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IN VITRO PRODUCTION OF PORCINE EMBRYOS: CURRENT STATUS AND POSSIBILITIES – A REVIEW*

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Abstract

This paper presents the current possibilities, state of knowledge and prospects of *in vitro* production (IVP) of pig embryos, which consists of *in vitro* oocyte maturation, *in vitro* fertilization and *in vitro* embryo culture. In pigs, oocyte maturation is one of the most important stages in the embryo IVP process. It determines the oocyte's fertilization ability as well as its embryonic development. Through many research studies of the proper selection of oocytes and appropriate maturation medium composition (especially the addition of various supplements), the *in vitro* maturation of pig oocytes has been significantly improved. Recent studies have demonstrated that modifications of the diluents and *in vitro* fertilization media can reduce polyspermy. Furthermore, several adjustments of the porcine culture media with the addition of some supplements have enhanced the embryo quality and developmental competence. These updates show the progress of IVP in pigs that has been achieved; however, many problems remain unsolved.

Key words: pig, oocyte maturation, *in vitro* fertilization, embryo, *in vitro* culture

In vitro production (IVP) of pig embryos remains the object of studies because there are still multiple unsolved problems related to this method. Regarding the IVP of embryos, the main issues are the quality of *in vitro* matured oocytes, semen quality after capacitation, polyspermy and *in vitro* obtained embryo quality.

Taking into consideration the constant development of biotechnological and biomedical studies performed on pigs, there is a steady increase in demand for mature porcine oocytes and embryos. Research in the field of *in vitro* production of porcine embryos was started over 30 years ago, and in 1986, the first piglets were born from *in vivo* matured oocytes (Cheng et al., 1986). However, the first piglets from embryos obtained after implementing the complex *in vitro* method, i.e., oocyte maturation,

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fertilization and embryo culture were born in 1993 (Yoshida et al., 1993). Despite visible progress in recent years, the effectiveness of the IVP method in pigs is still unsatisfactory. The *in vitro* production of embryos consists of *in vitro* oocyte maturation, *in vitro* fertilization and *in vitro* embryo culture.

In vitro oocyte maturation

The maturation of oocytes is one of the most important stages in the embryo IVP process. It determines the embryo's fertilization capability as well as its cellular development. During the maturation process, changes occur within the nucleus and cytoplasm. The primary oocyte is inhibited in prophase of the first meiotic division and remains in this phase until the period leading up to ovulation. In the first phase of oocyte maturation, condensation of chromosomes occurs in the nucleus, after which the chromosomes are attached to microtubules that make up the spindle apparatus and then form the equatorial plate of the first meiotic division. The first meiotic division is completed by telophase and the cytoplasm division into the secondary oocyte and the first polar body. The secondary oocyte quickly starts the second meiotic division. The spindle apparatus is formed, and the chromosomes line up to create the equatorial plate of the second meiotic division. At this time, the second meiotic division is stopped. The conclusion of meiosis is manifested by the ejection of the second polar body during fertilization after the oocyte is penetrated by the spermatozoon (Bielańska-Osuchowska, 2001). Successful *in vitro* maturation of pig oocytes and the subsequent ability to fertilize can be achieved when oocytes reach synchronous nuclear, cytoplasmic and genomic maturation. Nuclear maturity can be easily evaluated, however, cytoplasmic maturity can only be confirmed by the oocytes' ability to form the pronucleus (Niemann and Rath, 2001). During the oocyte maturation process, multiple interactions occur between the nucleus and cytoplasm. Nuclear maturity signifies the oocyte's reaching of the second meiotic division. On the other hand, cytoplasmic maturity determines the oocyte's ability to be fertilized and initiates further embryo development (Abeydeera, 2002). A high percentage of *in vitro* matured pig oocytes do not reach cytoplasmic maturity (Hyun et al., 2003). Cumulus cells play key roles during the cytoplasmic and nuclear maturation processes. During the maturation process, granulosa cells around the oocyte undergo expansion, which may correspond with reaching nuclear and cytoplasmic maturation. The expansion level of these granulosa cells is one of the indicators of the completion of the nuclear and cytoplasmic maturation processes (Abeydeera, 2002).

Oocyte maturation media components

Oocyte maturation media contain a variety of supplements, including hormones such as follicle-stimulating hormone (FSH), luteinizing hormone (LH), human chorionic gonadotropin (hCG), pregnant mare serum gonadotropin (PMSG) or equine chorionic gonadotropin (eCG), growth factors, foetal calf serum (FCS), porcine follicular fluid (pFF), various antioxidants, hyaluronic acid, and amino acids such as cysteine and leptin (Sato et al., 1990; Stankiewicz et al., 2009; Batista et al., 2016; Yuan et al., 2017).

Hormones and growth factors

Mattioli et al. (1991) observed a much higher percentage of mature oocytes in media that were supplemented with LH (76%) and FSH (86%) in comparison to oocytes maturing in media without these hormones. Other studies (Algriany et al., 2004) have also confirmed the positive effect of FSH and LH on the expansion of cumulus oophorus cells and therefore the increased percentage of oocytes reaching the metaphase II stage. Studies carried out in recent years have shown that the LH and FSH hormones are commonly used as standard supplements of media for *in vitro* porcine oocyte culture (Gil et al., 2017; Yuan et al., 2017).

It has been demonstrated that supplementation with epidermal growth factor (EGF) in the presence of gonadotropins affected the formulation of the male prenucleus without influencing oocyte nuclear maturation (Abeydeera, 2002). The positive effect of media supplementation with EGF along with FSH manifests as the obtainment of a higher percentage of blastocysts after *in vitro* fertilization of oocytes cultured in this way (Uhm, 2010). Recently, trials have been conducted using several growth factors, e.g., fibroblast growth factor (FGF2), insulin-like growth factor (IGF1) and leukaemia inhibitory factor (LIF) (Yuan et al., 2017). The addition of the abovementioned cytokines to oocyte maturation media increased oocyte developmental competences, simultaneously resulting in a higher percentage (up to 49%) of developing blastocysts.

Proteins

The component most commonly used to supplement media is a protein in the form of bovine serum albumin (BSA) or foetal calf serum (FCS). Serum proteins are responsible for acid–base homeostasis and osmotic pressure, stabilize pH and are a source of amino acids. For the first time, Zheng and Sirard (1992) and later Batista et al. (2016) concluded the positive effect of FCS on pig oocyte maturation. On the other hand, research performed by Funahashi and Day (1993) proved that FCS decreases pig oocytes' ability to form a male pronucleus. Based on the available research, the exact effects of serum are not conclusive. It is known that it contains enzymes, hormones and growth factors that are crucial during the oocyte maturing process. However, using animal protein in the form of BSA and FCS for media supplementation may create the risk of infection. Moreover, the use of these proteins makes it impossible to standardize culture conditions because they have a different concentration in subsequent product series. Taking that into consideration, efforts have been made to substitute serum with other compounds, such as polyvinylpyrrolidone (PVP), polyvinyl acid (PVA) or plant proteins (Sreenivas et al., 2013).

Porcine follicular fluid (pFF) is the optimal environment for the proper development of oocytes. It contains a mixture of proteins and other factors that positively affect the growth of developmentally competent oocytes. It has been determined that pFF contains substances that increase the expansion of oocyte granulosa cells as well as stimulate nuclear maturation and fertilization. Thanks to the presence of a highly concentrated superoxide dismutase enzyme, the follicle fluid protects the oocyte from oxidation stress. Moreover, superoxide dismutase stimulates development and affects cytoplasmic maturation and therefore increases oocyte developmental

competence after fertilization. It has been found that adding pFF to the pig embryo culture medium accelerates the formation of the male pronucleus and increases the percentage of oocytes fertilized monospermically (Ito et al., 2008).

Antioxidants

Despite many studies and improvements in porcine IVP, the efficiency of embryo production is still unsatisfactory. One of the most significant reasons for this low quality is the oxidative stress caused by the production of reactive oxygen species (ROS) during *in vitro* maturation. Therefore, specific supplements are needed to protect oocytes and inhibit the effect of ROS. To improve the quality of *in vitro* matured oocytes and embryos, various antioxidants are used (Qasim et al., 2019), e.g., phycocyanin, chlorogenic acid, zinc, resveratrol, melatonin, manganese, vitamin E, vitamin C, and laminarin.

Phycocyanin is a compound used in oocyte maturation media (Liang et al., 2018). This antioxidant obtained from microalgae positively affected oocyte maturation by increasing their antioxidant abilities, which had a positive effect on oocyte quality.

A similar antioxidant effect is demonstrated by chlorogenic acid (CGA), which, when added to oocyte maturation media, significantly increased the percentage of mature oocytes (Nguyen et al., 2017).

The antioxidant effect of zinc on maturing porcine oocytes, which was manifested by a lowered level of reactive oxygen species (ROS), was demonstrated by the research of Jeon et al. (2014). Moreover, zinc had a positive effect on the later development of parthenogenetic (PA) embryos as well as embryos obtained after IVF.

Another antioxidant used as a maturation medium supplement is resveratrol. This polyphenol found in fruit, mainly grapes, has antifungal and anti-inflammatory qualities and reduces apoptosis. It has been demonstrated that supplementing oocyte maturation media with resveratrol resulted in an increase in intercellular glutathione levels and a decrease in the level of ROS, which enhanced the developmental competences of oocytes (Itami et al., 2015). Other research (Lee et al., 2018) supports the reports of this antioxidant's positive effects and that its supplementation resulted in an increase in the percentage of mature oocytes. As has been shown by various studies, resveratrol, when added to porcine embryo culture media, had a positive effect on the early stage development of embryos (Lee et al., 2010). Moreover, it has been reported that resveratrol can improve embryonic development post-warming probably through the active removal of damaged mitochondria (Hara et al., 2018).

Melatonin is a multi-functional molecule with various biological activities. It is synthesized by the pineal gland and in other organs, such as the ovaries (Reiter et al., 2007). It controls many physiological processes, including seasonal reproduction. This hormone also has antioxidant and antiapoptotic properties and can reduce ROS generation (Zhao et al., 2016). Melatonin regulates oocyte maturation and pre-implantation embryo development in different mammalian species (Cruz et al., 2014). This hormone also protects porcine oocytes from heat stress during *in vitro* maturation by reducing ROS formation, enhancing GSH production and inhibiting apoptosis (Li et al., 2015; Lin et al., 2018). In pigs during prolonged *in vitro* maturation, the

addition of melatonin has a positive effect on the quality and development of poor quality porcine oocytes via antioxidative and antiapoptotic effects (Lin et al., 2018).

Manganese (Mn) is an antioxidant mineral naturally present in food. It influences many processes, such as lipid and protein metabolism as well as reproduction (Horning et al., 2015). Supplementation of Mn to the bovine maturation medium enhanced the competence of oocytes and decreased the frequency of apoptotic cumulus cells (Anchorodoquy et al., 2014). In pigs, it has been reported that the addition of Mn to IVM medium did not enhance nuclear maturation, but it can improve the developmental competence of porcine PA and somatic cell nuclear transfer (SCNT) embryos by increasing intercellular glutathione (GSH) and decreasing ROS levels (Qasim et al., 2019).

To enhance embryo culture media, vitamin E – an antioxidant dissolvable in lipids – was tested (Tappel, 1980). Porcine embryos are characterized by a high lipid level and therefore are very susceptible to peroxidation. Lipid peroxidation can inhibit cell functions and affect the triggering of apoptosis. As has already been mentioned, one of the factors that can inhibit lipid peroxidation is vitamin E. First, trials undertaken by Steele et al. (1974) demonstrated a positive effect of vitamin E on the maturation of rat embryos. Investigation of the effect of vitamin E on the *in vitro* development of porcine embryos has led to the conclusion that culture in media with vitamin E supplementation resulted in an increase in the percentage of blastocysts as well as an improvement in their quality in comparison to the control group (Gajda, 2009).

Vitamin C (ascorbic acid) is a water-soluble antioxidant that plays an important role in multiple biological processes (Camarena and Wang, 2016). The addition of vitamin C to the IVM medium enhances nuclear maturation (Tao et al., 2010), increases intracellular glutathione (GSH) levels, reduces ROS levels in porcine oocytes (Kere et al., 2013) and improves the developmental competence of porcine oocytes after parthenogenetic activation, *in vitro* fertilization (Tatemoto et al., 2001) and SCNT (Kere et al., 2013). Moreover, the research of Yu et al. (2018) supports these findings by demonstrating that supplementation of IVM medium with vitamin C improves oocyte meiotic maturation and the developmental competence of the obtained embryos. Supplementation of embryo culture medium with vitamin C decreased the number of apoptotic cells and increased the cell number in porcine blastocysts (Hu et al., 2012). These findings are supported by Rajska et al. (2018), who reported that vitamin C used as a supplement for embryo culture significantly enhanced porcine embryo development.

Another recently tested modification of culture media is supplementation with laminarin. It has been stated that this polysaccharide, which can be found in brown algae and in many other plants, fungi and bacteria, decreased the oxidative stress triggered by lipid peroxidation and significantly increased the cleavage and blastocyst percentage, obtained parthenogenetically in comparison to embryos cultured without this supplementation (Jiang et al., 2018).

Other supplements

One of the media supplements is hyaluronan (hyaluronic acid, HA). It is a polysaccharide that belongs to the glycosaminoglycans (GAGs). It was first isolated and

described by Karl Meyer and John Palmer in 1934. In vertebrates, molecules of hyaluronic acid were found in all tissues and fluids. In mammals, HA is synthetized with synthases (HAS 1,2,3) that are produced by plasmatic membranes (Itano and Kimata, 2002). In pigs, HAS2 and CD44 were localized in cumulus cells, and HAS3 was found only in oocytes (Kimura et al., 2002). Upon interaction with receptors on the cell surface (mainly CD44), hyaluronic acid is broken up by hyaluronidases (Hyal-1, Hyal-2, and Hyal-3) into smaller polymers. The interaction of Hyal-2 and CD-44 triggers the endocytosis of HA, which undergoes further divisions as a result of Hyal-1 and triggers migration, proliferation and mitosis (Stern et al., 2006). Hyaluronic acid plays a major role in the process of oocyte maturation (Palasz et al., 2006). As a result of intercellular hyaluronan synthesis, expansion of cumulus cells occurs. In pigs, the physiological concentration of HA in follicles and in the oviduct fluid varies between 0.04 and 1.83 mg/ml (Kano et al., 1998). A positive effect of hyaluronic acid has been confirmed on the maturation process of pig (Sato et al., 1990) and cattle oocytes (Ríos et al., 2015). It has been determined that hyaluronic acid localized in the oviduct fluid stimulates the *in vitro* capacitation of spermatozoa without triggering acrosomal reactions. It is speculated that HA may also play a role during spermatozoa penetration, thus regulating polyspermy levels (Abeydeera, 2002). Studies by Gajda et al. (2010), Smorąg et al. (2013), and Romek et al. (2017) have demonstrated the positive effect of HA supplementation of NCSU-23 medium on the development and quality of cultured pig embryos.

The latest reports demonstrate that various other compounds are being tested as supplements to IVM and IVC media. Among them are L-carnitine (Lowe et al., 2017), cysteamine (Wang et al., 2019), polyunsaturated fatty acids (Hoyos-Marulanda et al., 2019), baicalin (Guo et al., 2019), mogroside V (Nie et al., 2020), trehalose (Cai et al., 2020), asiatic acid (Qi et al., 2020) and thymosin (Poniedziałek-Kempny et al., 2020).

Conditions of oocyte *in vitro* maturation

Oocyte *in vitro* maturation requires the creation of a specific environment that is as close as possible to *in vivo* conditions. These conditions are created by various *in vitro* culture systems that include media with a very specific chemical composition that is one of the factors influencing the effectiveness of IVP. In the 1990s, it was determined that the cytoplasmic maturity of an oocyte is dependent on the selected culture medium (Funahashi et al., 1994). Based on these findings, studies concentrated on determining the optimal media composition, as well as various supplements that increase the *in vitro* maturation effectiveness of oocytes. Currently, the following media, with very specific chemical compositions, are used for pig oocyte culture: Tissue Culture Medium (TCM-199) (Mattioli et al., 1989), North Carolina State University Culture (NCSU-23) or NCSU-37 (Petters and Wells, 1993), and Whitten Medium (MWM) (Beckmann and Day, 1993).

Pig oocytes require a much longer culture (42–48 hours) than oocytes from other mammalian species. With regard to pig oocytes, a two-phase culture approach is used. This is related to the presence of dibutryl-cAMP (dbcAMP), which synchronizes the meiotic cycle in the first phase of the maturation process. This synchroni-

zation supports achieving cytoplasmic maturity and therefore influences the quality level of obtained embryos. In pig oocyte culture, the presence of dbcAMP from the very beginning in the maturation medium may lead to an increase in the number of obtained blastocysts (Gil et al., 2017).

The presence of oxygen in the atmosphere is also a very important factor in the process of oocyte cytoplasmic maturation. It is believed that an optimal amount of oxygen is dependent on the oocyte maturation medium used. Studies have shown that using a gas mixture, where the oxygen content has been lowered to 5%, was more beneficial for the oocyte maturation process than 5% CO_2 when the medium used was NCSU-37 (Kikuchi et al., 2002). Other studies concluded that pig embryos matured in NCSU-23 medium and an atmosphere of 5% CO_2 demonstrated a higher cell number than embryos obtained from oocytes maturing in this medium with a lowered oxygen level of 5% (Machaty et al., 1998).

Table 1. Most commonly used media for *in vitro* oocyte maturation

Medium	Medium components	References
mWM	0.57 mM cysteine + 1000 IU eCG + 1000 IU hCG in 5 µl H_2O + 10% pFF	Wang et al., 1997
NCSU-23	0.57 mM cysteine + 20 ng/ml EGF + 5 IU/ml eCG + 5 IU/ml hCG	Hong et al., 2004
	0.1 mg/ml cysteine +10 IU/ml eCG + 10 IU/ml hCG + 10% (v/v) pFF	Stankiewicz et al., 2009
NCSU-37	0.6 mM cysteine + 10 IU/ml eCG + 10 IU/ml hCG + 10% (v/v) pFF + 50 µg/ml gentamycin + 1 mM dbcAMP	Wongsrikeao et al., 2006
	0.6 mM cysteine + 10 IU/ml eCG + 10 IU/ml hCG + 1 mM dbcAMP + 10% pFF	Ito et al., 2008
	0.6 mM cysteine + 10 IU/ml eCG +10 IU/ml hCG + 1 mM dbcAMP +10% (v/v) pFF +100 units/ml penicillin + 0.1 mg/ml streptomycin	Nakamura et al., 2017
TCM-199	0.57 mM cysteine + 0.5 µg/ml LH + 10 ng/ml FSH + 0.91 mM sodium pyruvate + 10 ng/ml EGF + 3.05 mM glucose + 0.1% polyvinyl alcohol	Yuan et al., 2017
	0.6 mM cysteine + 10 IU/ml hCG + 10 IU/ml eCG + 1 µg/ml β-estradiol + 50 µM sodium pyruvate + 2 mg/ml D-sorbitol + 50 µg/ml gentamycin + 10% pFF	Nguyen et al., 2017
	0.5 µg/ml FSH + 0.5 µg/ml LH + 10 ng/ml EGF + 0.91 mM Na-pyruvate + 1 µg/ml insulin + 75 µg/ml kanamycin + 10% pFF	Liang et al., 2018
	0.57 mM cysteine + 0.5 µg/ml FSH + 0.5 µg/ml LH + 0.91 mM sodium pyruvate + 26 mM Na-bicarbonate +10 ng/ml EGF + 75 µg/ml penicillin + 50 ng/ml streptomycin + 10% pFF	Biswas et al., 2018
	1 mM cysteine + 1 mM dbcAMP + 10 IU/ml eCG + 10 IU/ml hCG + 10% pFF	Poniedziałek-Kempny et al., 2020

The methods of pig oocyte *in vitro* maturation have been considerably developed and optimized over the past twenty years. At the present stage of investigations, it is

possible to obtain efficiency ranging from 70% to 90% of nuclear maturation (metaphase II stage) (Zhang et al., 2015; Lee et al., 2016; Nguyen et al., 2017). However, as has been stated previously, to achieve oocyte *in vitro* maturity, a suitable environment needs to be created to resemble as much as possible *in vivo* conditions. This is achieved by, among other methods, the correct selection of the culture medium composition.

Oocyte selection

The evaluation of immature oocytes is a critically important phase in the *in vitro* maturation process. However, the selection of oocytes remains significantly problematic. The main selection criterion is the morphological evaluation of the nucleus and the granulosa cells surrounding the oocyte. It is speculated that one of the reasons for the low effectiveness of oocyte maturation is the imperfect selection process.

The polar body is an indicator of oocyte nuclear maturation; however, researchers using polarized light microscopy have concluded that oocytes displaying a polar body may still be immature (Hoshino, 2018).

Morphological evaluations of oocytes that are currently used are considered non-invasive methods and are subjective and controversial. They may not be correlated with the competence of oocytes. Nevertheless, the identification of non-invasive molecular markers that assess the quality and ability of oocytes is one of the most important research goals (Hoshino, 2018).

One of the selection methods of immature oocytes is non-invasive Brilliant Cresyl Blue staining (BCB). The BCB method describes the activity of the G6PDH enzyme, which is synthesized in the first part of S phase during the growth of the oocyte. In oocytes that have completed the growth phase, this enzyme is deactivated, and the oocytes turn blue (BCB+). On the other hand, oocytes that remain in the maturation stage are colourless (BCB-) because the active G6PDH reduces the blue colour in the BCB dye to a colourless compound (Opiela and Kątska-Książkiewicz, 2005). Our observations have shown a limited usefulness of BCB staining for the selection of porcine oocytes (Poniedziałek-Kempny, unpublished data).

Molecular methods have led to the creation of a new objective oocyte evaluation process to assess their developmental potential. The disadvantage of these methods is their invasiveness (Jaśkowski et al., 2010), which leads to a partial or complete loss of cell viability. Genomics is based on screening studies of gene expression. Techniques, such as expressive micro-matrices, give the possibility of analysing the entire transcriptome in the studied oocytes (Jaśkowski et al., 2010).

One of the latest studies reported that the intracellular temperature of oocytes may be influenced by the function of organelles (microtubules and mitochondria). Hoshino (2018) described intracellular temperature and its relationship to oocyte quality using a fluorescent polymer thermometer (FPT). The FPT is a molecular marker developed by Okabe et al. (2012) that can diffuse throughout cells and is able to assess their thermal profile. However, in the future, non-invasive methods for temperature measurement should be developed for visualization (Hoshino, 2018).

The evaluation of other invasive, biochemical and cytological parameters requires a biopsy of cumulus cells. It is a very complicated, expensive and time-con-

suming process. Moreover, this procedure might not impair COC developmental competence (Goovaerts et al., 2010).

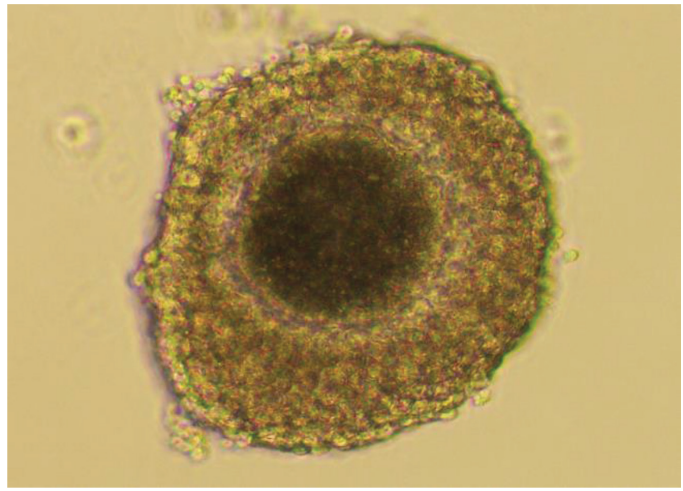


Figure 1. Immature porcine oocyte

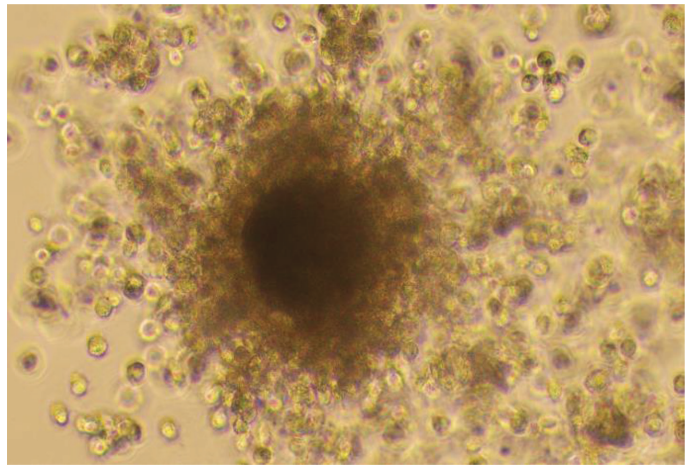


Figure 2. *In vitro* matured oocyte with expanded cumulus layers

***In vitro* fertilization**

In the *in vitro* production of embryos, *in vitro* fertilization (IVF) is carried out with cultured oocytes that reach the metaphase II stage. To prepare for *in vitro* fertilization, sperm are incubated in a capacitation medium that stimulates biochemical processes and morphological changes that trigger the fertilization ability of the oocyte.

Factors influencing the fertilization capabilities of spermatozoa include the spermatogenesis process as well as technological aspects related to semen conservation,

which is common when semen is used for pig insemination. Technological aspects play an even greater role when boar semen is used for *in vitro* fertilization. Despite many improvements in IVF protocols, the efficiency is still unsatisfactory. Factors such as the IVF medium, sperm concentration, ejaculated or epididymal, frozen or fresh spermatozoa, capacitation *in vitro*, and gametes co-incubation time during IVF may vary considerably. Moreover, parameters such as the penetration and polyspermy rate are difficult to compare, and these problems are still unsolved (Appeltant et al., 2016).

***In vitro* capacitation of semen**

During maturation, a number of changes occur in spermatozoa properties and kinetics in the epididymis. After leaving the epididymis, spermatozoa acquire movement abilities but are not yet capable of fertilization. Sperm reach full maturity during the capacitation process, resulting in changes in movement type called hyperactivation. These changes are manifested by increasing the movement range and frequency of the spermatic filament as well as inhibiting the circular movement around the spermatozoon's long axis. This type of movement is crucial for the spermatozoon to penetrate the zona pellucida. As a result of changes in the structure and composition of the cell membrane above the acrosome, the phospholipid membranes are discarded, and the cell membrane becomes penetrable for Ca^{2+}, K^{+} and protons (Bielańska-Osuchowska, 2001). Capacitation, which occurs in the female reproductive tract under physiological conditions, can also be triggered under *in vitro* conditions. Ejaculated semen or sperm obtained from the epididymis are incubated in a medium with a composition resembling the oviduct fluid. The medium contains, among other compounds, albumin, sodium bicarbonate ($NaHCO_3$) and calcium ions (Ca^{2+}). In boars, capacitation may also be triggered by hyaluronan, fertilization promoting peptide (FPP protein), adenosine (Gil et al., 2008) and caffeine (Funahashi et al., 2000). Funahashi and Romar (2004) proved that methylxanthines such as theophylline or caffeine can increase the *in vitro* sperm penetration abilities of mature porcine oocytes by stimulating phosphodiesterase inhibitors, which increases the cAMP concentration. Moreover, caffeine not only triggers capacitation but also stimulates boar sperm acrosomal reactions (Funahashi et al., 2000). One of the conditions of capacitation is the increase in phospholipids in relation to cholesterol. This is achieved by adding BSA, which eliminates cholesterol from the cell membrane of the spermatozoon. This procedure results in an increase in cell penetrability for Ca^{2+} ions (Kątska, 2000). In cattle, under *in vitro* conditions, capacitation may be triggered by adding to the medium heparin or oviduct fluid that contains glycosaminoglycans (GAGs). These polysaccharides bond with and eliminate seminal plasma proteins that stabilize the spermatozoon's cell membrane. Glucose also plays an important role in the capacitation process. Its activity is species specific (Kątska, 2000).

For the spermatozoon to penetrate the zona pellucida, further morphological changes need to take place during the acrosomal reaction. These changes start in the spermatozoon's head. First, the cell and acrosomal membranes become penetrable to Ca^{2+} ions, and then the cell membrane fuses with the external acrosomal membrane. This results in the fragmentation and breakup of the membrane into vesicles (ve-

siculation process). During this process, enzymes are released from the acrosome. The first enzyme to be activated is hyaluronidase, which dissolves the intercellular substance that binds corona radiata cells with cumulus oophorus cells. This enzyme loosens corona radiata and cumulus cells. Hyaluronidase is supported by esterase, which is present in semen plasma. Another crucial enzyme is acrosin, which triggers changes in membranes by increasing their penetrability, thus allowing spermatozoa to penetrate the zona pellucida (Kątska, 2000; Bielańska-Osuchowska, 2001). During the acrosome reaction, the internal acrosome membrane is exposed. This process takes place only in capacitated spermatozoa. It is believed that the acrosome reaction-inducing factor is blood serum albumin that is present in the oviduct fluid and the intercellular space. In *in vitro* conditions, the acrosome reaction is triggered by stimulating spermatozoa with different elements, e.g., catecholamines, steroids, heparin (Malik et al., 1997), progesterone, prostaglandins, sterols, glycosaminoglycans present in the follicle fluid and other non-physiological substances, e.g., calcimycin, lectin, and neoglycoprotein (Tulsiani et al., 1998).

It has been demonstrated that capacitated boar semen is capable of *in vitro* oocyte fertilization 2.5 hours from the start of the co-incubation of gametes. During this time, penetration of the zona pellucida and binding with the ooplasm take place (Nagai et al., 1993). It has been shown that spermatozoon chromatin decondensation occurs approximately 5 hours after the start of cell co-incubation and that both pronuclei are formed after 8 hours (Ding and Foxcroft, 1994). The optimal time for gamete incubation is approximately 4 hours (Coy et al., 1993). After *in vitro* fertilization of porcine oocytes, a result can be achieved ranging from approximately 30% to approximately 90% of potential zygotes (Jeon et al., 2014; Zhang et al., 2015; Gil et al., 2017; Lee et al., 2018).

Zona pellucida reaction

During penetration, the spermatozoon initiates physiochemical changes in the zona pellucida that block other spermatozoa from penetrating by hardening the zona pellucida. Moreover, changes occur in the cortical layer of the oocyte cytoplasm. This reaction is known as the cortical reaction and is triggered by the flux of Ca^{2+}. As a result of the fusion of cortical granule membranes with the oolemma, the mucopolysaccharide content of the granules is released, which in turn penetrates into the zona pellucida and triggers physiochemical changes. This results in blocking penetration by other spermatozoa. The polyspermy block occurs on two levels. The first is related to the cortical reaction, while the second is related to the changes occurring in the oolemma (Nagai, 1996).

Polyspermic fertilization

The main factors related to the penetration of the oocyte by more than one spermatozoon, i.e., polyspermic fertilization, are oocyte immaturity and low quality, inferior spermatozoa quality used for fertilization and the fertilization process conditions (Sirard et al., 1993; Gil et al., 2008). In *in vitro* conditions, a high correlation between polyspermy and spermatozoa concentration as well as the number of fertilized oocytes has been demonstrated (Gil et al., 2003). It has also been proven that

for pigs, prolonging the incubation period of spermatozoa with oocytes to 6–8 hours results in an increase in polyspermy occurrence (Gil et al., 2008). However, until now, studies have not fully explained the causes of polyspermy. This phenomenon is quite common in pigs and is the main reason for lethal embryo development abnormalities. In comparison to *in vivo* conditions, *in vitro* matured oocytes are exposed to a longer incubation period with a higher number of spermatozoa. These conditions increase the probability of oocyte penetration by more than one spermatozoon. An optimal fertilization process in *in vitro* conditions should result in a high penetration ratio (>80%) with a low polyspermy level (<10%). Theoretically, the lowering sperm concentration during the fertilization process should resolve the polyspermy issue, but it is also connected to a low penetration ratio (Abeydeera, 2002). Studies by Wang et al. (1997) have shown that *in vivo* matured oocytes are less susceptible to polyspermy than oocytes matured *in vitro*. It was stated that hyaluronic acid, apart from expanding cumulus cells, can also limit polyspermy in pigs without affecting the penetration ratio (Suzuki et al., 2000). By shortening the co-incubation period of gametes to 10 minutes and then placing them in pure IVF medium for 5–6 hours, a higher penetration percentage (80%) and a higher percentage of developing blastocysts (30%) were achieved in comparison to the standard procedure (57% and 3%, respectively) (Grupen and Nottle, 2000). It has been stated that under *in vivo* conditions, porcine oocyte cytoplasm penetration by more than one spermatozoon may occur in 30–40% of the cells (Xia et al., 2001). On the other hand, under *in vitro* conditions, a significantly higher percentage of polyspermy is observed (up to 65%) (Xia et al., 2001). The level of polyspermy can be reduced by controlling the number of capacitated spermatozoa and by the oocyte exocytosis reaction, which limits the binding of semen as well as oocyte penetration. Moreover, studies carried out by Nagai and Moor (1990) have shown that co-incubation of spermatozoa and *in vitro* matured oocytes with oviduct cells resulted in a significant decrease in polyspermy levels. However, these levels were still unacceptable. One of the latest studies reports the possibility of using frutalin (lectin with α-D-galactose-specific binding sites) to reduce the incidence of polyspermy during IVF in swine (Silva et al., 2018). The polyspermy issue can be solved by using the fertilization method of injecting a single spermatozoon or its head into the oocyte's cytoplasm (ICSI – intracytoplasmic sperm injection).

***In vitro* embryo culture**

The *in vitro* culture conditions are an important factor influencing the quality and number of porcine embryos obtained *in vitro*. Therefore, studies have been carried out for many years to optimize the composition of culture media as well as to optimize the culture conditions.

Media for embryo culture

The most commonly used porcine embryo culture media are modified Whitten medium (mWM) (Beckmann and Day, 1993), North Carolina State University medium (NCSU-23 or NCSU-37) (Petters, 1992; Petters and Wells, 1993), and porcine zygote medium (PZM) (Yoshioka et al., 2002). Regardless of composition, media are

a source of proteins, salts, energy (in the form of glucose), calcium, lactate, pyruvic acid and amino acids (Petters and Wells, 1993). The most commonly used proteins are bovine serum albumin or foetal calf serum. As previously mentioned, these media are a source of amino acids and energy, and they also stabilize the pH and medium osmolality (Gajda and Smorąg, 2002).

Culture medium supplements

Some researchers successfully used BSA or FCS supplementation, emphasizing their positive effect (Skrzyszowska et al., 2008; Mandryk, unpublished data). However, taking into consideration the risk of infection and difficulties with the standardization of culture conditions, efforts have been made to replace animal proteins with plant proteins. The first trials with the synthetic serum substitute (SSS) were undertaken by George et al. (2006). Later, Gajda et al. (2009) substituted BSA in NCSU-23 medium with plant proteins (PP). These tests did not determine differences in the percentage of blastocysts obtained after culture with the addition of PP in comparison to the embryos obtained after culture with BSA. There were also no differences in the number of cells in blastocysts cultured in plant and animal proteins (Gajda et al., 2010).

Another supplement of culture media is phenazine ethosulfate (PES), a cell metabolism regulator. It triggers an increase in glucose metabolism and reduces lipid accumulation in cultured embryos. Romek et al. (2011) reported that the triglyceride content in blastocysts obtained after *in vitro* culture with PES was low (57.2%). This result – the decrease in triglyceride content in pig blastocysts – may be explained by altered lipid metabolism in embryos. In an experiment by Gajda (2009), a slight increase in the cleavage, morulae and blastocyst percentages cultured in NCSU-23 medium with the addition of PES in comparison to the control group was observed.

Research involving glycine as a porcine embryo culture medium supplement (Redel et al., 2016) resulted in a lower apoptosis level in embryos cultured with this compound in comparison to embryos developed without it. Moreover, blastocysts obtained from a culture medium supplemented with glycine were characterized by a higher number of cells in comparison to control blastocysts. Glycine is an amino acid that is a precursor to protein and nucleic acid synthesis that influences swift cell proliferation.

A recent study showed that supplementing *in vitro* culture media with vascular endothelial growth factor (VEGF) may have beneficial effects on the development of porcine embryos *in vitro* (Biswas et al., 2018). VEGF is a potent mitogen used for the propagation of micro- and macro-vascular endothelial cells. It is also involved in numerous processes, such as ovulation, early embryonic implantation, and subsequent embryo development. VEGF also reduces apoptosis (Grasl-Kraupp et al., 1995). Biswas et al. (2018) suggest that adding VEGF to IVC media during *in vitro* culture of porcine embryos increases their developmental potential.

Other compounds used as supplements to IVC culture media are antioxidants that have been discussed in the *oocyte maturation media components* chapter.

The above-mentioned *in vitro* porcine embryo culture medium modifications can increase the number of developed embryos, and the percentage of obtained blastocysts is over 50% (Zhang et al., 2015; Nguyen et al., 2017; Gil et al., 2017; Biswas et al., 2018). In our *in vitro* embryo culture studies, we achieved results ranging from 25% to 50% blastocysts (Poniedziałek-Kempny et al., 2020).

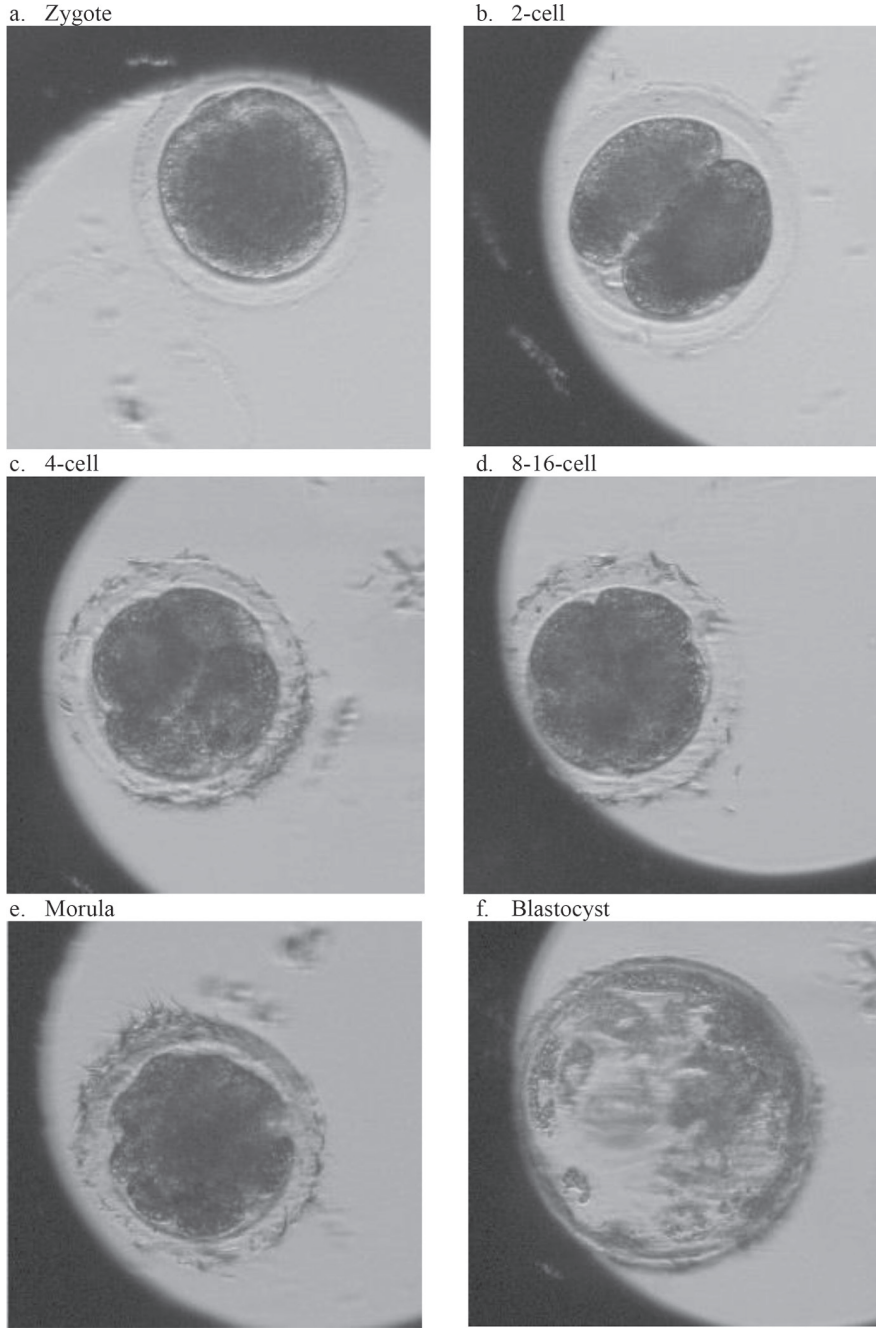
Table 2. Important *in vitro* porcine embryo culture results

Medium	Blastocyst rate (%)	References
mWM	5**	Funahashi et al., 1994
NCSU-23	23.8*	Wu et al., 2004
	32**	Gajda et al., 2015
	51.3**	Rajska et al., 2018
	30.1*	Wang et al., 2019
NCSU-37	36.8**	Ulloa Ulloa et al., 2008
	33*	Dang-Nguyen et al., 2018
PZM-3	38**	Redel et al., 2016
	49.7**	Yuan et al., 2017
	51.8*	Biswas et al., 2018
PZM-5	41.3*	Yoshioka et al., 2008
	21.6*	Nguyen et al., 2017

* blastocyst/oocytes.
** blastocyst/cleavage.

Conditions of embryo culture in vitro

During the culture process, the amount of oxygen in the atmosphere is an important factor that influences the effectiveness of embryo development (Kątska-Książkiewicz, 2006). In mammals, the percentage of oxygen in the uterus is approximately 5% (Fisher and Bavister, 1993). Under *in vitro* conditions, the standard content is 5% CO_2; therefore, the content of oxygen is approximately 20%. By decreasing the amount of oxygen from 20% to approximately 5% during *in vitro* culture, a higher percentage of developing cattle embryos (Liu and Foote, 1995), sheep embryos (Thompson et al., 1990) and porcine blastocysts after IVF was observed. At the same time, other authors did not note differences in the percentage of porcine blastocysts obtained after *in vitro* fertilization when cultured in 5% and 20% oxygen in the atmosphere (Yoneda et al., 2004).



a – zygote, b – 2-cell, c – 4-cell, d – 8-16-cell, e – morula, f –blastocyst

Figure 3. Porcine embryos obtained *in vitro* in various developmental stages; Time Lapse PrimoVision®

Conclusions

In recent years, in studies carried out on the optimization of *in vitro* production of pig embryos, an increase in the percentage of competent oocytes that reach the blastocyst stage after fertilization has been observed thanks to the usage of various *in vitro* maturation media modifications. The available *in vitro* oocyte maturation methods allow us to obtain a high percentage of good quality mature oocytes. However, the *in vitro* fertilization methods and techniques and *in vitro* embryo culture conditions still require more studies and research. At the same time, modifications of the culture media of *in vitro* obtained porcine embryos have led to an increase in the effectiveness of obtaining blastocysts from approximately 20% to over 50%. The abovementioned modifications have also resulted in the production of blastocysts of a better morphological quality. Despite this progress, the quality of porcine embryos obtained *in vitro* is still significantly lower than the quality of embryos obtained *in vivo*.

Therefore, the currently used methods of IVP, despite visible progress, still do not allow the implementation of this technology in breeding practice.

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