Chundekkad)

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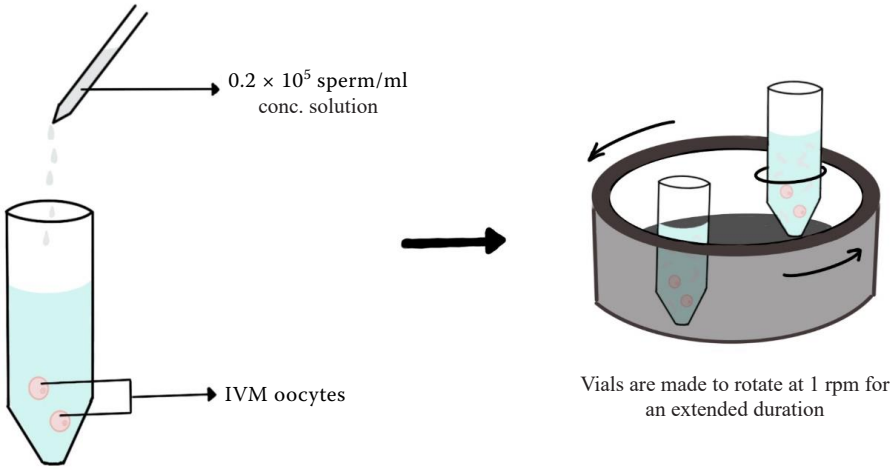
at the bottom of a vial had to swim upward towards the oocytes, across a 70 µm pore size barrier. This technique improved the monospermy and diploidy and increased the blastocyst formation (Figure 1).

Sano et al. (2009) studied the outcomes of using a microfluidic sperm sorter (MFSS) which is conventionally used in the sorting of human spermatozoa, for porcine IVF instead. It is of great significance as it utilises microfluidics and laminar flow, hence eliminating the need for centrifugation of the samples. Since only the motile sperm gradually accumulates in the exit chamber of the MFSS, the polyspermic fertilisation rates were greatly decreased and the efficiency of the blastocyst formation was considerably improved.

In the most recent study conducted by Martinez et al. (2019), it was observed that high pre-freezing sperm dilution of 20 × 10^6 sperm/ml as opposed to the conventional 1000 × 10^6 sperm/ml reduced the polyspermic fertilisation without affecting the *in vitro* penetration rate and, thereby, increased the IVF efficiency and blastocyst formation. It was also found that, although the sperm motility between the control and the experimental group was similar, the number of live spermatozoa with whole, undamaged acrosomes were greater in the experimental group than in the control.

Kitaji et al. (2015) performed another experiment where porcine IVM oocytes were mixed with spermatozoa at a concentration of 0.2 × 10^5 sperm/ml and made to constantly rotate at 1 rpm for an extended duration while parallelly, a control was used which was not kept in rotation. With this rolling culture-based method, it was found that although the sperm penetration rates were similar in both groups, the experimental group showed 50% higher rates of monospermy and 10% higher rates of blastocyst formation than the control (Figure 2).

Recently, Brogni et al. (2016) conducted a series of experiments to study the effect of pre-incubated semen on the polyspermic penetration rates in high- and low-quality porcine oocytes. They found that cleavage and blastocyst formation rates were higher in the superior quality oocytes while the cell density remained similar. They also pre-incubated the spermatozoa for different times and



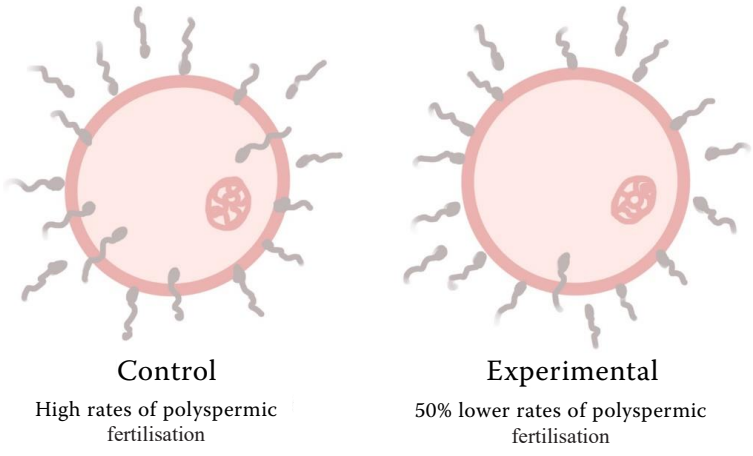


Figure 2. The spermatozoa, in lower concentrations, are cultured with the oocytes and are made to rotate at 1 rpm for an extended duration, leading to 50% lowered rates of the polyspermic fertilisation (developed by Kitaji et al. (2015); drawing made by P. Chundekkad)

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found that doing so had no significant effect on the fertilisation rates. However, the spermatozoa pre-incubated for 1.5 h showed improved rates of monospermy, cleavage and blastocyst formation with the low-quality oocytes. Even so, the rates of the blastocyst formation in the low-quality oocytes were not comparable to those in the superior quality oocytes.

Han et al. (2019) conducted a comparative study of *ex vivo* and *in vitro* models and found that although the monospermy rate was increased in the *ex vivo* model, it, in parallel, decreased the penetration rate and overall fertilisation efficiency. Polyspermy is reduced in the *ex vivo* model due to the presence of ciliated cells permitting only a small amount of sperm to reach the oocyte.

Romar et al. (2019) also refer to the selection of motile sperm being of paramount importance and, hence, they suggest that centrifugation of this sperm must be avoided and instead the sperm must be cultured in a viscous medium containing certain additional oviductal fluid compounds with the medium ideally having a pH around 7.8 to 8 and a temperature of around 37 °C to 38 °C (Soriano-Ubeda et al. 2017).

The method developed by Soriano-Ubeda et al. (2017) is where an *in vivo* IVF model was created which mimicked the pre-ovulatory oviductal environment. An IVF medium of a pH similar to that found in the oviducts (pH = 8.0) prior to ovulation was used, which was made up of a mixture of various oviductal fluid components like cumulus-oocyte complex secretions, a follicular fluid and an oviductal periovulatory fluid. Also, a device was contrived which acted as a physical barrier between the gametes. Combined, this IVF model was able to reduce the polyspermy and showed a drastic increase in the overall efficiency due to the lowered sperm motility and lower capacitating effects of the medium.

5. Conclusion

Since the conception of assisted reproductive technology, thousands of *in vitro* fertilised species and genetically modified ones have been born. This is due to the immense contributions made by scientists around the world that have greatly enhanced our knowledge about reproductive anatomy, embryology, endocrinology, genetics and much more. Today, extensive research is being undertaken to further this knowledge in order to apply it to the more complex and intricate problems that exist such as cloning, xenotransplantation, and the study of animals such as pigs or mice as models of human disease.

The many problems with respect to porcine *in vitro* fertilisation and reproduction are also being investigated with the help of genomics and bioinformatics. With a sound understanding of the pig genome coupled with precision technology contributing to the advancement of fields like genomics, proteomics, transcriptomics and metabolomics, this vision could soon become a reality.

Polyspermic fertilisation has been extensively studied, yet this problem continues to persist. The aforementioned techniques in this review along with the proper formulation of IVF media for the capacitation of the sperm and the maturation of the oocytes seek to provide a positive step towards a standardised procedure for *in vitro* fertilisation in pigs. Currently, methods used to reduce the incidence of polyspermy in porcine oocytes mostly involve improvising on the existing techniques and optimising them to achieve the best possible result. Although the benefits of the oviductal fluid for the prevention of polyspermic fertilisation are plentiful, the use of an oviductal fluid as a routine supplement in IVF media is not yet a reality. This is due to the need for a better understanding of its composition, proper purification and storage, along with ensuring its quality and keeping it sterile in order to improve the IVF efficiency (Ballester et al. 2014).

Sperm capacitation also depends heavily on various complex compounds found in the male and female reproductive systems. Replication of this process for the IVF setup remains a challenge for the future as sperm capacitation occurs in a very controlled manner with specific compounds and post capacitation, the lifespan of the sperm gets reduced significantly which only provides a short window for the fertilisation to occur. Hence, proper optimisation of the capacitation media can help reduce the polyspermic fertilisation rates significantly.

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