Modulation of physiological pathways to activate porcine oocytes

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"*Jumping from failure to failure with undiminished enthusiasm is the big secret to success*."

-Savas Dimopoulus

ABSTRACT

Oocyte activation is a physiological process triggered by the sperm during fertilization and is responsible for several events necessary for embryo development such as cortical granule exocytosis, elimination of the second polar body, cell cycle progression and pronuclear formation. Assisted oocyte activation is necessary for the production of porcine embryos through somatic cell nuclear transfer (SCNT), intracytoplasmic sperm injection (ICSI) and parthenogenetic activation. However, techniques such as SCNT and ICSI still show contradictory and low results. Although effective protocols for artificial oocyte activation have been developed, current protocols require long exposures to non-specific inhibitors, which do not mimic the physiological process and may have detrimental consequences for embryo development. In this research, we have explored physiological events induced following fertilization, through manipulation of calcium (Ca^{2+}) and zinc (Zn^{2+}) levels, and protein kinase C (PKC) and cyclin dependent kinase 1 (CDK1) activities, aiming at developing an improved protocol for activation of porcine oocytes. In the first experiment, matured oocytes were exposed to Ionomycin (Ion) for 5 min, and then treated with a specific CDK1 inhibitor (RO-3306) and/or PKC activator (OAG) for different times. The highest rate of pronuclear (PN) formation (58.8%) was obtained when oocytes were treated with CDK1i+PKCa for 4 h. Second, PN formation and embryo development were evaluated in oocytes exposed for different intervals to a Zn^{2+} chelator (TPEN) after Ion treatment. This revealed that 15 min was the minimal exposure time to TPEN required to maximize oocyte activation and embryo development. Next, we observed that treatment with CDK1i+PKCa for 4 h after TPEN for 15 min decreased embryo development compared with TPEN alone. Last, we compared the efficiency of the Ion (5 min) plus TPEN (15 min) protocol (IT-20) with a control protocol used in our laboratory (CT-245) for the production of PA, SCNT and ICSI embryos. In PA embryos, IT-20 resulted in

higher cleavage (72% vs. 49.2%) and blastocyst from cleaved embryo (65.5% vs. 46.2%) rates compared with CT-245. In ICSI embryos, higher PN rates were obtained with the IT-20 protocol. Moreover, the two protocols were equally efficient for activation of SCNT embryos. Based on these findings, we propose that IT-20 is a faster and effective protocol for activation of porcine oocytes.

RÉSUMÉ

L'activation de l'ovocyte est un processus physiologique déclenché par le spermatozoïde pendant la fécondation et elle est responsable pour plusieurs évènements nécessaires pour le développement de l'embryon, tel que l'exocytose des granules corticaux, l'expulsion du deuxième globule polaire, la progression du cycle cellulaire et la formation des pronoyaux (PN). Une activation assistée est nécessaire pour la production d'embryons porcins construits par transfert nucléaire de cellules somatiques (TNCS), injection intra-cytoplasmique de spermatozoïde (ICSI) et activation parthénogénétique (AP). Cependant, les méthodes telles que TNCS et ICSI démontrent toujours des résultats contradictoires et faibles. Bien que des protocoles efficaces pour l'activation artificielle d'ovocytes ont été développés, ces protocoles nécessitent une exposition prolongée à des inhibiteurs non-spécifiques, qui ne reproduit pas le processus physiologique, et peut même avoir des conséquences néfastes pour le développement de l'embryon. Cette recherche explore les évènements physiologiques provoqués suite à la fécondation, avec la manipulation des taux de Ca^{2+} et Zn^{2+}, et l'activité des enzymes protéine kinase C (PKC) et kinase dépendante des cyclins 1 (CDK1), avec le but de développer un protocole amélioré pour l'activation des ovocytes porcins. Dans la première expérience, des ovocytes matures ont été exposés à ionomycine (Ion) pour 5 minutes, et ensuite traités avec un inhibiteur spécifique pour CDK1 (RO-3306) et/ou un activateur de PKC (OAG) pour différentes durées de temps. Le taux le plus élevé de formation de PN (58.8%) a été obtenu lorsque les ovocytes ont été traités avec CDK1i+PKCa pour 4 heures. Deuxièmement, la formation de PN et le développement embryonnaire ont été évalués lorsque des ovocytes ont été exposés, après traitement d'ionomycine, avec un chélateur de Zn^{2+} (TPEN) pour différentes périodes de temps. Ceci a révélé que 15 minutes était la durée minimale d'exposition à TPEN pour maximiser l'activation d'ovocytes et le développement embryonnaire. Ensuite, nous avons observé

que le traitement avec CDK1i+PKCa pour 4 heures après TPEN pour 15 minutes a diminué le développement des embryons comparé avec seulement TPEN. Finalement, nous avons comparé l'efficacité du protocole d'Ion (5 min) suivi de TPEN (15 min) (IT-20) avec un protocole de contrôle utilisé dans notre laboratoire (CT-245) pour la production d'embryons par AP, TNCS et ICSI. IT-20 a augmenté le clivage (72% vs. 49.2%) et le taux de blastocyste selon clivage (65.5% vs. 46.2%) comparé à CT-245, dans les embryons développés par AP. Des taux plus élevés de formation PN ont été obtenu avec le protocole IT-20 lorsque les embryons ont été construits par ICSI. De plus, l'efficacité d'activation d'embryons construits par TNCS étaient pareilles avec les deux protocoles. Selon ces résultats, nous proposons que le protocole IT-20 est une méthode plus rapide et efficace pour l'activation des ovocytes porcins.

CONTRIBUTION OF AUTHORS

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Vilceu Bordignon and Hernan Baldassarre designed experiments, analyzed data, reviewed the manuscript and supervised the primary author. Mariana Priotto de Macedo conducted the experiments, analyzed data and wrote the manuscript. Werner Giehl Glanzner, Vitor Braga Rissi, Karina Gutierrez and Luke George Currin assisted on conducting the experiments and analyzing the data.

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LIST OF ABBREVIATIONS

6-DMAP: 6-(Dimethylamino)purine

AI: artificial insemination

AOA: assisted oocyte activation

APC/C: anaphase promotor factor

ART: assisted reproductive technology

BSA: bovine serum albumin

CaMKII: Ca^{2+}/calmodulin-dependent kinase II

cAMP: cyclic adenosine monophosphate

CatSper: sperm specific Ca^{2+} channels

CB:cytochalasin B

CDK1: catalytic cyclin-dependent kinase 1

CDK1i: RO-3306, a specific inhibitor of CDK1

cGMP : cyclic guanosine monophosphate

CHX: cycloheximide

COC: Cumulus-oocyte complex

cPKC: conventional protein kinase C

CRISPR/Cas9: clustered regularly interspaced short palindromic repeats/associated nuclease Cas9

CSF: cytostatic factor

DAG: 1,2-diacylglycerol

EdU: 5- ethynyl-2'-deoxyuridine

EGA: embryonic genome activation

EGF: epidermal growth factor

EMI2: early mitotic inhibitor 2

ER: endoplasmic reticulum

FSH: follicle-stimulating hormone

GVBD: germinal vesicle breakdown

ICSI: intracytoplasmic sperm injection

Ion: Ionomycin

IP3: inositol 1,4,5-trisphosphate

IVC: *in vitro* early embryo culture

IVEP: *in vitro* embryo production

IVF: *in vitro* fertilization

IVM: *in vitro* maturation

LH: luteinizing hormone

LOPU: laparoscopic ovum pick up

MAPK: mitogen-activated protein kinases

MOET: multiple ovulation and embryo transfer

MPF: maturation promoting factor

NBC: Na^+/HCO_3^- co-transporter

OAG: 1-Oleolyl-2-acetyl-sn-glycerol; PKC activator

OAM: outer acrosomal membrane

PA: parthenogenetic activation

PAWP: post-acrosomal sheath WW domain-binding protein

PDE3A: phosphodiesterase 3A

PGM: primordial germ cells

PIP2: phosphatidylinositol 4,5-bisphosphate

PKA: protein kinase A

PKCa: 1-Oleolyl-2-acetyl-sn-glycerol

PLCz: phospholipase C Z

PM: plasma membrane

PMA: phorbol 12-myristate 13-acetate

PVP: polyvinylpyrrolidone

sACY: soluble adenylyl cyclase

SCNT: somatic cell nuclear transfer

SOAF: sperm-born oocyte activating factor

$SrCl_2$: strontium chloride

TALEN: transcription activator-like effector nucleases

TPEN: N,N,N′,N′-Tetrakis(2-pyridylmethyl)ethylenediamine

ZFN: zinc-finger nucleases

ZP: zona pellucida

CHAPTER 1: INTRODUCTION

Advanced reproductive biotechnologies such as intracytoplasmic sperm injection (ICSI) and somatic cell nuclear transfer (SCNT) are important tools for a more efficient propagation of genetically valuable animals in order to accelerate genetic gain, enhance productivity and/or produce genetically modified animals with improved production and health traits. These techniques may also play an important participation in conservation of endangered species, contributing to the maintenance of biodiversity and the global ecosystem. The production of parthenogenetic activated (PA) embryos, i.e. embryos that develop without being fertilized, is an important resource as a study model for early embryo development in research. However, these techniques have low success rates and inconsistent results, and one possible cause for this is the absence of an optimized oocyte activation protocol. Oocyte activation is responsible for events essential for embryo development, such as cortical granule exocytosis, completion of meiosis including extrusion of the second polar body, pro nuclei formation and progression to subsequent mitosis and embryonic development [1, 2].

Oocyte activation is a physiological process triggered by fertilization. The sperm releases a specific factor, phospholipase C zeta (PLCz), which will cause the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to generate the second messengers inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG) [3, 4]. IP3 will then bind to its receptors, which are mostly located in the cortical area close to cytoplasmic membrane and in the endoplasmic reticulum (ER), causing opening of the Ca^{2+} channels, releasing Ca^{2+} to the cytoplasm [5, 6]. Zn^{2+} levels are also regulated in the oocyte following the Ca^{2+} oscillations. Zn^{2+} levels decrease intracellularly in response to fertilization, in exocytosis events termed Zn^{2+} sparks [7, 8]. The pathways regulated by Ca^{2+} and Zn^{2+} intracellular levels will decrease the maturation promoting

factor (MPF), which is also referred as cyclin dependent kinase 1 (CDK1) complex, leading to the events of oocyte activation.

Protocols for assisted oocyte activation have been widely applied and tested for the production of embryos by ICSI, SCNT and PA. Many protocols used for oocyte activation rely on modulators of intracellular Ca^{2+}, which often do not mimic the physiological patterns necessary for proper activation [9]. Other commonly used protocols are protein synthesis or phosphorylation inhibitors, which are mostly non-specific and may have several adverse effects [10, 11]. The development of an activation protocol that is simple, similar to the physiological mechanism and has an easy applicability would be beneficial for both practical uses in the industry and research. Therefore, the focus of this research was to develop an effective protocol for the activation of porcine oocytes following ICSI, SCNT and PA, by attempting to mimic physiological events that happen following fertilization through manipulation of Ca^{2+} and Zn^{2+}. This thesis is organized in chapters that present background information about the use of assisted reproductive technologies (ARTs), oocyte maturation, fertilization, oocyte activation and early embryo development followed by a manuscript, which has been submitted for publication in Theriogenology.

CHAPTER 2: REVIEW OF THE LITERATURE

2.1 Assisted Reproductive Technologies (ART)

2.1.2 ARTs in livestock

The development of assisted reproductive technologies (ART) represented a landmark in a variety of sectors around the world. Regarding livestock production, the ever-increasing world population continues to place a rising demand for livestock products, thereby requiring increased production efficiency and sustainability [12]. The requirements for greater and more efficient food production lead to an increase in the development and use of reproductive biotechnologies. Farmers are increasing the selection intensity for animals with greater meat and milk production and taking advantage of ARTs to improve the reproductive efficiency of these animals. Reproductive biotechnologies have been used for decades to accelerate genetic gain and enhance productivity, by means of having exponentially more offspring from the animals with the highest breeding value. In the future and subsequent to regulatory and consumers approval, it is anticipated that livestock productivity will be further and faster enhanced by generation of genome-edited animals with improved traits for enhanced productivity, disease resistance, prolificity, etc.

Artificial insemination (AI) was the first reproductive biotechnology developed to accelerate genetic gain and improve production of domestic animals. One of the first reports being back in 1897, using dogs, horses and rabbits [13]. However, it was not until the mid-1900's with the development of adequate semen extenders and semen cryopreservation protocols, that this technique became a common procedure used worldwide in cattle, pigs, horses and small ruminants (reviewed in [14]).

In the early 1970's, the commercial embryo transfer industry begun to develop [15]. The development of protocols for inducing multiple ovulations followed by artificial insemination and embryo transfer (MOET) started to be widely used in cattle and small ruminants, allowing for an

accelerated genetic gain, commercialization of embryos and reducing the risk of disease transmission [16-20]. However, the development and improvement of *in vitro* embryo production (IVEP) technology is advancing at high speed and has significant advantages over MOET. In small ruminants, despite the limitations, MOET is still the main technique used for embryo transfer [21]. However, in bovine, IVEP has recently surpassed MOET in the world statistics of embryo transfer [22].

2.2.2.1 *In Vitro* Embryo Production

The establishment of *in vitro* embryo production (IVEP) technique was also an important hallmark, especially in the bovine species, since it allowed a large-scale embryo production and marketing. This technique started being described in the late 70s - early 80s [23-27]. All of these studies used different approaches in order to improve the technique and make it viable, e.g. the utilization of heparin for sperm capacitation *in vitro* [27]. Finally, the first calf from *in vitro* fertilization (IVF) was born in 1982 [28], and the first calves produced entirely *in vitro* (IVM/IVF/IVC) were reported in 1990 [29]. In small ruminants, substantial progress has been made since the birth of the first lambs produced by IVF [30]. The efficiency of collecting the oocytes through laparoscopic ovum pick up (LOPU) is very high, which facilitates IVP is small ruminants. However, the commercial aspect of IVP is still low in small ruminants compared with bovine, partially due to the lack of specialized commercial laboratory services [31-35]. The first piglets from oocytes and embryos fertilized and cultured *in vitro* were born in 1989 [36]. Although it has been further demonstrated that live piglets can be produced by this technique [37-39], IVP in pigs is far less successful than in other species. High rates of polyspermy are a main limitation to the production of porcine embryos by IVF at commercial scale [40-42]. Overall, the main advantage of IVP over MOET in most species is that it can be repeated in the same animals more

frequently, thereby allowing for more procedures and more offspring born per donor/year. Moreover, oocyte collection followed by IVP can be applied to produce offspring from animals that cannot breed naturally, including prepubertal, early pregnant, early post-partum and injured females.

2.1.2.2 Somatic Cell Nuclear Transfer

The birth of Dolly, the first animal from somatic cell nuclear transfer (SCNT) [43], boosted the use of cloning in farm animals. SCNT can be used to produce genetic copies of animals with superior genetics, as well as to create genetically engineered animals for different purposes such as animal models for the study of diseases, the production of recombinant proteins of biomedical interest, to modify or introduce new desired production traits in food-producing animals (e.g. increased productivity, product quality, disease resistance, prolificacy, etc.), and to maintain genetic diversity in conservation programs [44-49].

Despite of all the advantages and the improvements made on the SCNT technique throughout the years, this technique still presents unsatisfactory results. Several factors are responsible for the low success rates of SCNT such as the substantial manipulation of oocytes, the stage of cellular differentiation [50] and coordination of the cell cycle between the donor cell and the oocyte [51-53]. Following SCNT, the epigenetic patterns of the somatic cell must be remodelled to become similar to an embryo, allowing the cell to acquire pluripotency to form all the cells necessary for the development of a normal fetus. However, the mechanisms behind this epigenetic remodelling are not yet fully understood. It has been established that pluripotency and cellular reprogramming in SCNT are regulated by epigenetic modifications like histone modifications and DNA demethylation [54-58]. In SCNT, following the transfer of the somatic cell into the enucleated metaphase II oocyte, the oocyte requires a stimulus to activate, mimicking the events

that happen following fertilization in order to start embryo development. Different activation protocols may affect the efficiency of SCNT regarding fusion, embryo development and pregnancy rates [59, 60].

Despite being legal in several countries, the use of cloned animals in the food chain is still banned in many countries, including Canada [61] and is strictly regulated, requiring government authorization in the European Union [62], limiting its use. Despite of that, SCNT has a big potential for growing and widespread utilization, however, the mechanisms behind it low success need to be further studied.

2.1.2.3 Intracytoplasmic Sperm Injection

An alternative for conventional IVF is the intracytoplasmic sperm injection (ICSI). In this technique, instead of incubating a large amount of spermatozoa with the oocytes (usually from 2 to $4x10^6$ spermatozoa/ml), one single spermatozoa is injected inside the oocyte, reducing drastically the number of sperm cells needed for fertilization. ICSI allows the production of more offspring from animals with high genetic value, maximizes the use of sexed semen, allows the production of embryos from prepubertal animals that present IVF issues, and is a tool for producing transgenic animals. It is also a valuable technique for research, since it allows the study of the early events of fertilization. After the first human babies were born with the assistance of this technique [63], ICSI became widely used and is now well stablished in human fertility clinics. ICSI is also well established in mice, and usually satisfactory rates of live born animals are obtained [64]. IVP in horses is done by ICSI not IVF, since the co-incubation of the female and male gametes is not enough for effective fertilization, and it is already performed at a commercial level [65-69].

In bovine, there is a huge discrepancy in the results of embryos produced by this technique, and the overall success rates remain very low. The two main concerns in bovine ICSI are oocyte

activation and sperm decondensation [70]. The majority of bovine oocytes are unable to produce adequate calcium (Ca^{2+}) oscillations and activate following ICSI, thus, requiring assisted activation protocols [71-79]. Sperm decondensation has been shown to be the main cause of failure in bovine ICSI, and it has been associated with the source of oocytes used for the procedure [80]. Sperm protamine content may also be associated with failure in sperm decondensation in bovine, since they only have type I protamines, which also occurs in the pig, another species in which decondensation is a problem, while mouse and primates have both type I and II protamines [81, 82]. Additionally, the plasma membrane of bovine spermatozoa was shown to be more stable than human and mice [83]. In order to overcome the lack of sperm decondensation following ICSI, several sperm pre-treatments have been tested including DTT [84-86] and glutathione [78, 87] in order to reduce the disulfide bonds in the spermatozoa. Substances that act decreasing integrity and destabilizing spermatozoa acrosome and membrane, like lysolecithin and Triton X-100 have also been tested [88-90].

In pigs, ICSI could play an important role in IVEP, since conventional IVF results in high rates of polyspermy [40-42]. Although there are still some contradictions regarding the need for assisted activation, a lack of Ca^{2+} oscillations have been demonstrated following the procedure [91]. Moreover, some papers reported improved embryo production rates when oocytes were submitted to activation following ICSI [81, 92-95]. Although not as significant as in the bovine, male pronuclear formation after ICSI in pigs remains a problem accounting for the low embryo production rates [81]. Contradictions still exist regarding if sperm pre-treatment is beneficial in this species. It has been shown that some sperm pre-treatments decrease the ability of the sperm to induce oocyte activation by decreasing the amounts of PLCz in the sperm [96]. On the contrary, some studies showed improved embryo production rates when sperm was pre-treated with disulfide bond reducing agents [93, 97, 98] or membrane destabilizing agents [93].

Minimal deviations in protocols used by different laboratories may be behind these differences, for example: oocyte source and quality, individual male variations, media used for sperm preparation and oocyte manipulations and culture, different treatments of spermatozoa before injection and different oocyte activation protocols. There are many specifications and enormous variation in results inside and between species, and a consensus for an effective protocol for ICSI, especially in bovine and porcine has not been established.

2.1.3 ARTs in wildlife conservation

ARTs also play a crucial role for maintaining genetic diversity in conservation strategies for endangered species. The extinction of such species can compromise the biodiversity and have negative effects on the global ecosystem [99]. Compared with farm animals, the use of these technologies in wildlife and endangered species is much more limited, and there are more failures than successful cases. The reasons include the lack of knowledge regarding basic reproductive biology of wild species, limited resources for research and economical relevance [100, 101].

Several ARTs have an important role in captive breeding programs. For instance, AI became a helpful technique that can overcome behaviour incompatibilities, allows natural breeding for animals that became physically disabled and facilitates breeding between two animals in different locations [102]. SCNT allows the propagation of species that cannot reproduce successfully when in captivity and it may even allow the 'resurrection' of extinct species from cryopreserved tissues [101]. Cryopreservation of gametes, embryos, ovarian tissue and somatic cell lines, allows the storage of genetic material from endangered species for several years, buying time to the development of new technologies and gain of knowledge about reproductive biology of wild animals [103, 104].

Some successful advances that achieved live births using ARTs in wild animals include the African Wildcat, produced by SCNT and transferred to a domestic cat [105], a white rhinoceros born by AI [106], offspring produced by SCNT using granulosa cells obtained from ovaries collected post-mortem from female mouflons [48], killer whales born by AI [107] and red deer born by IVEP [108]. Despite of the progress made using ARTs for preserving and rescuing endangered species, the efficiency of these techniques in wild animals are far from being acceptable at a level we can rely on to protect biodiversity [100].

2.1.4 Porcine as models in research

Pigs and humans share several physiological and genomic similarities, making the pig an excellent a model for biomedical research [46, 109-111]. The development of genome-editing techniques like zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN) and clustered regularly interspaced short palindromic repeats/associated nuclease Cas9 (CRISPR/Cas9) have already been applied successfully to several species and presented as a great advantage for the creation of animal models. The production of genome-edited animals has several benefits and applications such as improving livestock and livestock-products, and the production of proteins of pharmaceutical and biomedical interest. Moreover, it plays as very important role in research, for the study of gene function and regulation, in the production of animal models for the study of human diseases and for the generation of human-compatible organs for xenotransplantation [46, 112-114].

The heart of pigs is comparable to human's, regarding size, blood supply and physiology, making the pig a great model for the study of the cardiovascular system [115, 116]. A model of hypercholesterolemia and atherosclerosis has been produced in Yukatan mini-pigs through targeting the low-density lipoprotein receptor using recombinant adeno-associated virus-mediated

gene targeting [117]. The lung disease with highest morbidity and mortality in humans is cystic fibrosis. A model of cystic fibrosis was created in pigs by the disruption of the cystic fibrosis transmembrane conductance regulator gene, allowing scientists to obtain answers for several questions related to the pathogenesis of this disease [118]. A pig model for diabetes mellitus was also created, through the mutation of the human hepatocyte nuclear factor 1alpha gene, which is known to cause type 3 diabetes mellitus [119]. Genetically modified pigs can also serve as organ donors for xenotransplantation, i.e. by the inactivation of the GGTA1 gene, which catalyzes the formation of Galα(1,3)Gal epitopes, that are the main reason for acute rejection of the xenografts [47]. Reproductive biotechnologies such as SCNT and ICSI have played a decisive role in the successful production of these models, since they are used for the delivery of the genome editing tools and production of the genome-edited animals, being of great importance for the study of diseases that can affect animals and humans.

2.2 The beginning of life

2.2.1 Oocyte maturation

Up to the late stages of oogenesis, the oocytes are arrested at the end of prophase I of meiosis. They remain in this stage since before the animal is born until it reaches puberty. During folliculogenesis, the oocytes acquire meiotic competence, i.e. the ability of the oocytes to resume meiosis, which is related to the nuclear maturation. Developmental competence is related to the ability of the oocyte to develop into an embryo, and it depends on the accumulation of several factors [120]. Meiotic resumption can also be stimulated *in vitro* by the removal of the oocyte from the follicular environment [121]. However, in vivo, the preovulatory LH surge is the stimulus for meiotic resumption. The main factor involved in the maintenance of oocytes arrested in the of prophase I stage is the cyclic adenosine monophosphate (cAMP) [122]. Cyclic guanosine

monophosphate (cGMP) that passes from the surrounding cumulus cells through gap junctions also plays a role in maintaining the meiotic arrest through preventing degradation of cAMP by the oocyte's phophosphodiesterase PDE3A [123, 124]. The LH surge triggers a sequence of events that degrade maturation-inhibitory factors, thereby inducing oocyte maturation. The most important event is the stimulation of production of epidermal growth factors-like growth factors, which in turn will decrease the production of cGMP from the oocyte-surrounding cells. The decrease of cGMP influx to the oocyte will be accentuated by the closure of the gap junctions by mitogen-activated protein kinase *(*MAPK), reducing the communication of the oocyte and follicular cells, allowing the oocyte to resume meiosis through the decrease of intracellular cAMP [125, 126] (Figure 1). Decrease of intracellular cAMP will reduce protein kinase A (PKA) activity, dephosphorylating CDC25B, which in turn will remove inhibitory phosphates from CDK1 and thus, activating the MPF [127]. Protein kinase C also have a role acting in CDC25B phosphorylation [128].

Oocyte maturation is regulated through an interaction of the oocyte with the follicular cells and follicular environment [129]. Some of the main factors involved in this event are the MPF and the MAPK. MPF is composed by a regulatory cyclin b1 subunit and a catalytic cyclin-dependent kinase 1 (CDK1) subunit [130]. Immature oocytes are deficient in mRNA that will encode for MPF. However, at the end of the growing phase, there is an increase in the concentration and activity of MPF, being correlated with meiotic competence of the oocyte [131]. MPF levels are low when the oocyte is on germinal vesicle stage, reaches a peak in metaphase I, decreases in the other stages and have another peak on metaphase II, which is maintained for several hours or until it is degraded following fertilization [130, 132]. The MAPK family is also activated during oocyte maturation, most specifically the isoforms ERK1/2. MAPK pathway plays a role in GVBD acting on cumulus cells [133]. During maturation, MAPK is important for microtubule reorganization,

formation of the meiotic spindle, chromosome morphology, polar body formation and maintenance of the oocyte in metaphase II [134-136]. Several factors are involved in oocyte maturation, and all of them are important for an adequate maturation and subsequent embryo development.

The nuclear maturation begins with the breakdown of the germinal vesicle (GVBD) and condensation of the chromatin, followed by metaphase I, anaphase I and telophase I, which is the final phase of maturation when the segregation of the homolog chromosomes occurs by the extrusion of the first polar body. The oocyte is then maintained in the second meiotic arrest until the moment of fertilization [2].

Cytoplasmic maturation can be divided in modification and redistribution of cytoplasmic organelles, dynamic of the cytoskeleton and molecular maturation [137]. During cytoplasmic maturation there are changes in the number, size and localization of the organelles, in order to provide the machinery required for each phase of the oocyte maturation. There is an increase in lipid content, a decrease in the size of the Golgi apparatus, nucleoli compaction, alignment of the cortical granules close to the membrane and mitochondrial redistribution[138, 139]. The cytoskeleton, through its filaments and microtubules, is responsible for the spatial rearrangement of the organelles during the different phases of maturation [137]. The molecular regulation of maturation refers to cessation of transcription, degradation of mRNAs no longer needed (e.g. those involved in oocyte growth), and processing of mRNA transcribed and stored during oogenesis, important for upcoming stages including maturation, fertilization and embryo development up to the stage when the embryo genome is activated (EGA) [120, 140-142].

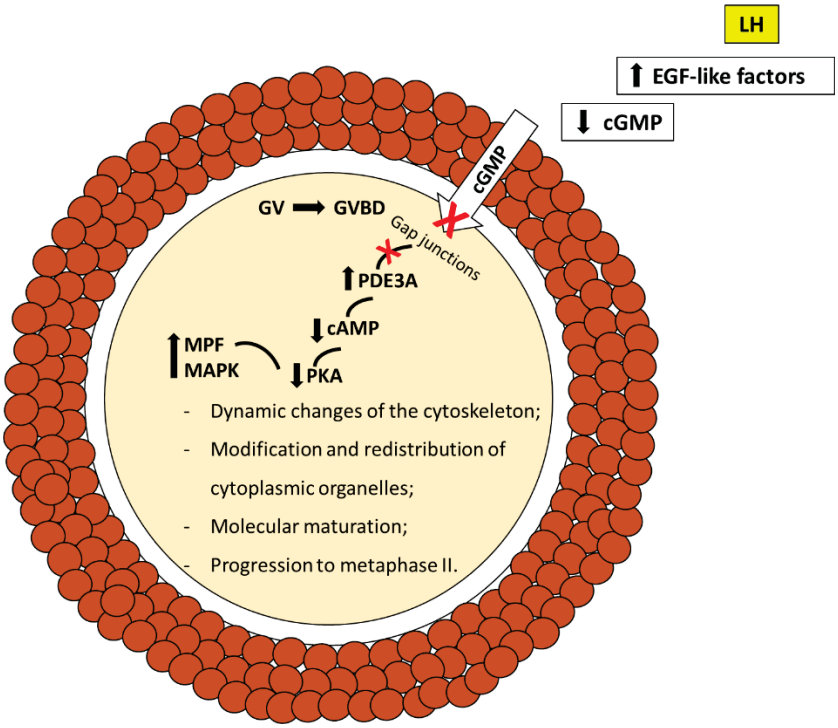

Figure 1. Some of the main mechanisms involved in resumption of meiosis and oocyte maturation.

2.2.2 Fertilization

2.2.2.1 Sperm capacitation

Following ejaculation, in order to be able to fertilize the egg, the sperm has to go through some changes. These changes include the events of sperm capacitation and acrosome reaction. Sperm capacitation happens naturally in the female reproductive tract and can be defined as a series of biochemical changes that ultimately will allow the sperm to bind to the zona pellucida (ZP) and undergo acrosome exocytosis. It includes the loss of proteins from seminal plasma, but most importantly, the removal of cholesterol from the sperm membrane, which is stimulated by serum albumin. This will cause increased membrane fluidity, unmasking of surface proteins necessary for oocyte interactions, and facilitation of transmembrane movement of signalling molecules [143-145]. The membrane fluidity causes an influx of Ca^{2+}, through sperm specific Ca^{2+} channels

(CatSper), and HCO_3^- ions, through Na^+/HCO_3^- co-transporter (NBC). There is decrease in intracellular Na^+, activation of K^+ channels and increase in the pH. Decrease in Na^+ and activation of K^+ channels will lead to plasma membrane potential hyperpolarization [146-149].There is an increase in cAMP concentration, through activation of soluble adenylyl cyclase (sACY), activation of protein kinase A, activation of protein kinase A and proteins tyrosine phosphorylation. These events will activate several pathways in the sperm that will trigger all the events associated with capacitation such as the hyperactivated motility pattern, ZP binding and preparation for the acrosome exocytosis [144, 146, 150] (Figure 2).

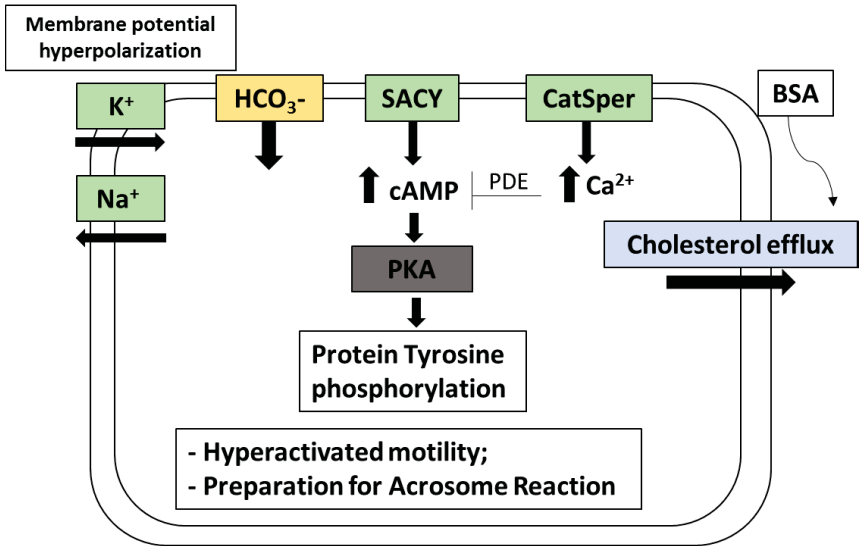

Figure 2. Events involved in sperm capacitation. Adapted from [151].

2.2.2.2 Sperm-oocyte binding

Once the sperm is capacitated, it is attracted to the oocyte by chemoattractant substances from the follicular fluid [152, 153]. The sperm will then bind to the glycoproteins at the ZP of the oocyte. The ZP of porcine and bovine oocytes is composed of three types of glycoproteins, ZP2, ZP3 and ZP4, and it is a complex formed by ZP3 and ZP4 that is responsible for sperm binding [154-156]. However, this mechanism is not conserved and may vary among species, regarding the

type and number of the glycoproteins present and which of them presents the sperm- binding activity.

2.2.2.3 Acrosome reaction and oocyte plasma membrane fusion

While previous findings established that the binding of the sperm to the ZP-glycoproteins induced the acrosome reaction [157], more recently reports indicate this is not an exclusive mechanism. For example, recent research indicates that mammalian sperm undergoes acrosome reaction before interaction with the ZP [158, 159]. It has also been speculated that acrosome reaction is induced by factors present in the oviduct or cumulus cells [160]. More recently, studies in mice showed that the acrosome reaction takes place in the upper isthmus, showing that it is not stimulated by or close to the ZP [161]. Currently, there is consensus that the sperm must be capacitated to be able to undergo acrosome reaction, and that binding to the ZP is not sufficient to induce it, however where this events takes place is still undetermined [162]. Acrosome reaction has several effects in the sperm such as: activation of G proteins, membrane depolarization, activation of Ca^{2+} channels and increase in intracellular Ca^{2+} and pH [163].

The following phase comprises the fusion of the sperm plasma membrane (PM) and sperm outer acrosomal membrane (OAM), which will cause a release of the acrosomal content. Degradation of the ZP is induced mainly by hydrolytic activity of the enzymes released from the acrosome into the ZP, specially acrosin. In addition, sperm motility will allow the sperm to penetrate the ZP [164]. Recent studies indicate that sperm metalloproteases and proteasome are also involved in this step [165]. Changes in the acrosome will alter the surface of the sperm and will expose the inner acrosomal membrane, preparing the cell for binding and fusion [166]. After the sperm has penetrated the ZP, it binds to the oocyte plasma membrane. The interaction of the

sperm immunoglobulin Izumo1 with its egg receptor Juno was shown to be essential for the sperm-oocyte membrane fusion [167, 168].

2.2.3 Oocyte activation

Once fused with the oocyte, the sperm will introduce a sperm-born oocyte-activating factor (SOAF) that will trigger the activation of the oocyte. Currently, there is a debate regarding which SOAF is in fact responsible for inducing activation [169]. While the most studied and accepted hypothesis indicates that the testis-specific phospholipase C isozyme or PLCz is the main SOAF [170], there are studies suggesting that the post-acrosomal sheath WW domain-binding protein or PAWP is a required factor for activation [171].

The release of PLCz, or the activation of an ooplasmic PLC by PAWP, will cause the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) generating the second messengers inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG) [3, 4, 172, 173]. IP3 will then bind to its receptor, located mostly in the cortical area close to cytoplasmic membrane and in endoplasmic reticulum (ER) causing the opening of Ca^{2+} channels and releasing Ca^{2+} to the cytoplasm. This process is termed IP3-induced Ca^{2+} release [5, 174]. Ca^{2+} oscillations are caused by a feedback system, in which IP3 receptors are also regulated by Ca^{2+} levels. Low Ca^{2+} levels cause a positive regulation for further Ca^{2+} release, and when the Ca^{2+} levels are high there is a negative regulation for decreasing Ca^{2+} release, causing the oscillations [173, 175]. Ca^{2+} oscillations following fertilization are conserved among different species [176]. DAG, another second messenger originated from hydrolysis of PIP2, together with Ca^{2+}, will activate conventional protein kinase C (cPKC) translocating it for the plasma membrane and activating it. PKC has a role controlling long lasting Ca^{2+} oscillations by regulating Ca^{2+} influx from the extracellular space [177, 178].

Oocyte meiotic arrest is mainly maintained by the cytostatic factor (CSF), which is composed by mediators, such as early mitotic inhibitor 2 (EMI2), MAPK, Mos protein kinase, and p90 protein kinase [179-181]. CSF maintains metaphase II arrest through EMI2 inhibition of anaphase promotor factor (APC/C). The release of Ca^{2+} in the cytoplasm activates Ca^{2+}/calmodulin-dependent kinase II (CaMKII). CAMKII will act phosphorylating the EMI2, which is subsequently targeted for degradation. After EMI2 degradation, the APC/C becomes active and will target cyclin B (the regulatory component of MPF) for degradation , thereby reducing MPF activity[182].

While the role of Ca^{2+} in oocyte activation and meiotic progression is well known and stablished, the importance of Zn^{2+} has gained attention in the past few years. Zn^{2+} has been shown to regulate intracellular signaling events in a Ca^{2+} influx dependent way [183]. It was later proposed that Zn^{2+} concentration also has an important role in regulating oocyte activation [8]. This happens by the activation of EMI2 in the meiotic arrest and inactivation of EMI2 during oocyte activation, being Zn^{2+} a component of the CSF itself [184]. Contrary to Ca^{2+}, Zn^{2+} levels decrease intracellularly in response to fertilization, in exocytosis events termed Zn^{2+} sparks. Mouse oocytes showed 1 to 5 Zn^{2+} events, preceded by the Ca^{2+} waves, during the first 90 minutes from fertilization [8]. The Zn^{2+} sparks are originated from thousands of vesicles containing around 10^6 Zn^{2+} atoms each, and the oocyte total Zn^{2+} levels drop by 20% during the first 6 hours post-activation [185]. Zn^{2+} intracellular concentration acts like a switch to EMI2, which has a zinc-binding region, activating APC/C and decreasing MPF activity [184] (Figure 3).

The pathways regulated by Ca^{2+} and Zn^{2+} will lead to the events of oocyte activation, necessary for subsequent embryo development, that can be divided in early and late events of activation. One of the first changes that happen after fertilization is the exocytosis of the cortical granules and release of their content into the perivitelline space, modifying the ZP and its

glycoproteins and resulting in inhibition of penetration by additional sperm, thereby blocking polyspermy [186]. The oocyte will resume meiosis and eliminate half of the sister chromatids into the second polar body. The female and male pronuclei will be formed and DNA synthesis will start. Because the sperm chromatin is highly compacted, it needs to go through extensive changes that include replacement of the protamines by maternal histones and decondensation, forming the male pronucleus [187]. Both pronuclei will migrate towards the center of the oocyte, their DNA will be duplicated, their membranes will breakdown and their genomes will undergo syngamy [1, 2].

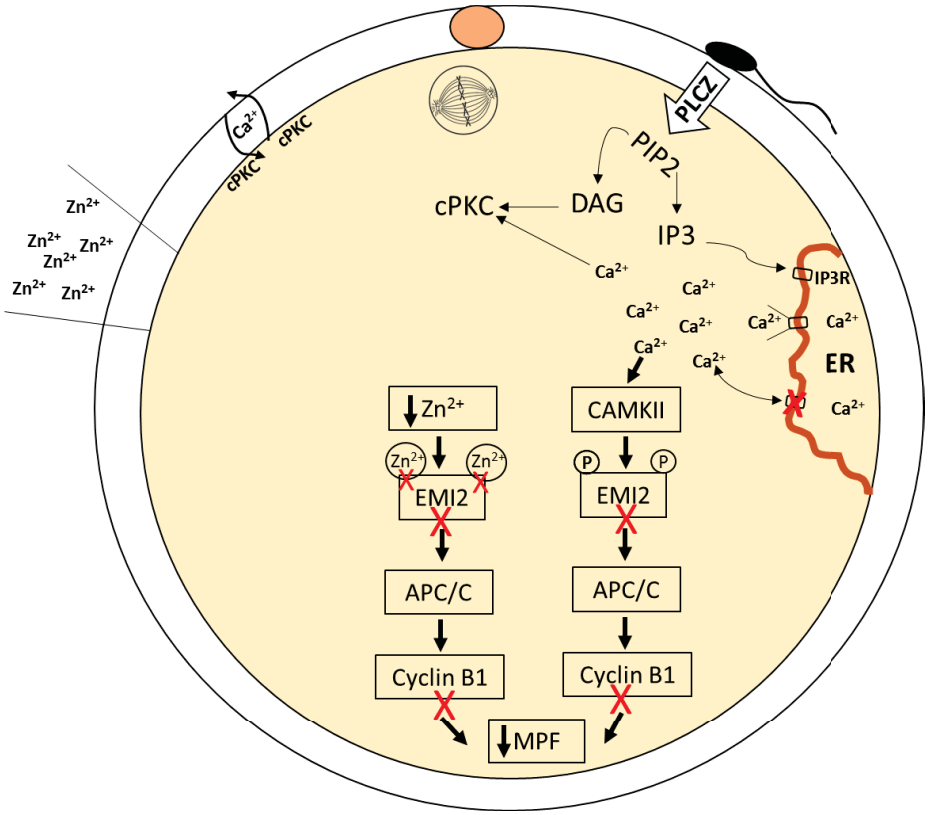

Figure 3. Molecular events triggered by fertilization that will lead to oocyte activation.

2.2.4 Early embryo development

2.2.4.1 Epigenetic reprograming

After syngamy, the embryo needs to acquire a state of totipotency. The epigenetic patterns inherited from the parents are destabilized in waves of DNA demethylation and remethylation in two main stages of development. First, when gametes give rise to the zygote, and second, during the formation of primordial germ cells (PGM). Epigenetic reprograming during these stages reduces global DNA methylation in developing embryos to a ground state, with the exception of imprinted genes. Cells of early stage embryos are totipotent, i.e., they can differentiate and become any cell type in the developing embryo or placental tissue. Throughout development, embryonic cells become differentiated presenting a specific gene expression pattern. Cellular differentiation during development is due to specific changes in gene expression, which are mainly regulated by epigenetic events and its modulations. Therefore, during all the stages of development, the embryo, fetus, and neonate are under constant effects of environment, which can lead to epigenetic modifications, and consequently alterations in gene regulation [188, 189].

2.2.4.2 Maternal to embryo transition

Another important event that happens during early embryo development is the transition of the gene expression control from the mother to the embryo. In order for this to occur, maternal mRNAs must be degraded and the zygotic transcription must begin. In mice, there is evidence that the maternal transcripts are degraded by the phosphorylation of MYS2, an RNA binding protein, making the maternal mRNA unstable [190]. EGA occurs from zygote to two-cells on mice, eight to sixteen cells in bovine and four to eight cells in porcine species [191-193]. Epigenetics plays a key role in controlling EGA. Changes in the chromatin state through DNA methylation and histone modifications are extremely important in controlling the expression of genes that need to be expressed or repressed [188, 194].

2.2.4.3 Blastocyst formation

Following completion of meiosis, the progression of the cell cycle will make the embryo go through several mitotic divisions, named cleavage, until it reaches the blastocyst stage. The cells originated from these divisions are named blastomeres. After several cleavages, the embryo will go through compaction, a process in which there is an increased contact and adhesion between the blastomeres, until it becomes a cell mass named morula [195]. The cadherin superfamily is important for cell adhesion and morula compaction, and the E-Cadherin or CDH1, which is dependent of extracellular Ca^{2+}, seems to be the most important member [196, 197]. Formation of the fluid-filled cavity of the blastocyst, or blastocoel, happens through the action of Na/K-ATPase in the trophoblast [195]. Some of the blastomeres will become positioned in one of the poles of the embryo, forming the inner cell mass, or embryoblast, while the trophoblast cells will be in an outer layer in the entire embryo perimeter. The accumulation of fluid in the blastocoel and the multiplication of cells will cause the embryo to expand, making the ZP thinner and eventually the blastocyst will hatch from it [198]. During hatching, the embryo will already be in the uterine lumen and the trophoblast will be responsible for implantation of the embryo in the uterine wall [199].

2.3 Assisted oocyte activation (AOA)

While activation is a spontaneous event that happens following fertilization, when techniques such as ICSI or SCNT are performed, the oocyte needs an exogenous stimulus in order to activate. AOA is also necessary for the production of PA embryos, which are widely used as a research model for the study of early embryo development. In SCNT, following the transfer of the somatic cell into the enucleated metaphase II oocyte, the oocyte requires a stimulus to activate and start embryo development.

Since in ICSI the spermatozoa is directly injected in the cytoplasm, skipping many physiological oocyte–spermatozoa interaction steps, there is possibly a lack of proper stimulus for oocyte activation through the procedure. To assist in this problem, in some species, the use of exogenous oocyte activation following the procedure is necessary. In equine, exogenous oocyte activation did not increase embryo production [200] Equally, in mouse there is no need for exogenous activation [64]. In humans, in general, there is no need for exogenous oocyte activation; however, it can be necessary in cases when there is fertilization failure [201]. Bovine presents low pronuclear formation rates following ICSI possibly due to the fact that bovine oocytes are less sensitive to the injection, and require an additional activation following the procedure [71-73]. Pig oocytes also fail to activate spontaneously following ICSI, requiring assisted activation [91, 94]. With the aim of increasing the efficacy of ICSI, SCNT and PA embryo production, many protocols for assisted oocyte activation have been tested, including physiological, electrical, chemical and combined protocols.

2.3.1 Electrical stimulus

Electrical stimulus involves the application of high-voltage pulses that will cause the formation of pores in the oocyte membrane, allowing the influx of Ca^{2+} from the extracellular medium. This method has successfully activated porcine oocytes following SCNT [59] and following ICSI, while maintaining the expected chromosomal configuration [94]. However, there is a limit for the stimulation, and depending on the voltage used may have negative effects on embryo development [202, 203]. Electrical pulses has been related with the generation of reactive oxygen species, depending on the intensity and duration of the stimulus and the amount of Ca^{2+} in the electroporation medium. However, this did not affect embryo development rates [204]. PA

embryos also showed high apoptotic rates when activated with electrical stimulus, hampering embryo development [205].

2.3.2 Modulators of Calcium

Despite oocyte activation using electrical activation has been successfully applied for producing embryos in several species, the most used method remains the use of chemical agents. Modulators of Ca^{2+} are widely used in order to try mimicking the events that happen physiologically. However, they often do not mimic correctly the Ca^{2+} oscillations. Ca^{2+} ionophores, such as ionomycin, increase intracellular Ca^{2+} by releasing it from internal stores [206], but, in general induce a single Ca^{2+} wave [9]. Ethanol also causes one single Ca^{2+} increase through both release from intracellular stores and extracellular influx [207, 208]. Because these agents are not capable of inducing multiple Ca^{2+} oscillations, they are often combined with other treatments in order to improve activation rate in bovine and porcine oocytes [209-211]

Strontium chloride ($SrCl_2$) is frequently used in mice to activate oocytes and as it induces several Ca^{2+} oscillations by acting on IP3 receptors [212]. However, embryo development rates in bovine are low when this protocol is used [213, 214]. For porcine oocytes, $SrCl_2$ needs to be combined with other agents in order to obtain adequate activation rates [215, 216]. Thimerosal, a sulfhydryl reagent, also induces multiple Ca^{2+} oscillations. However, its use has been associated with negative effects on the meiotic spindle [217, 218]. Early embryo development using thimerosal for activation has only been successful described in pigs [217].

2.3.3 Physiological activation

Another method of oocyte activation, here described as physiological activation, since it involves the use of two candidates for the SOAF, is the delivery of recombinant PLCz or PAWP.

The injection of recombinant PLCz was effective to cause Ca^{2+} oscillations and activate bovine [219, 220], porcine [221] and mouse oocytes [170]. The effective use of PAWP as an oocyte-activation inducer has been demonstrated in porcine, bovine, macaque and *Xenopus* oocytes [171, 222]. Mouse and human oocytes injected with PAWP also presented Ca^{2+} oscillations and were activated [223]. While it remains to be determined weather PLCz or PAWP is the real SOAF, their use for oocyte activation is very promising since it recreates the molecular events stimulated by the sperm.

2.3.3 Protein phosphorylation or synthesis inhibitors

In order of increasing activation efficiency, agents that manipulate Ca^{2+} levels are frequently used in combination with inhibitors of protein phosphorylation or synthesis. In general, protein phosphorylation or synthesis inhibitors aim to prevent MPF activity. 6-(Dimethylamino)purine (6-DMAP) is a non-specific protein kinase inhibitor that inactivates CDK1 through blocking activation of cdc25 [224, 225]. This protocol has been widely used for the production of parthenogenetic [209, 226] and SCNT embryos [227-229]. 6-DMAP also inhibits extrusion of the second polar body, possibly by inhibiting contractile activity in the oocyte cortex [230], and by inhibiting the spindle assembly, which results in one diploid pronucleus [136]. Inhibition of the second polar body extrusion makes this protocol unsuitable for ICSI, since it would induce the development of polyploid embryos. Cycloheximide is a protein synthesis inhibitor [231] that blocks the translocation step during elongation [232]. Although cycloheximide does not have such a detrimental effect as high rates of polyploidy [10], bovine PA embryos activated with cycloheximide showed a delay in cleavage, probably due to a temporary inhibition of proteins important for cleavage [233]. Despite protein inhibitors are widely used and effective

as activation agents, its use has several limitations such as interference at phosphorylation and synthesis of molecules critical to embryo development or chromosomal abnormalities [11, 234].

2.3.4 Zinc chelator

As described above, it has been shown that oocyte intracellular Zn^{2+} levels decrease, in exocytosis events, in response to fertilization [7, 8, 235]. Therefore, activation protocols based on the use of a Zn^{2+} chelator, N,N,N′,N′-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), have been tested in pigs [236, 237], humans [238] and mice [7, 184]. In pigs, electroporation in a medium with low Ca^{2+} followed by exposure to 200μM of TPEN for 30 minutes showed to be efficient to produce PA embryos. In addition, treating activated oocytes with a low concentration of TPEN (5μM) increased the rate of blastocysts produced by SCNT, leading to the birth of live animals [237]. In mice, decreasing intracellular Zn^{2+} levels was shown to be not only necessary but also sufficient for inducing activation and full-term development [7, 184]. However, long exposure times to TPEN decreased embryo development due to toxic effects, indicating that basal Zn^{2+} levels in the oocyte are still necessary [237]. The exploration of this physiological route has great potential to optimize current oocyte activation protocols, since it does not involve undesired effects such as protein inhibitor protocols.

2.3.5 Specific cyclin-dependent kinases inhibitors or protein kinases activators

Specific cyclin-dependent kinases inhibitors or protein kinases activators can be used for oocyte activation to avoid unspecific effects induced with general protein synthesis inhibitors. Bohemine, a cyclin-dependent kinases inhibitor successfully activated bovine PA embryos, however it also inhibits CDK2, which plays a role in cell cycle progression [239]. Studies in porcine observed that the cyclin kinase inhibitors AZD5438 and butyrolactone I are effective to produce

PA and SCNT embryos. However, the use of these inhibitors may prevent second polar body extrusion [60, 240], which is a problem in embryos generated by ICSI. Another study showed that, phorbol 12-myristate 13-acetate (PMA), a PKC activator, is helpful in promoting pronuclear formation following ICSI [241]. However, long exposure to PMA may also prevent second polar body extrusion [242]. Finally, a DAG analogue, 1-Oleolyl-2-acetyl-sn-glycerol, was shown to effectively induce cortical granules exocytosis, but not oocyte activation [243].

CHAPTER 3: RATIONALE, HYPOTHESIS AND OBJECTIVES

Current protocols used for assisted oocyte activation are based on the manipulation of Ca^{2+} levels and inhibition of protein synthesis/phosphorilation [9]. These approaches do not mimic the physiological patterns necessary for activation and may have adverse effects for embryo development [11, 234]. The establishment of a protocol for oocyte activation that is simple, effective and closely simulate physiological mechanisms is beneficial for both industry and research, since it is essential for technologies such as PA, SCNT and ISCI.

It has been demonstrated that intracellular Ca^{2+} oscillations and decrease in Zn^{2+} levels are triggered by the fertilising sperm and induce oocyte activation [8, 244-246]. Therefore, our hypothesis is that an activation protocol that mirrors the physiological events of fertilization, involving the manipulation of Ca^{2+} with ionomycin and Zn^{2+} levels with TPEN will be efficient to activate porcine oocytes following PA, SCNT and ISCI.

Therefore, the objectives of this study were to:

1. Evaluate if inducing a single Ca^{2+} transient with ionomycin, and then exposing the oocytes to a CDK1 inhibitor, a PKC activator, or both would effectively activate porcine oocytes.

2. Determine the required time of exposure to the Zn^{2+} chelator TPEN following ionomycin on oocyte activation and embryo development.

3. Evaluate if there was an additive effect TPEN and CDK1 inhibition plus PKC activation on embryo development.

4. Compare the efficiency of a 20 min protocol based on ionomycin and TPEN with a 245 min control protocol based on ionomycin plus cycloheximide and strontium chloride for the production of porcine embryos by PA, SCNT and ICSI.

CHAPTER 4: ARTICLE

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A fast and reliable protocol for activation of porcine oocytes

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Short title: Porcine oocyte activation with ionomycin and TPEN

4.1 Abstract

Oocyte activation is physiologically triggered by the sperm during fertilization, however, production of porcine embryos by somatic cell nuclear transfer (SCNT), intracytoplasmic sperm injection (ICSI) or parthenogenetic activation (PA) requires artificial oocyte activation. Although effective protocols for artificial oocyte activation have been developed, current protocols require long exposures to non-specific inhibitors, which do not mimic the physiological process and may have detrimental consequences for embryo development. In this study, we explored the physiological events induced following fertilization, through the manipulation of Ca^{2+} and Zn^{2+} levels, and protein kinase C (PKC) as well as cyclin dependent kinase 1 (CDK1) activities, with the aim of developing an improved protocol for activation of porcine oocytes. In the first experiment, matured oocytes were exposed to ionomycin (Ion) for 5 min, and then treated with a specific CDK1 inhibitor (RO-3306) and/or PKC activator (OAG) for different time intervals. The highest rate of pronuclear (PN) formation (58.8%) was obtained when oocytes were treated with PKCa+CDK1i for 4 h. Second, PN formation and embryo development were evaluated in oocytes exposed for different times to a Zn^{2+} chelator (TPEN) following Ion treatment. This revealed that 15 min was the minimal exposure time to TPEN required to maximise oocyte activation and embryo development. Next, we observed that treatment with PKCa+CDK1i for 4 h after TPEN for 15 min decreased embryo development compared to TPEN alone. Lastly, we compared the efficiency of the Ion (5 min) plus TPEN (15 min) protocol (IT-20) with a control protocol used in our laboratory (CT-245) for production of PA, SCNT and ICSI embryos. In PA embryos, IT-20 resulted in higher cleavage (72% vs 49.2%) and blastocyst from cleaved embryos (65.5% vs 46.2%) compared to CT-245. In ICSI embryos, higher PN rates were obtained with the IT-20 protocol. Moreover, the two protocols were equally efficient for activation of SCNT embryos.

Based on these findings, we propose that IT-20 is a faster and effective protocol for activation of porcine oocytes.

4.2 Introduction

Assisted reproductive technologies (ARTs) have been extensively applied in humans for treatment of infertility, as well as in many animal species for a variety of reasons including accelerated genetic improvement, conservation and generation of animal models for research. Therefore, research efforts to develop ARTs protocols that are more efficient, simple and representative of natural physiological processes have been a continued endeavor. Oocyte activation is an essential step for the application of certain ARTs including somatic cell nuclear transfer (SCNT) and intracytoplasmic sperm injected (ICSI), as well as research programs involving embryo production by parthenogenetic activation (PA). However, the efficient application of these technologies in some animal species remains a challenge due to low success rates and contradictory results, which are part due to the lack of optimized protocols for oocyte activation. This is especially true for porcine, since, the development of an effective protocol for ICSI would be an important alternative for *in vitro* embryo production, because conventional *in vitro* fertilization (IVF) often results in high rates of polyspermy [40, 41].

Oocyte activation comprises essential events required for normal embryo development, such as cortical granule exocytosis, meiotic resumption, extrusion of the second polar body, cell cycle progression and pronuclear formation [1, 2]. Physiologically, oocyte activation is induced by the sperm-egg interaction, which results in transient oscillations in intracellular Ca^{2+} levels that culminate with the degradation of cyclin B1 [5, 173-175]. This in turn, reduces maturation promoting factor (MPF), i.e. cyclin B1-CDK1 complex activity, which leads to oocyte activation

and meiotic progression [130]. Following sperm-egg binding, protein kinase C (PKC) is activated and participates in the control of Ca^{2+} oscillations and other events of activation [177, 178, 247]. While the role of Ca^{2+} in oocyte activation and meiotic progression has been well established, the importance of Zn^{2+} has gained attention more recently. It has been shown that Zn^{2+} regulates intracellular signaling events in a Ca^{2+} influx-dependent way [183]. Intracellular Zn^{2+} levels decrease during exocytosis events, which are preceded by the Ca^{2+} oscillations, and also act by decreasing MPF activity [8, 184]. Moreover, it was shown that exposure of oocytes to the Zn^{2+} chelator N,N,N',N'-Tetrakis(2-pyridylmethyl) ethylenediamine (TPEN) induced activation [7, 184, 236, 237].

Commonly used protocols for oocyte activation are based on the exposure to agents that promote increase in cytoplasmic Ca^{2+} levels, such as Ca^{2+} ionophore, ethanol, thimerosal, strontium chloride ($SrCl_2$) and electric stimulation. However, most of these agents induce a single increase in Ca^{2+} levels, which do not mimic the repetitive physiological oscillations induced by fertilization [9, 214]. Two exceptions are thimerosal and $SrCl_2$, which can induce multiple Ca^{2+} oscillations. However, thimerosal is not often used for oocyte activation because it has negative effects on the meiotic spindle [217, 218]. On the other hand, $SrCl_2$ is very effective for activation of mouse oocytes [248], but it needs to be associated with other agents in order to successfully activate porcine oocytes [215, 216]. To increase activation efficiency, after exposure to Ca^{2+} inducing agents, oocytes are often treated with inhibitors of protein synthesis (e.g. cycloheximide - CHX) or kinase activity (e.g. 6-dimethylaminopurine - 6-DMAP) to prevent reactivation of MPF [224]. However, despite improving activation, these inhibitors are not specific and may have detrimental consequences on embryos such as inducing chromosomal abnormalities [10, 11, 234]. Specific

cyclin kinases inhibitors, such as bohemine, roscovitine and RO-3306, are available and have been used to promote activation and/or prevent meiotic resumption in oocytes [239, 249-251].

This study aimed at developing an effective protocol for activation of porcine oocytes based on the manipulation of Ca^{2+}, Zn^{2+}, PKC and MPF activity, which are key regulators of the oocyte activation cascade. The specific objectives were to: i) evaluate if CDK1 inhibition and/or PKC activation after inducing a Ca^{2+} wave would effectively activate porcine oocytes; ii) determine the effect of exposure time to the Zn^{2+} chelator TPEN on oocyte activation and embryo development; iii) test if TPEN combined with PKC activation and CDK1 inhibition can improve oocyte activation; and iv) compare the effect of oocyte activation with TPEN vs. CHX+$SrCl_2$ on activation, development and quality of porcine embryos produced by PA, SCNT and ICSI.

4.3 Material and methods

4.3.1 Chemicals

Unless otherwise indicated, chemicals and reagents were purchased from Sigma Chemical Company (Sigma-Aldrich; Oakville, ON, Canada).

4.3.2 Oocytes collection and *in vitro* maturation

Ovaries from prepubertal gilts were collected from a local abattoir (Olymel S.E.C/L.P.) and transported to the laboratory in saline solution (0.9% NaCl), supplemented with 100 IU/mL penicillin and 10 mg/mL streptomycin, at 30-35°C. Cumulus-oocyte complex (COCs) were aspirated from follicles with diameter ranging from 3 to 6 mm using a 21 G needle. Only COCs with at least 3 layers of cumulus cells and homogeneous granulated cytoplasm were selected for *in vitro* maturation (IVM). Groups of 30 COCs were matured in 90 µL drops of maturation medium covered with mineral oil, in an incubator with an atmosphere of 5% CO_2 in air, at 38.5°C.

Maturation medium consisted of TCM199 (Life technologies, Burlington, ON, Canada), supplemented with 0.5 μg/mL luteinizing hormone (LH; SIOUX Biochemical Inc, Sioux Center, IA, United States), 0.5 μg/mL follicle stimulating hormone (FSH; Sioux Biochemical Inc.), 1 mM cyclic adenosine monophosphate (cAMP), 100 ng/mL of epidermal growth factor (EGF; Life technologies), 100 µg/mL cysteine, 0.91 mM sodium pyruvate, 3.05 mM D-glucose, 20 μg/mL gentamicin (Life technologies) and 20% porcine follicular fluid. After 22 h, the COCs were washed and transferred to a new drop of IVM media, which was not supplemented with cAMP, LH and FSH, and cultured for an additional 22-24 h. After approximately 44 h of IVM, cumulus cells were removed by vortexing in TCM199 HEPES-buffered medium supplemented with 0.1% hyaluronidase, and selected oocytes were used in the different experiments as described below.

4.3.3 Embryo culture

Embryos in all experiments were cultured in 60 µl drops of PZM-3 medium supplemented with 3 mg/mL bovine serum albumin (BSA) at 38.5°C in an atmosphere of 5% CO_2 in air. Culture medium was supplemented with 10% fetal bovine serum (FBS) on day 5. Embryo cleavage and blastocyst rates were determined at 48 h and 7 days after activation, respectively. Embryos that developed to the blastocyst stage were fixed and stained for cell counting.

4.3.4 Somatic Cell Nuclear Transfer (SCNT)

Matured oocytes were incubated in TCM199 medium supplemented with 0.4 µg/mL demecolcine and 0.05 M sucrose for 1 h. This treatment resulted in a small protrusion in the ooplasmic membrane that contained the metaphase chromosomes. The oocytes were then enucleated in TCM199 HEPES-buffered medium supplemented with 2 mg/mL BSA (fatty acid free), 20 µg/mL gentamicin, and 7.5 µg/mL cytochalasin B (CB) by removing the protruded oocyte chromatin with the first polar body. A nuclear donor cell was transferred into the perivitelline space of each enucleated oocyte. A single DC pulse of 35 V for 50 μsec was applied in a 0.28 M mannitol

solution, supplemented with 50 μM $CaCl_2$, 100 μM $MgSO_4$, and 0.1% BSA, to induce oocyte/cell fusion. Oocytes were then transferred to TCM-199 medium supplemented with 3 mg/mL BSA for 1 h to allow cell fusion.

4.3.5 Intracytoplasmic Sperm Injection

Fresh semen from fertile boars (supplied by CIPQ Inc.; Roxton Falls, QC, Canada) was washed through centrifugation in TCM199 HEPES-buffered medium supplemented with 2 mg/mL BSA (fatty acid free) at 2000 rpm for 2 min. A small fraction of spermatozoa was then transferred to a 5 µl drop of 10% polyvinylpyrrolidone (PVP) in HEPES-buffered TCM199. Mature oocytes were manipulated in HEPES-buffered TCM199 supplemented with 2 mg/mL BSA. A random moving spermatozoon was immobilized by compressing the tail with a micropipette and applying a piezo pulse. Using a piezo-micromanipulator (Prime Tech, Tsuchiura, Ibaraki, Japan), the zona pellucida and membrane were perforated, a small volume of ooplasm was aspirated into the micropipette to ensure the membrane was perforated, and then the spermatozoon was injected.

4.3.6 Assessment of DNA synthesis

Detection of DNA synthesis was performed using the Click-iT EdU Imaging Kit (Invitrogen, Life Technologies). One-cell stage embryos produced by PA were incubated with 10 mM 5- ethynyl-2'-deoxyuridine (EdU; DNA synthesis) for 4 h, starting at 6, 8 or 10 h after the beginning of oocyte activation treatment. After EdU exposure, embryos were fixed in 4% paraformaldehyde, stained according to manufacturer's instructions, mounted on microscope slides, and evaluated to determine DNA synthesis in an epifluorescence microscope (Nikon eclipse 80i - Nikon).

4.3.7 Pronuclear evaluation and differential cell counting

For pronuclear evaluation, one cell stage embryos were fixed in 4% paraformaldehyde 15 h after the beginning of oocyte activation treatment, and then permeabilized in PBS containing

0.3% BSA and 0.1% Triton X-100 at 37C° for 30 min. Embryos were later exposed to 10 µg/mL 4',6-diamidino-2-phenylindole (DAPI; Life Technologies) in blocking solution (BSA 3% and Tween 20 0,2%) for 20 min, mounted on slides using Mowiol, and evaluated in an epifluorescence microscope to assess chromatin decondensation and PN formation. For differential cell number analysis, blastocysts at day 7 of development were fixed and permeabilized, as described above, and then incubated overnight with anti-SOX2 primary antibody (Santa Cruz biotechnologies sc-17320) diluted in blocking solution (1:200), and incubated for 1 h at room temperature with anti-goat Alexa Fluor 488 secondary antibody (Invitrogen), diluted in blocking solution (1:500). Nuclei were stained by exposing the embryos to 10 µg/mL DAPI for 20 min. Embryos were then mounted into slides using Mowiol, and total cell number and $Sox2^+$ nuclei were counted in each embryo using an epifluorescence microscope.

4.3.8 Oocyte activation

Four experiments were performed to test different conditions for oocyte activation. In all the experiments, oocytes were first exposed to 15 µM Ion for 5 min and then submitted to different treatments according to the objectives of each experiment. TPEN, CDK1i and PKCa were diluted in PZM-3 media supplemented with 3 mg/mL BSA. $SrCl_2$, CHX and CB were diluted in Ca^{2+} free PZM-3 media supplemented with 3 mg/mL BSA.

Experiment 1

In this experiment, the rate of activation was determined by assessing PN formation in oocytes exposed to the following treatments: a) Ion for 5 min; b) Ion for 5 min followed by 400 µM OAG, a diacylglycerol analogue and PKC activator (PKCa), for 1, 2, 3 or 4 h; c) Ion for 5 min followed by 10 µM RO-3306, a specific inhibitor of CDK1 (CDK1i), for 1, 2, 3 or 4 h; and d) Ion for 5 min followed by PKCa+CDK1i for 1, 2, 3 or 4 h. Oocytes were fixed 15 h after Ion treatment, stained with DAPI and evaluated using an epifluorescence microscope.

Experiment 2

This experiment was conceived to evaluate the effect of exposure time to TPEN on oocyte activation and embryo development. The concentration of TPEN was based on previous studies by Lee et al. [237]. Oocytes were exposed to Ion for 5 min followed by 200 μM TPEN for 5, 10, 15, 20, 25 or 30 min. A proportion of the oocytes were fixed 15 h after Ion treatment to evaluate PN formation. To prevent extrusion of the second polar body, the oocytes that were kept for embryo development evaluation, were cultured in the presence of 7.5 μg/mL CB for 4 h after exposure to TPEN.

Experiment 3

This experiment was designed to evaluate if exposure of oocytes to TPEN for 15 min followed by CDK1i plus PKCa would improve embryo development. The time of exposure to TPEN was elected based on the results obtained in the second experiment. The following treatments were compared: a) Ion for 5 min followed by TPEN for 15 min (IT-20) vs. b) IT-20 followed by PKCa+CDK1i for 4 h. In both treatments, oocytes were cultured for 4 h in the presence of 7.5 μg/mL CB starting after TPEN exposure to prevent extrusion of the second polar body.

Experiment 4

The last experiment was conducted to compare development and quality of PA, SCNT and ICSI embryos activated with the new established protocol (IT-20), as determined based on the best results of the first three experiments, by comparing with a control protocol routinely used in our laboratory for the production of PA and SCNT embryos. The control protocol (CT-245) consisted of 15 μM Ion for 5 min followed by 10 mM $SrCl_2$ and 10 μg/mL CHX for 4 h, i.e. a total duration of 245 minutes [215]. For ICSI-derived embryos, a control group without activation treatment was also included. For SCNT or PA embryos, the oocytes were maintained in 7.5 μg/mL CB for 4 h, starting after Ion exposure, to prevent extrusion of the second polar body. In PA embryos,

beginning of the first S-phase was evaluated by assessing DNA synthesis between 6-10 h, 8-12 h and 10-14 h after Ion exposure (beginning of activation treatment), and PN formation was concomitantly determined. In ICSI embryos, PN formation was determined around 15 h from the sperm injection and only embryos that contained 2 PNs and 2 polar bodies were considered normally activated. For all groups (PA, SCNT and ICSI), cleavage rate was determined at 48 h and blastocyst rate at day 7.

4.3.9 Statistical analysis

All data was analyzed using the JMP software (SAS Institute Inc., Cary, NC). In each experiment, data was tested for normal distribution with Shapiro-Wilk test. Means were compared using Student's t-test for single comparisons or LSMeans Student t-test for multiple comparisons. Results are presented as means ± standard error of the mean (SEM) and $P<0.05$ was considered statistically different. All experiments were performed at least in three individual replicates.

4.4 Results

4.4.1 Oocyte activation with CDK1i and PKCa

In the first experiment, the effect of CDK1 inhibition (CDK1i) and PKC (PKCa) activation for different times was evaluated on activation (PN formation) of porcine oocytes. As showed in Figure 1, exposure of oocytes to CDK1i and PKCa alone or combined increased oocyte activation, but the effect was time-dependent. However, the numeric increase in PN formation induced by PKCa alone was not statistically different from control group exposed to Ion alone. Oocytes exposed for 3 or 4 h to CDK1i, and 2, 3 or 4 to PKCa+CDK1i had higher PN rates than those exposed to Ion alone. The highest numeric rate of PN formation was obtained when oocytes were treated with PKCa+CDK1i for 4 h (58.8%), which was significantly higher than all other exposure

times in the same treatment, but not statistically different than oocytes treated with CDK1i alone, which produced the second highest rate (41.7%) of PN formation.

4.4.2 Oocyte activation with TPEN

In the second experiment, we evaluated the effect of exposure time to TPEN after Ion on oocyte activation and embryo development. As showed in Table 1, PN formation tended to increase with exposure to TPEN from 5 to 15 min (22.3% higher), but remained similar between 15 and 30 min. However, the only statistical difference on PN formation was observed between 5 and 25 min. Cleavage rate also tended to increase with exposure to TPEN from 5 to 15 min (14.3% higher), but was only 6.4% higher when oocytes were treated for 30 min compared with 15 min. The only statistical difference in the cleavage rate was between 5 min and 30 min of exposure to TPEN. There were no statistical differences between all time of exposure to TPEN on blastocyst development and average number of cells per blastocyst. However, blastocyst rates from oocytes exposed to TPEN for shorter times (5, 10 and 15 min) were all above 60%, while those exposed to longer times (20, 25 and 30 min) had blastocysts rates below 60%. Based on the findings in this experiment, we elected 15 min as the exposure time to TPEN for the subsequent experiments. To confirm that the high rates of activation obtained in this experiment were due to a synergist effect of Ion and TPEN, oocytes were either exposed to Ion for 5 min alone or TPEN for 15 min alone and then fixed 15 h later to determine PN formation. The PN rate for TPEN (37% ± 7, n=52) was significantly higher than Ion (8.7% ± 4, n=57), but both were much lower than when oocytes were exposed to the chemicals combined, confirming the synergistic effect.

4.4.3 Treatment with TPEN followed by CDK1i and PKCa did not enhance embryo development

In this experiment, we assessed whether the exposure of oocytes to CDK1i and PKCa for 4 h would further improve embryo development from oocytes treated with Ion for 5 min followed by TPEN for 15 min. The goal was to verify if the best two treatments elected from the first two

experiments could have a synergistic effect on blastocyst development. As showed in Figure 2, cleavage rates were similar, but blastocyst development from cleaved embryos (79.8% vs. 51.8%), and average number of cells per blastocyst (63±7 vs. 40±6) were significantly lower in embryos treated with CDK1i and PKCa for 4 h after TPEN. Based on these results, we established that porcine oocytes are effectively activated by this 20-min treatment, i.e. 5 min Ion plus 15 min TPEN. This short protocol was named IT-20.

4.4.4 Efficiency of the IT-20 protocol for production of PA, SCNT and ICSI embryos.

Our final goal in this study was to compare the efficiency of the developed IT-20 protocol to the CT-245 protocol routinely used in our laboratory. In PA embryos, IT-20 resulted in higher cleavage (72% vs 49.2%) and blastocyst from cleaved embryo (65.5% vs 46.2%) rates compared with CT-245. The average number of total cells and proportion of ICM cells in blastocysts was similar between IT-20 and CT-245 treatments (Figure 3). To evaluate if the activation protocol affected cell cycle progression and time to the first cleavage, DNA synthesis was assessed at three time-points following activation and cleavage at 24 and 48 h after activation. As shown in Figure 4, a similar proportion of oocytes activated with either protocol had a positive signal for DNA synthesis at the three different time-points. The rate of early embryo cleavage at 24 h after activation was also similar, but total cleavage at 48h was higher in oocytes activated with IT-20 compared with CT-245 protocol. The two protocols were equally efficient for activation following SCNT, since no differences were observed in cleavage and blastocyst rates, total cells and proportion of ICM/total cells on day 7 blastocysts (Figure 5). In ICSI-derived embryos, total PN formation was statistically higher in the IT-20 protocol ($P<0.05$), but correct PN formation (2 PN + 2 polar bodies) was only numerically higher (11.5%, $P>0.05$) in the IT-20 protocol (Table 2). Cleavage and blastocyst rates in ICSI-derived embryos were not statistically different between activation protocols, but both protocols resulted in higher cleavage and blastocyst rates compared

with oocytes that were not activated after ICSI. Total cell number and proportion of ICM/total cells in ICSI blastocysts were not affected by the activation protocol (Table 2).

4.5 Discussion

In addition to their importance as main animal protein source in human diet, pigs are an excellent animal model for biomedical and translational research [46]. Thus, effective reproductive technologies are crucial for genetic improvement in swine production, as well as for the creation of unique research models including gene-edited animals, using either fertilized or SCNT embryos. Contrary to other species such as bovine, where production of IVF embryos is a well-established technology, IVF in pigs has many issues, including higher rates of polyspermy [40, 41]. Mechanical fertilization by ICSI could solve the polysemy issue, but an effective protocol for ICSI in swine remains to be developed. Artificial oocyte activation is a key component for the production of SCNT embryos, and seems to be also a necessary step for the production of ICSI embryos in swine. Therefore, our main goal in this study was to develop an improved protocol for porcine oocyte activation, through the modulation of pivotal elements in the molecular signaling cascade for oocyte activation, i.e. Ca^{2+}, PKC, CDK1 and Zn^{2+}.

Commonly used protocols for oocyte activation are based on the use of agents that increase Ca^{2+} levels, such as Ca^{2+} ionophores, ethanol and electric stimulation [9]. However, these agents are unable to mimic physiological Ca^{2+} oscillations to promote sufficient activation, and are normally associated with inhibitors of protein synthesis or phosphorylation to inhibit MPF activity [215-218]. In addition to requiring longer and more complex protocols, the use of non-specific inhibitors, such as 6-DMAP and CHX, may have detrimental effects to embryos, e.g. through interference with phosphorylation and synthesis of molecules critical to embryo development or causing chromosomal abnormalities [10, 11, 234]. Our first goal in this study was to determine if

good activation rates could be obtained by specifically activating PKC and/or inactivating CDK1 after increasing Ca^{2+} levels with Ion. We observed that CDK1i but not PKCa alone significantly improved PN formation. However, the numerically highest rate of PN formation was obtained when oocytes were treated with both CDK1i and PKCa for 4 h, which suggests a synergistic effect. Previous studies observed that exposure to other cyclin kinase inhibitors (e.g., AZD5438, butyrolactone I), after an electric pulse, were effective in producing PA and SCNT porcine embryos [60, 240]. However, those inhibitors prevented second polar body extrusion, which is a problem for production of ICSI embryos. In agreement with our findings, another study conducted using the same PKCa (OAG) observed a benefit in cortical granule exocytosis, but not in PN formation [243]. On the other hand, treatment with another PKCa (phorbol 12-myristate 13-acetate), promoted PN formation following ICSI [241]. However, long exposure to PKCa may also prevent second polar body extrusion [242].

The importance of Zn^{2+} for oocyte activation was proposed more recently [7, 8, 184, 235, 236, 238]. Intracellular Zn^{2+} levels decrease after fertilization in exocytosis events termed zinc sparks [235]. It was showed that Zn^{2+} regulates intracellular signaling events in a Ca^{2+} -influx dependent way [183], and Zn^{2+} levels act as a switch to EMI2, which has a zinc-binding region, resulting in the decrease of MPF activity [8, 184]. Moreover, it was showed that application of an electrical pulse in medium with low Ca^{2+} followed by exposure to 200 μM of TPEN for 30 min was an efficient treatment to produce porcine PA embryos. In addition, treatment with a low concentration of TPEN (5 μM) increased blastocysts rates of porcine SCNT embryos [237]. Based on the results of those previous studies, our second objective in this study was to test if exposure to TPEN (200 μM) after Ca^{2+} induction with Ion would effectively activate porcine oocytes, and to determine if the exposure time to TPEN would affect PN formation and development of PA embryos. We determined 15 min to be the minimal required time in the presence of TPEN to

maximise PN formation, cleavage and development to the blastocyst stage of porcine PA embryos. We next evaluated if exposure to CDK1i and PKCa after treatment with Ion and then TPEN for 15 min would further improve development of PA embryos. We observed that this association, despite of not affecting cleavage, decreased both blastocyst development and quality. This suggests that either one or both CDKi and PKCa, had detrimental consequences on embryo cells at post-cleavage stages, despite of promoting PN formation, as shown in our first experiment. It is thereby possible that the agents used in this study (RO3306 and OAG) may also regulate kinases other than CDK1 and PKC.

Our last objective in this study was to compare the efficiency of the best treatment identified in the first experiments, i.e. Ion 5 min followed by TPEN 15 or IT-20, with the CT-245 protocol for the production of PA, SCNT and ICSI embryos. The main differences between the IT-20 and CT-245 protocols are the activating agents (TPEN vs. $SrCl_2$ + CHX), and the exposure time after Ion (15 min vs. 240 min, respectively). Even though the CT-245 protocol has been effectively used for production of PA and SCNT [194, 252, 253], higher cleavage and blastocyst rates of PA embryos were obtained with the IT-20 protocol. Because this was not due to superior PN formation rate, it is possible to hypothesize that 4 h inhibition of protein synthesis in the CT-245 protocol interferes with first cell cycle progression and subsequent cell cleavages during embryo development [224]. However, we observed that beginning of first S-phase, as assessed by DNA synthesis, and proportion of early-cleaved embryos were similar in oocytes activated with either protocols. In embryos produced by SCNT, similar cleavage and blastocyst rates were obtained with the two protocols. Many factors can account for the discrepancies between PA and SCNT results. This includes the application of an electrical pulse to induce cell fusion, which is known to increase intracellular calcium release and help activation. Another possibility is that inhibition of protein synthesis may have other effects on SCNT embryos, such as favoring chromatin remodeling and/or

suppressing translation of somatic transcripts from the donor cell that may be unnecessary or perturb early embryo development. Lastly, activation of ICSI embryos revealed that IT-20 increased total PN formation compared with the CT-245 protocol. Moreover, the rate of normal pronuclear formation after ICSI was more than 10% higher in the IT-20 compared to the CT-245 protocol. Despite of not be statistically different, this numerical increase suggests that TPEN treatment may enhance male PN formation in porcine ICSI embryos, which is one of the main issues for ICSI success in pigs [81, 97]. The importance of Zn^{2+} depletion following fertilization is supported by previous reports describing a positive correlation between the magnitude of Zn^{2+} sparks and embryo development and quality [254], and the effects of Zn^{2+} sparks on zona pellucida hardening and prevention of polyspermy [255]. As expected, we observed that both activation protocols increased PN formation, embryo cleavage and development compared with controls, which confirms the importance of activation following ICSI in porcine species [92, 256, 257].

In summary, findings from this study demonstrated that after exposure of porcine oocytes to 15 μM Ion for 5 min: i) treatment with CDK1i in association or not with PKCa improved PN formation; ii) 15 min incubation in the presence of TPEN is the required time to promote PN formation, cleavage and development to the blastocyst stage; iii) treatment with CDK1i and PKCa following exposure to TPEN for 15 min decreased embryo development compared to TPEN alone; and iv) treatment with TPEN for 15 min resulted in higher cleavage and blastocysts rates in PA embryos, high PN rates in ICSI embryos and similar embryo development and quality in SCNT embryos compared with treatment with $SrCl_2$ plus CHX for 240 min. Based on these findings, we propose that the IT-20 protocol is a simple, fast and reliable protocol for activation of porcine oocytes for production of PA, SCNT and ICSI embryos.

4.6 Aknowledgements

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4.7 Figures and legends

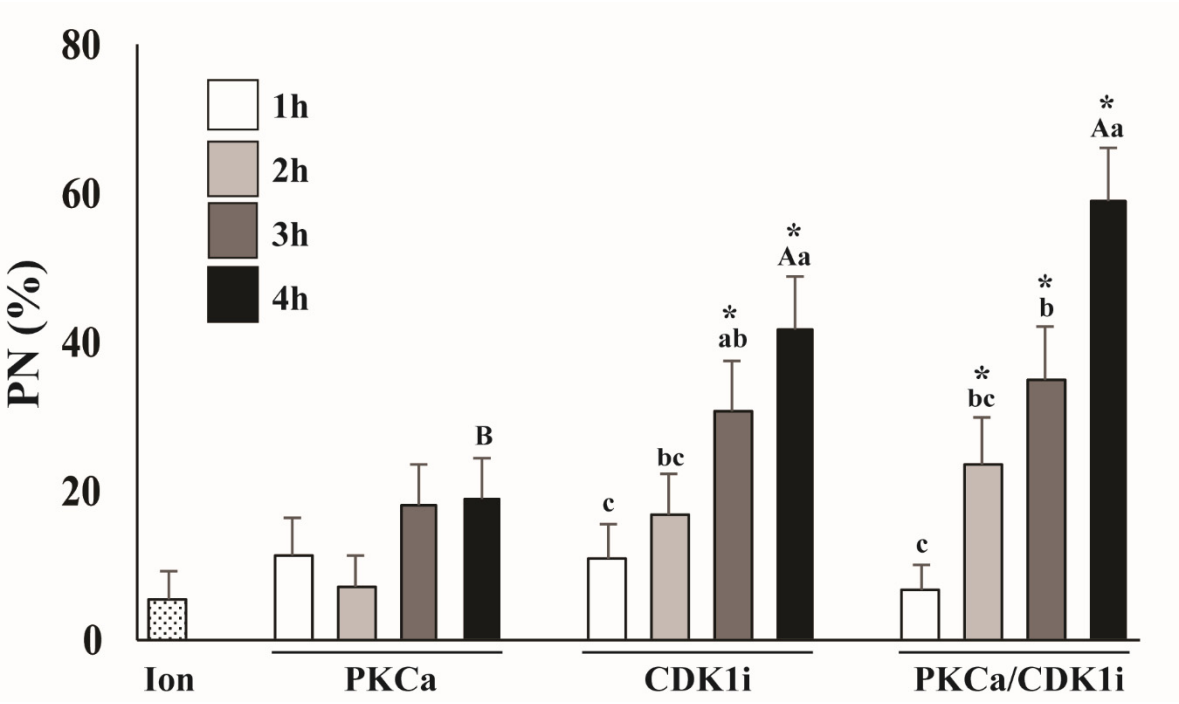

Figure 1. Pronuclear formation rates of porcine oocytes treated with Ion followed by activation of PKC and/or inhibition of CDK1. Capital letters indicate statistical differences (P<0.05) between different treatments in the same exposure time. Lower case letters indicate statistical differences (P<0.05) between different times in the same treatment. For pronuclear formation, 40 to 55 oocytes were analyzed in each treatment. Asterisks indicate statistical differences (P<0.05) from control group exposed to Ion alone.

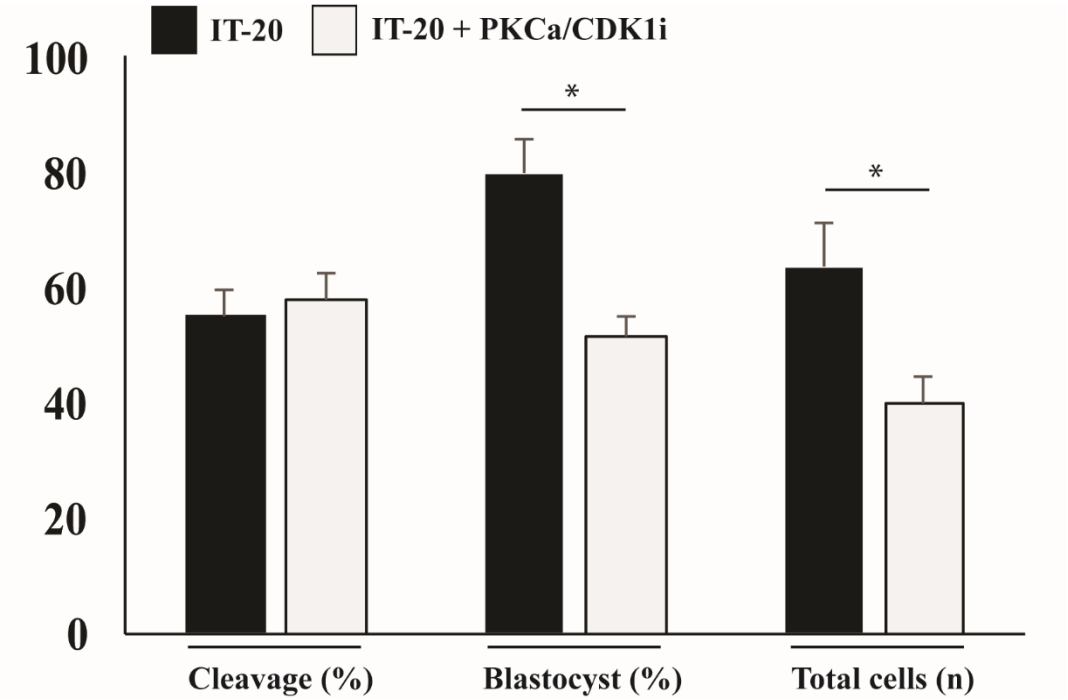

Figure 2. Development and total cells in blastocysts from oocytes exposed or not to CDK1i and PKCa for 4 h following TPEN. For TPEN and TPEN+CDK1i+PKCa treatments, the respective number of embryos used were 105 and 116 to assess cleavage, 58 and 67 to assess blastocyst and 46 and 35 to assess cell number. Asterisks indicate statistical differences between groups.

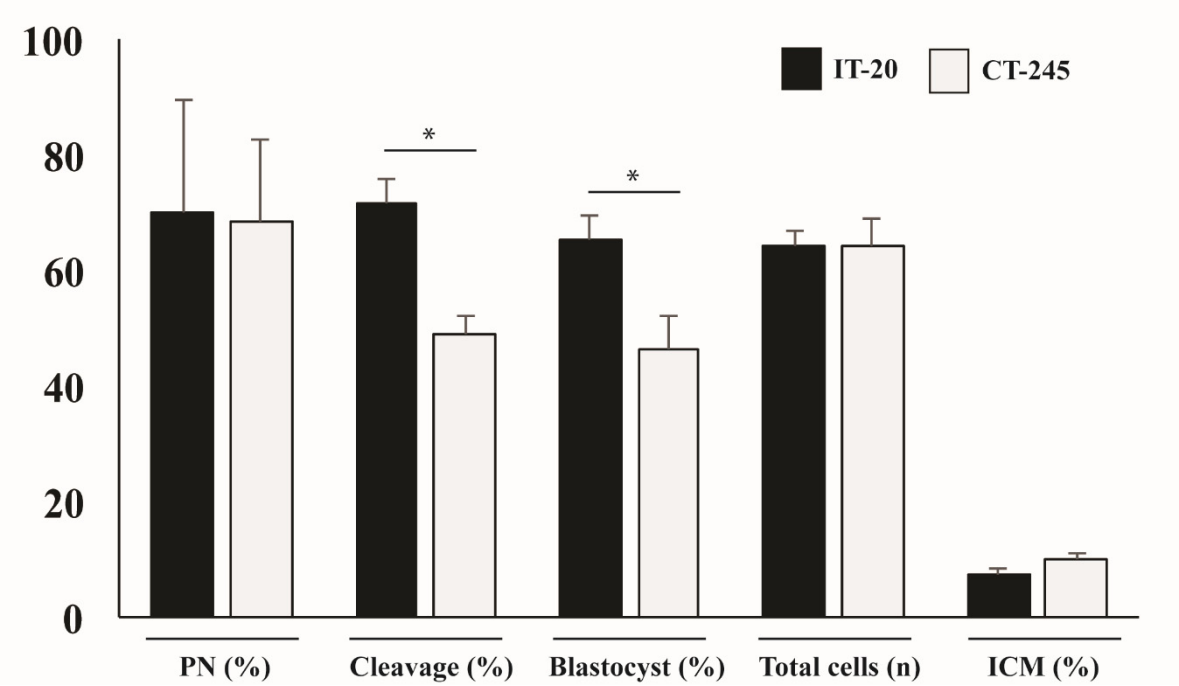

Figure 3. Pronuclear formation, cleavage and development rates and cell numbers in PA embryos activated with IT-20 and CT-245 protocols. For treatments IT-20 and CT-245 the respective number of oocytes or embryos used were 43 and 44 to assess activation, 199 and 205 to assess cleavage, 142 and 101 to assess development to blastocyst, and 93 and 47 to assess cell numbers. Asterisks indicate statistical differences between groups.

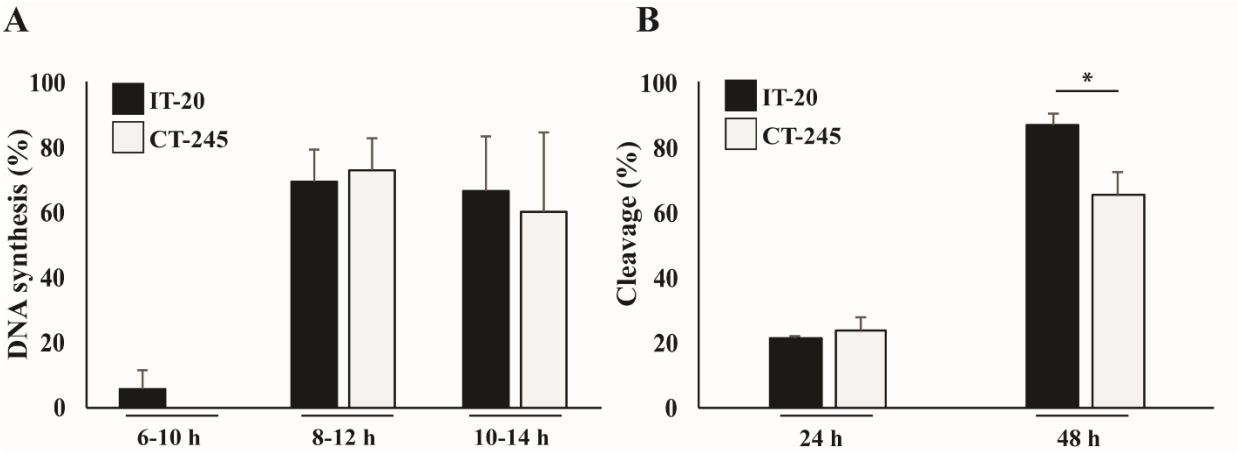

Figure 4. Beginning of S-phase and cleavage in PA embryos activated with IT-20 and CT-245 protocols. A) Proportion of embryos with positive signal for DNA synthesis at different times after activation. B) Proportion of early (24 h after activation) and total cleavage (48 h after activation). For treatments IT-20 and CT-245 the respective number of oocytes or embryos used were 159 and 172 to assess cleavage, 18 and 10 to assess DNA synthesis at 6 to 10 h, 23 and 22 to assess DNA synthesis at 8 to 12 h and 9 and 5 to assess DNA synthesis at 10 to 14 h. Asterisk indicates statistical differences between groups.

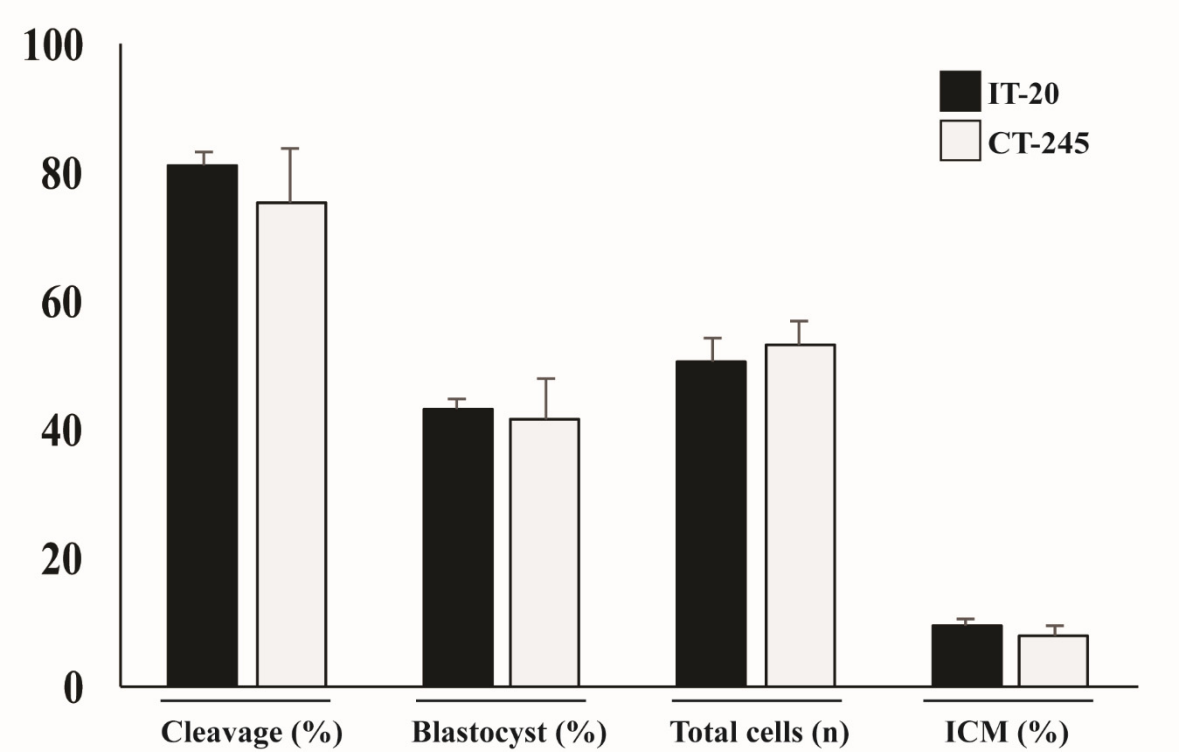

Figure 5. Development and cell numbers in SCNT embryos activated with IT-20 and CT-245 protocols. For treatments IT-20 and CT-245 the respective number of embryos used were 95 and 88 to assess cleavage, 77 and 65 to assess blastocyst, and 33 and 27 to assess cell numbers.

4.8 Tables

Table 1. Pronuclear formation, cleavage and blastocyst rates, and average number of cells in blastocyst from oocytes exposed to Ionomycin followed by TPEN.

Exposure time to TPEN (min)	PN % - (n)	Cleavage % - (n)	Blastocyst of cleaved % - (n)	Total cells (mean ± SEM) - (n)
5	69.4±14^{b} (39)	60.2± 6^{c} (124)	62.5±4 (75)	49±4 (47)
10	84.4±6^{ab} (36)	69.3±2^{bc} (123)	63.3±8 (85)	46±3 (54)
15	91.7±8^{ab} (30)	74.5±3^{ab} (115)	63.2±3 (86)	44±4 (55)
20	90.2±6^{ab} (29)	78.4±3^{ab} (113)	52.6±8 (89)	46±3 (49)
25	97.2±3^{a} (32)	75.3±2^{ab} (117)	59.3±5 (88)	45±4 (52)
30	91.7±4^{ab} (27)	80.9±4^{a} (107)	56.5±11 (87)	45±6 (52)

Different superscripts in the same column indicate significant differences between times ($P<0.05$).

Table 2. Activation, development and cell numbers in ICSI-derived embryos following different activation protocols.

Treatment	PN		Cleavage	Blastocyst	Number of cells	
	Correct (%)	Total (%)	(%)	(%)	Total	ICM (%)
IT-20	48.3±7[a]	88.6±3[a]	75.2±7[a]	19.5±3[a]	37±9[a]	14.3±2[a]
CT-245	37.8±5[ab]	61.5±5[b]	62.4±4[a]	14.4±3[a]	30±10[a]	11.9±2[a]
N-A	17±3[b]	21.5±6[c]	34.6±6[b]	0[b]	0[b]	0[b]

N-A, non-activated (control group); Correct PN formation: 2 pronuclei (PN) and 2 polar bodies (PB); total PN formation: 2PN-2PB + 1PN-2PB with a non-decondensed sperm head. Blastocyst rates are based on cleaved embryos. Total number of cells represent average ± SEM of total cells in blastocysts, and ICM (%) indicate the average proportion of SOX2-positive cells per blastocyst. For treatments IT-20, CT-245 and N-A, the respective number of oocytes or embryos used were 55, 54 and 48 to assess activation, 102, 98 and 66 to assess cleavage, 76, 61 and 23 to assess blastocyst, and 15, 9 and 0 to assess cell numbers. Different superscripts in the same column indicate significant differences between treatments.

CHAPTER 5: CONCLUSION

The need for AOA following these techniques, and the inconsistency in its results in different studies leads to a demand for the development of an AOA protocol that is fast, reliable and closer to physiological events, decreasing the risks of undesired outcomes. In this study, our goal was to develop a protocol for activation of porcine oocytes through manipulation of Ca^{2+}, Zn^{2+}, PKC and CDK1 activity, which are key regulators of the oocyte activation cascade.

Findings reported in this thesis demonstrated that:

1) Treatment with a CDK1 inhibitor in association or not with a PKC activator after exposure to ionomycin improved oocyte activation.

2) After treatment with ionomycin, exposure for 15 min to the zinc chelator TPEN is the ideal time to promote PN formation, cleavage and development to the blastocyst stage.

3) Treatment with CDK1i and PKCa following exposure to TPEN for 15 min decreased embryo development compared to TPEN alone.

4) After exposure to ionomycin, treatment with TPEN for 15 min resulted in higher cleavage and blastocysts rates in PA embryos, high PN rates in ICSI embryos and similar embryo development and quality in SCNT embryos compared with treatment with $SrCl_2$ plus CHX for 240 min.

5) Based on these findings, we propose that treatment for 5 minutes with ionomycin followed by 15 minutes with TPEN (IT-20 protocol) is a reliable method for oocyte activation and production of PA, SCNT and ICSI porcine embryos.

CHAPTER 6: REFERENCES

[1] Ducibella T, Schultz RM, Ozil JP. Role of calcium signals in early development. Semin Cell Dev Biol. 2006;17.
[2] Clift D, Schuh M. Restarting life: fertilization and the transition from meiosis to mitosis. Nature reviews Molecular cell biology. 2013;14:549-62.
[3] Machaty Z. Signal transduction in mammalian oocytes during fertilization. Cell and Tissue Research. 2016;363:169-83.
[4] Sanders JR, Swann K. Molecular triggers of egg activation at fertilization in mammals. Reproduction. 2016;152:R41-50.
[5] Miyazaki S, Shirakawa H, Nakada K, Honda Y. Essential role of the inositol 1,4,5-trisphosphate receptor/Ca2+ release channel in Ca2+ waves and Ca2+ oscillations at fertilization of mammalian eggs. Dev Biol. 1993;158:62-78.
[6] Miyazaki S, Ito M. Calcium signals for egg activation in mammals. J Pharmacol Sci. 2006;100:545-52.
[7] Suzuki T, Yoshida N, Suzuki E, Okuda E, Perry AC. Full-term mouse development by abolishing Zn2+-dependent metaphase II arrest without Ca2+ release. Development. 2010;137:2659-69.
[8] Kim AM, Vogt S, O'Halloran TV, Woodruff TK. Zinc availability regulates exit from meiosis in maturing mammalian oocytes. Nat Chem Biol. 2010;6:674-81.
[9] Ferrer-Buitrago M, Bonte D, De Sutter P, Leybaert L, Heindryckx B. Single Ca(2+) transients vs oscillatory Ca(2+) signaling for assisted oocyte activation: limitations and benefits. Reproduction. 2018;155:R105-r19.
[10] Alexander B, Coppola G, Berardino DD, Rho GJ, John ES, Betts DH, et al. The effect of 6-dimethylaminopurine (6-DMAP) and cycloheximide (CHX) on the development and chromosomal complement of sheep parthenogenetic and nuclear transfer embryos. Molecular Reproduction and Development. 2006;73:20-30.
[11] Simili M, Pellerano P, Pigullo S, Tavosanis G, Ottaggio L, de Saint-Georges L, et al. 6DMAP inhibition of early cell cycle events and induction of mitotic abnormalities. Mutagenesis. 1997;12:313-9.
[12] Thornton PK. Livestock production: recent trends, future prospects. Philosophical Transactions of the Royal Society B: Biological Sciences. 2010;365:2853-67.
[13] Heape W. The Artificial Insemination of Mammals and Subsequent Possible Fertilisation or Impregnation of Their Ova. Proceedings of the Royal Society of London. 1897;61:52-63.
[14] Foote R. The history of artificial insemination: Selected notes and notables. American Society of Animal Science. 2002.
[15] Phillips PE, Jahnke Marianna M. Embryo Transfer (Techniques, Donors, and Recipients). Veterinary Clinics of North America: Food Animal Practice. 2016;32:365-85.
[16] Looney CR, Bondioli KR, Hill KG, Massey JM. Superovulation of donor cows with bovine Follicle-Stimulating Hormone (bFSH) produced by recombinant DNA technology. Theriogenology. 1988;29:271.
[17] Philippo M, Rowson L, E. A. Prostaglandins and superovulation in the bovine. Ann Biol anim Bioch Biophys. 1975;15:233-40.
[18] Murphy BD, Mapletoft RJ, Manns J, Humphrey WD. Variability in gonadotrophin preparations as a factor in the superovulatory response. Theriogenology. 1984;21:117-25.

[19] Armstrong DT, Evans G. Factors influencing success of embryo transfer in sheep and goats. Theriogenology. 1983;19:31-42.
[20] González F, Calero P, Beckers JF. Induction of Superovulation in Domestic Ruminants. In: Renaville R, Burny A, editors. Biotechnology in Animal Husbandry. Dordrecht: Springer Netherlands; 2002. p. 209-23.
[21] Paramio MT. In vivo and in vitro embryo production in goats. Small Ruminant Research. 2010;89:144-8.
[22] Society I-IET. 2016 Data Retrieval Report - Statistics of embryo collection and transfer in domestic farm animals. 2017.
[23] Iritani A, Niwa K. Capacitation of bull spermatozoa and fertilization in vitro of cattle follicular oocytes matured in culture. Journal of reproduction and fertility. 1977;50:119-21.
[24] Brackett BG, Oh YK, Evans JF, Donawick WJ. In vitro fertilization of cow ova. Theriogenology. 1978;9:89.
[25] Ball GD, Leibfried ML, Ax RL, First NL. Maturation and fertilization of bovine oocytes in vitro. Journal of dairy science. 1984;67:2775-85.
[26] Parrish JJ, Susko-Parrish JL, Leibfried-Rutledge ML, Critser ES, Eyestone WH, First NL. Bovine in vitro fertilization with frozen-thawed semen. Theriogenology. 1986;25:591-600.
[27] Parrish JJ, Susko-Parrish J, Winer MA, First NL. Capacitation of bovine sperm by heparin. Biol Reprod. 1988;38:1171-80.
[28] Brackett BG, Bousquet D, Boice ML, Donawick WJ, Evans JF, Dressel MA. Normal development following in vitro fertilization in the cow. Biol Reprod. 1982;27:147-58.
[29] Fukuda Y, Ichikawa M, Naito K, Toyoda Y. Birth of normal calves resulting from bovine oocytes matured, fertilized, and cultured with cumulus cells in vitro up to the blastocyst stage. Biol Reprod. 1990;42:114-9.
[30] Crozet N, Huneau D, Desmedt V, Theron MC, Szollosi D, Torres S, et al. In vitro fertilization with normal development in the sheep. Gamete research. 1987;16:159-70.
[31] Paramio MT, Izquierdo D. Current status of in vitro embryo production in sheep and goats. Reprod Domest Anim. 2014;49 Suppl 4:37-48.
[32] Cognie Y, Poulin N, Locatelli Y, Mermillod P. State-of-the-art production, conservation and transfer of in-vitro-produced embryos in small ruminants. Reprod Fertil Dev. 2004;16:437-45.
[33] Amiridis GS, Cseh S. Assisted reproductive technologies in the reproductive management of small ruminants. Anim Reprod Sci. 2012;130:152-61.
[34] Baldassarre H, Karatzas CN. Advanced assisted reproduction technologies (ART) in goats. Anim Reprod Sci. 2004;82-83:255-66.
[35] de Souza-Fabjan JMG, Panneau B, Duffard N, Locatelli Y, de Figueiredo JR, de Figueirêdo Freitas VJ, et al. In vitro production of small ruminant embryos: Late improvements and further research. Theriogenology. 2014;81:1149-62.
[36] Mattioli M, Bacci ML, Galeati G, Seren E. Developmental competence of pig oocytes matured and fertilized in vitro. Theriogenology. 1989;31:1201-7.
[37] Somfai T, Ozawa M, Noguchi J, Kaneko H, Nakai M, Maedomari N, et al. Live piglets derived from in vitro-produced zygotes vitrified at the pronuclear stage. Biol Reprod. 2009;80:42-9.
[38] Nagashima H, Hiruma K, Saito H, Tomii R, Ueno S, Nakayama N, et al. Production of live piglets following cryopreservation of embryos derived from in vitro-matured oocytes. Biol Reprod. 2007;76:900-5.
[39] Abeydeera LR. In vitro production of embryos in swine. Theriogenology. 2002;57:256-73.

[40] Suzuki H, Saito Y, Kagawa N, Yang X. In vitro fertilization and polyspermy in the pig: factors affecting fertilization rates and cytoskeletal reorganization of the oocyte. Microsc Res Tech. 2003;61:327-34.
[41] Gil MA, Cuello C, Parrilla I, Vazquez JM, Roca J, Martinez EA. Advances in swine in vitro embryo production technologies. Reprod Domest Anim. 2010;45 Suppl 2:40-8.
[42] Romar R, Funahashi H, Coy P. In vitro fertilization in pigs: New molecules and protocols to consider in the forthcoming years. Theriogenology. 2016;85:125-34.
[43] Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. Viable offspring derived from fetal and adult mammalian cells. Nature. 1997;385:810-3.
[44] Niemann H, Lucas-Hahn A. Somatic cell nuclear transfer cloning: practical applications and current legislation. Reprod Domest Anim. 2012;47 Suppl 5:2-10.
[45] Bordignon V, El-Beirouthi N, Gasperin BG, Albornoz MS, Martinez-Diaz MA, Schneider C, et al. Production of cloned pigs with targeted attenuation of gene expression. PLoS One. 2013;8:e64613.
[46] Gutierrez K, Dicks N, Glanzner WG, Agellon LB, Bordignon V. Efficacy of the porcine species in biomedical research. Front Genet. 2015;6:293.
[47] Phelps CJ, Koike C, Vaught TD, Boone J, Wells KD, Chen SH, et al. Production of alpha 1,3-galactosyltransferase-deficient pigs. Science. 2003;299:411-4.
[48] Loi P, Ptak G, Barboni B, Fulka J, Jr., Cappai P, Clinton M. Genetic rescue of an endangered mammal by cross-species nuclear transfer using post-mortem somatic cells. Nature biotechnology. 2001;19:962-4.
[49] Niemann H, Kues WA, Petersen B, Carnwath JW. 4.37 - Transgenesis A2 - Moo-Young, Murray. Comprehensive Biotechnology (Second Edition). Burlington: Academic Press; 2011. p. 457-67.
[50] Heyman Y, Zhou Q, Lebourhis D, Chavatte-Palmer P, Renard JP, Vignon X. Novel approaches and hurdles to somatic cloning in cattle. Cloning and stem cells. 2002;4:47-55.
[51] Bordignon V, Smith LC. Telophase enucleation: an improved method to prepare recipient cytoplasts for use in bovine nuclear transfer. Mol Reprod Dev. 1998;49:29-36.
[52] Bordignon V, Smith LC. Telophase-stage host ooplasts support complete reprogramming of roscovitine-treated somatic cell nuclei in cattle. Cloning and stem cells. 2006;8:305-17.
[53] Rissi VB, Glanzner WG, Mujica LK, Antoniazzi AQ, Goncalves PB, Bordignon V. Effect of Cell Cycle Interactions and Inhibition of Histone Deacetylases on Development of Porcine Embryos Produced by Nuclear Transfer. Cell Reprogram. 2016;18:8-16.
[54] Papp B, Plath K. Epigenetics of Reprogramming to Induced Pluripotency. Cell. 2013;152:1324-43.
[55] Jullien J, Vodnala M, Pasque V, Oikawa M, Miyamoto K, Allen G, et al. Gene Resistance to Transcriptional Reprogramming following Nuclear Transfer Is Directly Mediated by Multiple Chromatin-Repressive Pathways. Molecular cell. 2017;65:873-84.e8.
[56] Hormanseder E, Simeone A, Allen GE, Bradshaw CR, Figlmuller M, Gurdon J, et al. H3K4 Methylation-Dependent Memory of Somatic Cell Identity Inhibits Reprogramming and Development of Nuclear Transfer Embryos. Cell stem cell. 2017.
[57] Xie B, Zhang H, Wei R, Li Q, Weng X, Kong Q, et al. Histone H3 lysine 27 trimethylation acts as an epigenetic barrier in porcine nuclear reprogramming. Reproduction. 2016;151:9-16.
[58] Matoba S, Liu Y, Lu F, Iwabuchi KA, Shen L, Inoue A, et al. Embryonic development following somatic cell nuclear transfer impeded by persisting histone methylation. Cell. 2014;159:884-95.

[59] Whitworth KM, Li R, Spate LD, Wax DM, Rieke A, Whyte JJ, et al. Method of oocyte activation affects cloning efficiency in pigs. Mol Reprod Dev. 2009;76:490-500.
[60] Li XC, Guo Q, Zhu HY, Jin L, Zhang YC, Zhang GL, et al. Parthenogenetic activation and somatic cell nuclear transfer of porcine oocytes activated by an electric pulse and AZD5438 treatment. Zygote. 2017;25:453-61.
[61] Canada H. Food Directorate Interim Policy on Foods from Cloned Animals. 2003.
[62] Parliament E. Regulation (EU) 2015/2283 of the European Parliament and of the Council of 25 November 2015 on novel foods, amending Regulation (EU) No 1169/2011 of the European Parliament and of the Council and repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001. 2013.
[63] Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet (London, England). 1992;340:17-8.
[64] Kimura Y, Yanagimachi R. Intracytoplasmic sperm injection in the mouse. Biology of Reproduction. 1995;52:709-20.
[65] Hinrichs K. Update on equine ICSI and cloning. Theriogenology. 2005;64:535-41.
[66] Hinrichs K. In vitro production of equine embryos: state of the art. Reprod Domest Anim. 2010;45 Suppl 2:3-8.
[67] Sessions-Bresnahan DR, Graham JK, Carnevale EM. Validation of a heterologous fertilization assay and comparison of fertilization rates of equine oocytes using in vitro fertilization, perivitelline, and intracytoplasmic sperm injections. Theriogenology. 2014;82:274-82.
[68] McKinnon AO, Lacham-Kaplan O, Trounson AO. Pregnancies produced from fertile and infertile stallions by intracytoplasmic sperm injection (ICSI) of single frozen-thawed spermatozoa into in vivo matured mare oocytes. Journal of reproduction and fertility Supplement. 2000:513-7.
[69] Cochran R, Meintjes M, Reggio B, Hylan D, Carter J, Pinto C, et al. Production of live foals from sperm-injected oocytes harvested from pregnant mares. Journal of reproduction and fertility Supplement. 2000:503-12.
[70] Salamone DF, Canel NG, Rodriguez MB. Intracytoplasmic sperm injection in domestic and wild mammals. Reproduction. 2017;154:F111-f24.
[71] Keefer CL, Younis AI, Brackett BG. Cleavage development of bovine oocytes fertilized by sperm injection. Mol Reprod Dev. 1990;25:281-5.
[72] Chung JT, Keefer CL, Downey BR. Activation of bovine oocytes following intracytoplasmic sperm injection (ICSI). Theriogenology. 2000;53:1273-84.
[73] Rho GJ, Kawarsky S, Johnson WH, Kochhar K, Betteridge KJ. Sperm and oocyte treatments to improve the formation of male and female pronuclei and subsequent development following intracytoplasmic sperm injection into bovine oocytes. Biol Reprod. 1998;59:918-24.
[74] Chen SH, Seidel GE. Effects of oocyte activation and treatment of spermatozoa on embryonic development following intracytoplasmic sperm injection in cattle. Theriogenology. 1997;48:1265-73.
[75] Suttner R, Zakhartchenko V, Stojkovic P, Muller S, Alberio R, Medjugorac I, et al. Intracytoplasmic sperm injection in bovine: effects of oocyte activation, sperm pretreatment and injection technique. Theriogenology. 2000;54:935-48.
[76] Hwang S, Lee E, Yoon J, Yoon B-K, Lee J-H, Choi D. Effects of Electric Stimulation on Bovine Oocyte Activation and Embryo Development in Intracytoplasmic Sperm Injection Procedure. Journal of Assisted Reproduction and Genetics. 2000;17:310-4.
[77] Galli C, Vassiliev I, Lagutina I, Galli A, Lazzari G. Bovine embryo development following ICSI: effect of activation, sperm capacitation and pre-treatment with dithiothreitol. Theriogenology. 2003;60:1467-80.

[78] Lee JW, Chang HC, Wu HY, Liu SS, Wang CH, Chu CY, et al. Effects of sperm pretreatment and embryo activation methods on the development of bovine embryos produced by intracytoplasmic sperm injection. Reprod Biol. 2015;15:154-62.
[79] Aguila L, Zambrano F, Arias ME, Felmer R. Sperm capacitation pretreatment positively impacts bovine intracytoplasmic sperm injection. Mol Reprod Dev. 2017;84:649-59.
[80] Aguila L, Felmer R, Arias ME, Navarrete F, Martin-Hidalgo D, Lee HC, et al. Defective sperm head decondensation undermines the success of ICSI in the bovine. Reproduction. 2017;154:207-18.
[81] Lee J-W, Tian XC, Yang X. Failure of Male Pronucleus Formation Is the Major Cause of Lack of Fertilization and Embryo Development in Pig Oocytes Subjected to Intracytoplasmic Sperm Injection. Biology of Reproduction. 2003;68:1341-7.
[82] Balhorn R. The protamine family of sperm nuclear proteins. Genome Biology. 2007;8:227-.
[83] Perreault SD, Barbee RR, Elstein KH, Zucker RM, Keefer CL. Interspecies differences in the stability of mammalian sperm nuclei assessed in vivo by sperm microinjection and in vitro by flow cytometry. Biol Reprod. 1988;39:157-67.
[84] Arias ME, Sanchez R, Risopatron J, Perez L, Felmer R. Effect of sperm pretreatment with sodium hydroxide and dithiothreitol on the efficiency of bovine intracytoplasmic sperm injection. Reprod Fertil Dev. 2014;26:847-54.
[85] Chankitisakul V, Am-In N, Tharasanit T, Somfai T, Nagai T, Techakumphu M. Sperm pretreatment with dithiothreitol increases male pronucleus formation rates after intracytoplasmic sperm injection (ICSI) in swamp buffalo oocytes. J Reprod Dev. 2013;59:66-71.
[86] Oikawa T, Itahashi T, Numabe T. Improved embryo development in Japanese black cattle by in vitro fertilization using ovum pick-up plus intracytoplasmic sperm injection with dithiothreitol. J Reprod Dev. 2016;62:11-6.
[87] Sekhavati MH, Shadanloo F, Hosseini MS, Tahmoorespur M, Nasiri MR, Hajian M, et al. Improved bovine ICSI outcomes by sperm selected after combined heparin-glutathione treatment. Cell Reprogram. 2012;14:295-304.
[88] Garcia-Mengual E, Garcia-Rosello E, Alfonso J, Salvador I, Cebrian-Serrano A, Silvestre MA. Viability of ICSI oocytes after caffeine treatment and sperm membrane removal with Triton X-100 in pigs. Theriogenology. 2011;76:1658-66.
[89] Zambrano F, Aguila L, Arias ME, Sanchez R, Felmer R. Improved preimplantation development of bovine ICSI embryos generated with spermatozoa pretreated with membrane-destabilizing agents lysolecithin and Triton X-100. Theriogenology. 2016;86:1489-97.
[90] Zambrano F, Aguila L, Arias ME, Sanchez R, Felmer R. Effect of sperm pretreatment with glutathione and membrane destabilizing agents lysolecithin and Triton X-100, on the efficiency of bovine intracytoplasmic sperm injection. Reproduction in Domestic Animals. 2017:n/a-n/a.
[91] Nakai M, Ito J, Suzuki SI, Fuchimoto DI, Senbon S, Suzuki M, et al. Lack of calcium oscillation causes failure of oocyte activation after intracytoplasmic sperm injection in pigs. J Reprod Dev. 2016.
[92] Cheng WM, Wu ZH, Zhang X, Zhu YB, Pang YW, Guo M, et al. Effects of different activation regimens on pronuclear formation and developmental competence of in vitro-matured porcine oocytes after intracytoplasmic sperm injection. Reprod Domest Anim. 2012;47:609-14.
[93] Tian JH, Wu ZH, Liu L, Cai Y, Zeng SM, Zhu SE, et al. Effects of oocyte activation and sperm preparation on the development of porcine embryos derived from in vitro-matured oocytes and intracytoplasmic sperm injection. Theriogenology. 2006;66:439-48.

[94] Yoo JG, Hur CG, Park MR, Park JY, Hwang KC, Kim JH, et al. Electrical activation enhances pre-implantation embryo development following sperm injection into in vitro matured pig oocytes. The Journal of veterinary medical science. 2012;74:429-34.
[95] Nakai M, Ito J, Kashiwazaki N, Men NT, Tanihara F, Noguchi J, et al. Treatment with protein kinase C activator is effective for improvement of male pronucleus formation and further embryonic development of sperm-injected oocytes in pigs. Theriogenology. 2016;85:703-8.
[96] Nakai M, Ito J, Sato K, Noguchi J, Kaneko H, Kashiwazaki N, et al. Pre-treatment of sperm reduces success of ICSI in the pig. Reproduction. 2011;142:285-93.
[97] Cheng WM, An L, Wu ZH, Zhu YB, Liu JH, Gao HM, et al. Effects of disulfide bond reducing agents on sperm chromatin structural integrity and developmental competence of in vitro matured oocytes after intracytoplasmic sperm injection in pigs. Reproduction. 2009;137:633-43.
[98] Watanabe H, Fukui Y. Effects of dithiothreitol and boar on pronuclear formation and embryonic development following intracytoplasmic sperm injection in pigs. Theriogenology. 2006;65:528-39.
[99] Margules CR, Pressey RL. Systematic conservation planning. Nature. 2000;405:243-53.
[100] Paris MC, Mastromonaco GF, Paris DB, Krisher RL. A perspective on the role of emerging technologies for the propagation of companion animals, non-domestic and endangered species. Reprod Fertil Dev. 2007;19:iii-vii.
[101] Andrabi SMH, Maxwell WMC. A review on reproductive biotechnologies for conservation of endangered mammalian species. Animal Reproduction Science. 2007;99:223-43.
[102] Pukazhenthi BS, Wildt DE. Which reproductive technologies are most relevant to studying, managing and conserving wildlife? Reprod Fertil Dev. 2004;16:33-46.
[103] Leibo SP, Songsasen N. Cryopreservation of gametes and embryos of non-domestic species. Theriogenology. 2002;57:303-26.
[104] Santos RR, Amorim C, Cecconi S, Fassbender M, Imhof M, Lornage J, et al. Cryopreservation of ovarian tissue: An emerging technology for female germline preservation of endangered species and breeds. Animal Reproduction Science. 2010;122:151-63.
[105] Gomez MC, Pope CE, Giraldo A, Lyons LA, Harris RF, King AL, et al. Birth of African Wildcat cloned kittens born from domestic cats. Cloning and stem cells. 2004;6:247-58.
[106] Hildebrandt TB, Hermes R, Walzer C, Sos E, Molnar V, Mezosi L, et al. Artificial insemination in the anoestrous and the postpartum white rhinoceros using GnRH analogue to induce ovulation. Theriogenology. 2007;67:1473-84.
[107] Robeck TR, Steinman KJ, Gearhart S, Reidarson TR, McBain JF, Monfort SL. Reproductive Physiology and Development of Artificial Insemination Technology in Killer Whales (Orcinus orca)1. Biology of Reproduction. 2004;71:650-60.
[108] Locatelli Y, Cognié Y, Vallet JC, Baril G, Verdier M, Poulin N, et al. Successful use of oviduct epithelial cell coculture for in vitro production of viable red deer (Cervus elaphus) embryos. Theriogenology. 2005;64:1729-39.
[109] Humphray SJ, Scott CE, Clark R, Marron B, Bender C, Camm N, et al. A high utility integrated map of the pig genome. Genome Biol. 2007;8:R139.
[110] Panepinto LM. Miniature Swine Breeds used Worldwide in Research. In: Tumbleson ME, Schook LB, editors. Advances in Swine in Biomedical Research: Volume 2. Boston, MA: Springer US; 1996. p. 681-91.
[111] Swanson KS, Mazur MJ, Vashisht K, Rund LA, Beever JE, Counter CM, et al. Genomics and clinical medicine: rationale for creating and effectively evaluating animal models. Experimental biology and medicine (Maywood, NJ). 2004;229:866-75.

[112] Maksimenko OG, Deykin AV, Khodarovich YM, Georgiev PG. Use of Transgenic Animals in Biotechnology: Prospects and Problems. Acta Naturae. 2013;5:33-46.
[113] Ruan J, Xu J, Chen-Tsai RY, Li K. Genome editing in livestock: Are we ready for a revolution in animal breeding industry? Transgenic research. 2017;26:715-26.
[114] Gun G, Kues WA. Current progress of genetically engineered pig models for biomedical research. BioResearch open access. 2014;3:255-64.
[115] Swindle MM, Makin A, Herron AJ, Clubb FJ, Jr., Frazier KS. Swine as models in biomedical research and toxicology testing. Veterinary pathology. 2012;49:344-56.
[116] Cui J, Li J, Mathison M, Tondato F, Mulkey SP, Micko C, et al. A clinically relevant large-animal model for evaluation of tissue-engineered cardiac surgical patch materials. Cardiovascular revascularization medicine : including molecular interventions. 2005;6:113-20.
[117] Davis BT, Wang XJ, Rohret JA, Struzynski JT, Merricks EP, Bellinger DA, et al. Targeted disruption of LDLR causes hypercholesterolemia and atherosclerosis in Yucatan miniature pigs. PLoS One. 2014;9:e93457.
[118] Rogers CS, Stoltz DA, Meyerholz DK, Ostedgaard LS, Rokhlina T, Taft PJ, et al. Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs. Science. 2008;321:1837-41.
[119] Umeyama K, Watanabe M, Saito H, Kurome M, Tohi S, Matsunari H, et al. Dominant-negative mutant hepatocyte nuclear factor 1alpha induces diabetes in transgenic-cloned pigs. Transgenic research. 2009;18:697-706.
[120] Sánchez F, Smitz J. Molecular control of oogenesis. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease. 2012;1822:1896-912.
[121] Pincus G, Enzmann EV. THE COMPARATIVE BEHAVIOR OF MAMMALIAN EGGS IN VIVO AND IN VITRO : I. THE ACTIVATION OF OVARIAN EGGS. The Journal of experimental medicine. 1935;62:665-75.
[122] Dekel N, Beers WH. Rat oocyte maturation in vitro: relief of cyclic AMP inhibition by gonadotropins. Proc Natl Acad Sci U S A. 1978;75:4369-73.
[123] Vaccari S, Weeks JL, 2nd, Hsieh M, Menniti FS, Conti M. Cyclic GMP signaling is involved in the luteinizing hormone-dependent meiotic maturation of mouse oocytes. Biol Reprod. 2009;81:595-604.
[124] Norris RP, Ratzan WJ, Freudzon M, Mehlmann LM, Krall J, Movsesian MA, et al. Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte. Development. 2009;136:1869-78.
[125] Sun QY, Miao YL, Schatten H. Towards a new understanding on the regulation of mammalian oocyte meiosis resumption. Cell cycle (Georgetown, Tex). 2009;8:2741-7.
[126] Norris RP, Freudzon M, Nikolaev VO, Jaffe LA. EGF receptor kinase activity is required for gap junction closure and for part of the decrease in ovarian follicle cGMP in response to luteinizing hormone. Reproduction (Cambridge, England). 2010;140:655-62.
[127] Pirino G, Wescott MP, Donovan PJ. Protein kinase A regulates resumption of meiosis by phosphorylation of Cdc25B in mammalian oocytes. Cell cycle (Georgetown, Tex). 2009;8:665-70.
[128] Tischer T, Schuh M. The Phosphatase Dusp7 Drives Meiotic Resumption and Chromosome Alignment in Mouse Oocytes. Cell Reports. 2016;17:1426-37.
[129] Eppig JJ. Oocyte control of ovarian follicular development and function in mammals. Reproduction. 2001;122:829-38.
[130] Jones KT. Turning it on and off: M-phase promoting factor during meiotic maturation and fertilization. MHR: Basic science of reproductive medicine. 2004;10:1-5.

[131] de Vant'ery C, Gavin AC, Vassalli JD, Schorderet-Slatkine S. An accumulation of p34cdc2 at the end of mouse oocyte growth correlates with the acquisition of meiotic competence. Dev Biol. 1996;174:335-44.
[132] Adhikari D, Liu K. The regulation of maturation promoting factor during prophase I arrest and meiotic entry in mammalian oocytes. Mol Cell Endocrinol. 2014;382:480-7.
[133] Liang C-G, Huo L-J, Zhong Z-S, Chen D-Y, Schatten H, Sun Q-Y. Cyclic Adenosine 3′,5′-Monophosphate-Dependent Activation of Mitogen-Activated Protein Kinase in Cumulus Cells Is Essential for Germinal Vesicle Breakdown of Porcine Cumulus-Enclosed Oocytes. Endocrinology. 2005;146:4437-44.
[134] Fan H-Y, Sun Q-Y. Involvement of Mitogen-Activated Protein Kinase Cascade During Oocyte Maturation and Fertilization in Mammals1. Biology of Reproduction. 2004;70:535-47.
[135] Choi T, Fukasawa K, Zhou R, Tessarollo L, Borror K, Resau J, et al. The Mos/mitogen-activated protein kinase (MAPK) pathway regulates the size and degradation of the first polar body in maturing mouse oocytes. Proceedings of the National Academy of Sciences. 1996;93:7032-5.
[136] Liu L, Ju JC, Yang X. Differential inactivation of maturation-promoting factor and mitogen-activated protein kinase following parthenogenetic activation of bovine oocytes. Biol Reprod. 1998;59:537-45.
[137] Ferreira EM, Vireque AA, Adona PR, Meirelles FV, Ferriani RA, Navarro PA. Cytoplasmic maturation of bovine oocytes: structural and biochemical modifications and acquisition of developmental competence. Theriogenology. 2009;71:836-48.
[138] Mao L, Lou H, Lou Y, Wang N, Jin F. Behaviour of cytoplasmic organelles and cytoskeleton during oocyte maturation. Reproductive BioMedicine Online. 2014;28:284-99.
[139] Watson AJ. Oocyte cytoplasmic maturation: A key mediator of oocyte and embryo developmental competence1. Journal of animal science. 2007;85:E1-E3.
[140] Sirard MA. Resumption of meiosis: mechanism involved in meiotic progression and its relation with developmental competence. Theriogenology. 2001;55:1241-54.
[141] Yamashita M, Mita K, Yoshida N, Kondo T. Molecular mechanisms of the initiation of oocyte maturation: general and species-specific aspects. Progress in cell cycle research. 2000;4:115-29.
[142] De La Fuente R, Viveiros MM, Burns KH, Adashi EY, Matzuk MM, Eppig JJ. Major chromatin remodeling in the germinal vesicle (GV) of mammalian oocytes is dispensable for global transcriptional silencing but required for centromeric heterochromatin function. Dev Biol. 2004;275:447-58.
[143] Manjunath P, Therien I. Role of seminal plasma phospholipid-binding proteins in sperm membrane lipid modification that occurs during capacitation. Journal of reproductive immunology. 2002;53:109-19.
[144] Bailey JL. Factors regulating sperm capacitation. Systems biology in reproductive medicine. 2010;56:334-48.
[145] Stival C, Puga Molina Ldel C, Paudel B, Buffone MG, Visconti PE, Krapf D. Sperm Capacitation and Acrosome Reaction in Mammalian Sperm. Advances in anatomy, embryology, and cell biology. 2016;220:93-106.
[146] Yeste M. Boar Spermatozoa Within the Oviductal Environment (II): Sperm Capacitation. In: Bonet S, Casas I, Holt WV, Yeste M, editors. Boar Reproduction: Fundamentals and New Biotechnological Trends. Berlin, Heidelberg: Springer Berlin Heidelberg; 2013. p. 347-405.
[147] Escoffier J, Krapf D, Navarrete F, Darszon A, Visconti PE. Flow cytometry analysis reveals a decrease in intracellular sodium during sperm capacitation. J Cell Sci. 2012;125:473-85.

[148] Zeng Y, Clark EN, Florman HM. Sperm membrane potential: hyperpolarization during capacitation regulates zona pellucida-dependent acrosomal secretion. Dev Biol. 1995;171:554-63.
[149] Ritagliati C, Baro Graf C, Stival C, Krapf D. Regulation mechanisms and implications of sperm membrane hyperpolarization. Mechanisms of Development. 2018.
[150] Abou-haila A, Tulsiani DRP. Signal transduction pathways that regulate sperm capacitation and the acrosome reaction. Archives of Biochemistry and Biophysics. 2009;485:72-81.
[151] Visconti PE. Understanding the molecular basis of sperm capacitation through kinase design. Proc Natl Acad Sci U S A. 2009;106:667-8.
[152] Serrano H, Canchola E, Garcia-Suarez MD. Sperm-attracting activity in follicular fluid associated to an 8.6-kDa protein. Biochemical and biophysical research communications. 2001;283:782-4.
[153] Sun F, Giojalas LC, Rovasio RA, Tur-Kaspa I, Sanchez R, Eisenbach M. Lack of species-specificity in mammalian sperm chemotaxis. Developmental Biology. 2003;255:423-7.
[154] Takahashi K, Kikuchi K, Uchida Y, Kanai-Kitayama S, Suzuki R, Sato R, et al. Binding of Sperm to the Zona Pellucida Mediated by Sperm Carbohydrate-Binding Proteins is not Species-Specific in vitro between Pigs and Cattle. Biomolecules. 2013;3:85-107.
[155] Yonezawa N, Kanai-Kitayama S, Kitayama T, Hamano A, Nakano M. Porcine zona pellucida glycoprotein ZP4 is responsible for the sperm-binding activity of the ZP3/ZP4 complex. Zygote. 2011;20:389-97.
[156] Saeko K, Naoto Y, Yuichiro I, Masaru T, Minoru N. Recombinant bovine zona pellucida glycoproteins ZP3 and ZP4 coexpressed in Sf9 cells form a sperm-binding active hetero-complex. The FEBS Journal. 2007;274:5390-405.
[157] Saling PM, Sowinski J, B BTS. An ultrastructural study of epididymal mouse spermatozoa binding to zonae pellucidae in vitro: Sequential relationship to the acrosome reaction. Journal of Experimental Zoology. 1979;209:229-38.
[158] Inoue N, Satouh Y, Ikawa M, Okabe M, Yanagimachi R. Acrosome-reacted mouse spermatozoa recovered from the perivitelline space can fertilize other eggs. Proceedings of the National Academy of Sciences of the United States of America. 2011;108:20008-11.
[159] Jin M, Fujiwara E, Kakiuchi Y, Okabe M, Satouh Y, Baba SA, et al. Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during in vitro fertilization. Proceedings of the National Academy of Sciences. 2011;108:4892-6.
[160] Buffone MG, Hirohashi N, Gerton GL. Unresolved Questions Concerning Mammalian Sperm Acrosomal Exocytosis. Biology of Reproduction. 2014;90:112-.
[161] La Spina FA, Puga Molina LC, Romarowski A, Vitale AM, Falzone TL, Krapf D, et al. Mouse sperm begin to undergo acrosomal exocytosis in the upper isthmus of the oviduct. Dev Biol. 2016;411:172-82.
[162] Yeste M, Jones C, Amdani SN, Coward K. Oocyte Activation and Fertilisation: Crucial Contributors from the Sperm and Oocyte. In: Arur S, editor. Signaling-Mediated Control of Cell Division : From Oogenesis to Oocyte-to-Embryo Development. Cham: Springer International Publishing; 2017. p. 213-39.
[163] Wassarman PM, Jovine L, Litscher ES. A profile of fertilization in mammals. Nat Cell Biol. 2001;3:E59-64.
[164] Abou-Haila A, Tulsiani DRP. Mammalian Sperm Acrosome: Formation, Contents, and Function. Archives of Biochemistry and Biophysics. 2000;379:173-82.
[165] Saldívar-Hernández A, González-González ME, Sánchez-Tusié A, Maldonado-Rosas I, López P, Treviño CL, et al. Human sperm degradation of zona pellucida proteins contributes to fertilization. Reproductive Biology and Endocrinology : RB&E. 2015;13:99.

[166] Stein KK, Primakoff P, Myles D. Sperm-egg fusion: events at the plasma membrane. Journal of Cell Science. 2004;117:6269-74.
[167] Bianchi E, Doe B, Goulding D, Wright GJ. Juno is the egg Izumo receptor and is essential for mammalian fertilization. Nature. 2014;508:483-7.
[168] Inoue N, Ikawa M, Isotani A, Okabe M. The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. Nature. 2005;434:234-8.
[169] Anifandis G, Messini CI, Dafopoulos K, Daponte A, Messinis IE. Sperm contributions to oocyte activation: more that meets the eye. Journal of Assisted Reproduction and Genetics. 2016;33:313-6.
[170] Saunders CM, Larman MG, Parrington J, Cox LJ, Royse J, Blayney LM, et al. PLC zeta: a sperm-specific trigger of Ca(2+) oscillations in eggs and embryo development. Development. 2002;129.
[171] Wu AT, Sutovsky P, Manandhar G, Xu W, Katayama M, Day BN, et al. PAWP, a sperm-specific WW domain-binding protein, promotes meiotic resumption and pronuclear development during fertilization. J Biol Chem. 2007;282:12164-75.
[172] Sutovsky P. Sperm Capacitation, the Acrosome Reaction, and Fertilization. In: Carrell DT, Peterson CM, editors. Reproductive Endocrinology and Infertility: Integrating Modern Clinical and Laboratory Practice. New York, NY: Springer New York; 2010. p. 389-421.
[173] Williams CJ. Signalling mechanisms of mammalian oocyte activation. Hum Reprod Update. 2002;8:313-21.
[174] Miyazaki S, Yuzaki M, Nakada K, Shirakawa H, Nakanishi S, Nakade S, et al. Block of Ca2+ wave and Ca2+ oscillation by antibody to the inositol 1,4,5-trisphosphate receptor in fertilized hamster eggs. Science. 1992;257:251-5.
[175] Schuster S, Marhl M, Hofer T. Modelling of simple and complex calcium oscillations. From single-cell responses to intercellular signalling. European journal of biochemistry. 2002;269:1333-55.
[176] Stricker SA. Comparative Biology of Calcium Signaling during Fertilization and Egg Activation in Animals. Developmental Biology. 1999;211:157-76.
[177] Halet G. PKC signaling at fertilization in mammalian eggs. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2004;1742:185-9.
[178] Halet G, Tunwell R, Parkinson SJ, Carroll J. Conventional PKCs regulate the temporal pattern of Ca²⁺ oscillations at fertilization in mouse eggs. The Journal of Cell Biology. 2004;164:1033-44.
[179] Maller JL, Schwab MS, Gross SD, Taieb FE, Roberts BT, Tunquist BJ. The mechanism of CSF arrest in vertebrate oocytes. Mol Cell Endocrinol. 2002;187:173-8.
[180] Tunquist BJ, Maller JL. Under arrest: cytostatic factor (CSF)-mediated metaphase arrest in vertebrate eggs. Genes Dev. 2003;17:683-710.
[181] Schmidt A, Rauh NR, Nigg EA, Mayer TU. Cytostatic factor: an activity that puts the cell cycle on hold. J Cell Sci. 2006;119:1213-8.
[182] Rauh NR, Schmidt A, Bormann J, Nigg EA, Mayer TU. Calcium triggers exit from meiosis II by targeting the APC/C inhibitor XErp1 for degradation. Nature. 2005;437:1048-52.
[183] Yamasaki S, Sakata-Sogawa K, Hasegawa A, Suzuki T, Kabu K, Sato E, et al. Zinc is a novel intracellular second messenger. J Cell Biol. 2007;177:637-45.
[184] Bernhardt ML, Kong BY, Kim AM, O'Halloran TV, Woodruff TK. A zinc-dependent mechanism regulates meiotic progression in mammalian oocytes. Biol Reprod. 2012;86:114.

[185] Que EL, Bleher R, Duncan FE, Kong BY, Gleber SC, Vogt S, et al. Quantitative mapping of zinc fluxes in the mammalian egg reveals the origin of fertilization-induced zinc sparks. Nat Chem. 2015;7:130-9.
[186] Liu M. The biology and dynamics of mammalian cortical granules. Reproductive Biology and Endocrinology : RB&E. 2011;9:149-.
[187] McLay DW, Clarke HJ. Remodelling the paternal chromatin at fertilization in mammals. Reproduction. 2003;125:625-33.
[188] Cantone I, Fisher AG. Epigenetic programming and reprogramming during development. Nature structural & molecular biology. 2013;20:282-9.
[189] O'Neill C. The epigenetics of embryo development. Animal Frontiers. 2015;5:42-9.
[190] Medvedev S, Yang J, Hecht NB, Schultz RM. CDC2A (CDK1)-mediated phosphorylation of MSY2 triggers maternal mRNA degradation during mouse oocyte maturation. Dev Biol. 2008;321:205-15.
[191] Schultz RM. Regulation of zygotic gene activation in the mouse. Bioessays. 1993;15:531-8.
[192] Barnes FL, First NL. Embryonic transcription in in vitro cultured bovine embryos. Mol Reprod Dev. 1991;29:117-23.
[193] Hyttel P, Laurincik J, Rosenkranz C, Rath D, Niemann H, Ochs RL, et al. Nucleolar Proteins and Ultrastructure in Preimplantation Porcine Embryos Developed In Vivo1. Biology of Reproduction. 2000;63:1848-56.
[194] Glanzner WG, Rissi VB, de Macedo MP, Mujica LKS, Gutierrez K, Bridi A, et al. Histone 3 lysine 4, 9 and 27 demethylases expression profile in fertilized and cloned bovine and porcine embryos. Biol Reprod. 2018.
[195] Watson AJ, Barcroft LC. Regulation of blastocyst formation. Frontiers in bioscience : a journal and virtual library. 2001;6:D708-30.
[196] Watson AJ, Natale DR, Barcroft LC. Molecular regulation of blastocyst formation. Anim Reprod Sci. 2004;82-83:583-92.
[197] Riethmacher D, Brinkmann V, Birchmeier C. A targeted mutation in the mouse E-cadherin gene results in defective preimplantation development. Proceedings of the National Academy of Sciences of the United States of America. 1995;92:855-9.
[198] Hardarson T, Van Landuyt L, Jones G. The blastocyst. Hum Reprod. 2012;27 Suppl 1:i72-91.
[199] Keefe DL, Winkler N. Chapter 1 - Embryology A2 - Sokol, Andrew I. In: Sokol ER, editor. General Gynecology. Philadelphia: Mosby; 2007. p. 1-20.
[200] Choi YH, Velez IC, Macias-Garcia B, Riera FL, Ballard CS, Hinrichs K. Effect of clinically-related factors on in vitro blastocyst development after equine ICSI. Theriogenology. 2016;85:1289-96.
[201] Heindryckx B, De Gheselle S, Gerris J, Dhont M, De Sutter P. Efficiency of assisted oocyte activation as a solution for failed intracytoplasmic sperm injection. Reproductive BioMedicine Online. 2008;17:662-8.
[202] Fissore RA, Robl JM. Intracellular Ca2+ response of rabbit oocytes to electrical stimulation. Mol Reprod Dev. 1992;32:9-16.
[203] Collas P, Fissore R, Robl JM, Sullivan EJ, Barnes FL. Electrically induced calcium elevation, activation, and parthenogenetic development of bovine oocytes. Molecular Reproduction and Development. 1993;34:212-23.
[204] Koo OJ, Jang G, Kwon DK, Kang JT, Kwon OS, Park HJ, et al. Electrical activation induces reactive oxygen species in porcine embryos. Theriogenology. 2008;70:1111-8.

[205] Hao Y, Lai L, Mao J, Im GS, Bonk A, Prather RS. Apoptosis in parthenogenetic preimplantation porcine embryos. Biol Reprod. 2004;70:1644-9.
[206] Morgan AJ, Jacob R. Ionomycin enhances Ca2+ influx by stimulating store-regulated cation entry and not by a direct action at the plasma membrane. The Biochemical journal. 1994;300 (Pt 3):665-72.
[207] Shiina Y, Kaneda M, Matsuyama K, Tanaka K, Hiroi M, Doi K. Role of the extracellular Ca2+ on the intracellular Ca2+ changes in fertilized and activated mouse oocytes. Journal of reproduction and fertility. 1993;97:143-50.
[208] Rybouchkin A, Dozortsev D, de Sutter PD, Dhont M. Factors affecting the role of the spindle during early response of mouse oocytes to ethanol stimulation. The Journal of experimental zoology. 1996;275:469-75.
[209] Yi YJ, Park CS. Parthenogenetic development of porcine oocytes treated by ethanol, cycloheximide, cytochalasin B and 6-dimethylaminopurine. Anim Reprod Sci. 2005;86:297-304.
[210] Wang Z-g, Wang W, Yu S-d, Xu Z-r. Effects of different activation protocols on preimplantation development, apoptosis and ploidy of bovine parthenogenetic embryos. Animal Reproduction Science. 2008;105:292-301.
[211] Cheng WM, Sun XL, An L, Zhu SE, Li XH, Li Y, et al. Effect of different parthenogenetic activation methods on the developmental competence of in vitro matured porcine oocytes. Animal biotechnology. 2007;18:131-41.
[212] Zhang D, Pan L, Yang L-H, He X-K, Huang X-Y, Sun F-Z. Strontium promotes calcium oscillations in mouse meiotic oocytes and early embryos through InsP3 receptors, and requires activation of phospholipase and the synergistic action of InsP3. Human Reproduction. 2005;20:3053-61.
[213] Meo SC, Leal CL, Garcia JM. Activation and early parthenogenesis of bovine oocytes treated with ethanol and strontium. Anim Reprod Sci. 2004;81:35-46.
[214] Meo SC, Yamazaki W, Ferreira CR, Perecin F, Saraiva NZ, Leal CL, et al. Parthenogenetic activation of bovine oocytes using single and combined strontium, ionomycin and 6-dimethylaminopurine treatments. Zygote. 2007;15:295-306.
[215] Che L, Lalonde A, Bordignon V. Chemical activation of parthenogenetic and nuclear transfer porcine oocytes using ionomycin and strontium chloride. Theriogenology. 2007;67:1297-304.
[216] Okada K, Miyano T, Miyake M. Activation of pig oocytes by intracytoplasmic injection of strontium and barium. Zygote. 2003;11:159-65.
[217] Machaty Z, Wang WH, Day BN, Prather RS. Complete activation of porcine oocytes induced by the sulfhydryl reagent, thimerosal. Biol Reprod. 1997;57:1123-7.
[218] Cheek TR, McGuinness OM, Vincent C, Moreton RB, Berridge MJ, Johnson MH. Fertilisation and thimerosal stimulate similar calcium spiking patterns in mouse oocytes but by separate mechanisms. Development. 1993;119:179-89.
[219] Ross PJ, Beyhan Z, Iager AE, Yoon SY, Malcuit C, Schellander K, et al. Parthenogenetic activation of bovine oocytes using bovine and murine phospholipase C zeta. BMC Dev Biol. 2008;8:16.
[220] Ross PJ, Rodriguez RM, Iager AE, Beyhan Z, Wang K, Ragina NP, et al. Activation of bovine somatic cell nuclear transfer embryos by PLCZ cRNA injection. Reproduction. 2009;137:427-37.
[221] Yoneda A, Kashima M, Yoshida S, Terada K, Nakagawa S, Sakamoto A, et al. Molecular cloning, testicular postnatal expression, and oocyte-activating potential of porcine phospholipase Czeta. Reproduction. 2006;132.

[222] Aarabi M, Qin Z, Xu W, Mewburn J, Oko R. Sperm-borne protein, PAWP, initiates zygotic development in Xenopus laevis by eliciting intracellular calcium release. Mol Reprod Dev. 2010;77:249-56.
[223] Aarabi M, Balakier H, Bashar S, Moskovtsev SI, Sutovsky P, Librach CL, et al. Sperm-derived WW domain-binding protein, PAWP, elicits calcium oscillations and oocyte activation in humans and mice. The FASEB Journal. 2014;28:4434-40.
[224] Alberio R, Zakhartchenko V, Motlik J, Wolf E. Mammalian oocyte activation: lessons from the sperm and implications for nuclear transfer. The International journal of developmental biology. 2001;45.
[225] Wakai T, Ito J, Fissore RA. Chapter 1 - Artificial Activation of Mammalian Oocytes for Cloning: Present Status and Future Perspectives. Principles of Cloning (Second Edition). San Diego: Academic Press; 2014. p. 3-10.
[226] Susko-Parrish JL, Leibfried-Rutledge ML, Northey DL, Schutzkus V, First NL. Inhibition of Protein Kinases after an Induced Calcium Transient Causes Transition of Bovine Oocytes to Embryonic Cycles without Meiotic Completion. Developmental Biology. 1994;166:729-39.
[227] Keefer CL, Baldassarre H, Keyston R, Wang B, Bhatia B, Bilodeau AS, et al. Generation of dwarf goat (Capra hircus) clones following nuclear transfer with transfected and nontransfected fetal fibroblasts and in vitro-matured oocytes. Biol Reprod. 2001;64:849-56.
[228] Galli C, Lagutina I, Vassiliev I, Duchi R, Lazzari G. Comparison of microinjection (piezo-electric) and cell fusion for nuclear transfer success with different cell types in cattle. Cloning and stem cells. 2002;4:189-96.
[229] Betthauser J, Forsberg E, Augenstein M, Childs L, Eilertsen K, Enos J, et al. Production of cloned pigs from in vitro systems. Nature biotechnology. 2000;18:1055-9.
[230] Szollosi MS, Kubiak JZ, Debey P, de Pennart H, Szollosi D, Maro B. Inhibition of protein kinases by 6-dimethylaminopurine accelerates the transition to interphase in activated mouse oocytes. Journal of Cell Science. 1993;104:861.
[231] Obrig TG, Culp WJ, McKeehan WL, Hardesty B. The mechanism by which cycloheximide and related glutarimide antibiotics inhibit peptide synthesis on reticulocyte ribosomes. J Biol Chem. 1971;246:174-81.
[232] Nussbaum DJ, Prather RS. Differential effects of protein synthesis inhibitors on porcine oocyte activation. Mol Reprod Dev. 1995;41:70-5.
[233] Holm P, Booth PJ, Callesen H. Developmental kinetics of bovine nuclear transfer and parthenogenetic embryos. Cloning and stem cells. 2003;5:133-42.
[234] Felmer R, Arias ME. Activation treatment of recipient oocytes affects the subsequent development and ploidy of bovine parthenogenetic and somatic cell nuclear transfer (SCNT) embryos. Mol Reprod Dev. 2015;82:441-9.
[235] Kim AM, Bernhardt ML, Kong BY, Ahn RW, Vogt S, Woodruff TK, et al. Zinc sparks are triggered by fertilization and facilitate cell cycle resumption in mammalian eggs. ACS Chem Biol. 2011;6:716-23.
[236] Zhao MH, Kim NH, Cui XS. Zinc depletion activates porcine metaphase II oocytes independently of the protein kinase C pathway. In Vitro Cell Dev Biol Anim. 2014;50:945-51.
[237] Lee K, Davis A, Zhang L, Ryu J, Spate LD, Park KW, et al. Pig oocyte activation using a Zn(2)(+) chelator, TPEN. Theriogenology. 2015;84:1024-32.
[238] Duncan FE, Que EL, Zhang N, Feinberg EC, O'Halloran TV, Woodruff TK. The zinc spark is an inorganic signature of human egg activation. Sci Rep. 2016;6:24737.
[239] Alberio R, Kubelka M, Zakhartchenko V, Hajduch M, Wolf E, Motlik J. Activation of bovine oocytes by specific inhibition of cyclin-dependent kinases. Mol Reprod Dev. 2000;55:422-32.

[240] Dinnyes A, Hirao Y, Nagai T. Parthenogenetic activation of porcine oocytes by electric pulse and/or butyrolactone I treatment. Cloning. 1999;1:209-16.
[241] Nakai M, Ito J, Kashiwazaki N, Men NT, Tanihara F, Noguchi J, et al. Treatment with protein kinase C activator is effective for improvement of male pronucleus formation and further embryonic development of sperm-injected oocytes in pigs. Theriogenology. 2016;85:703-8.
[242] Quan HM, Fan HY, Meng XQ, Huo LJ, Chen DY, Schatten H, et al. Effects of PKC activation on the meiotic maturation, fertilization and early embryonic development of mouse oocytes. Zygote. 2003;11:329-37.
[243] Sun QY, Wang WH, Hosoe M, Taniguchi T, Chen DY, Shioya Y. Activation of protein kinase C induces cortical granule exocytosis in a Ca(2+)-independent manner, but not the resumption of cell cycle in porcine eggs. Development, growth & differentiation. 1997;39:523-9.
[244] Jones KT. Ca2+ oscillations in the activation of the egg and development of the embryo in mammals. The International journal of developmental biology. 1998;42:1-10.
[245] Malcuit C, Kurokawa M, Fissore RA. Calcium oscillations and mammalian egg activation. J Cell Physiol. 2006;206.
[246] Krauchunas AR, Wolfner MF. Molecular changes during egg activation. Curr Top Dev Biol. 2013;102:267-92.
[247] Gallicano GI, McGaughey RW, Capco DG. Activation of protein kinase C after fertilization is required for remodeling the mouse egg into the zygote. Mol Reprod Dev. 1997;46:587-601.
[248] Ma SF, Liu XY, Miao DQ, Han ZB, Zhang X, Miao YL, et al. Parthenogenetic activation of mouse oocytes by strontium chloride: a search for the best conditions. Theriogenology. 2005;64:1142-57.
[249] Jang W-I, Lin Z-L, Lee SH, Namgoong S, Kim N-H. A specific inhibitor of CDK1, RO-3306, reversibly arrests meiosis during in vitro maturation of porcine oocytes. Animal Reproduction Science. 2014;144:102-8.
[250] Iba T, Yano Y, Umeno M, Hinokio K, Kuwahara A, Irahara M, et al. Roscovitine in combination with calcium ionophore induces oocyte activation through reduction of M-phase promoting factor activity in mice. Zygote. 2012;20:321-5.
[251] Perecin F, Méo SC, Leal CLV, Garcia JM. Oocyte activation and preimplantation development of bovine embryos obtained by specific inhibition of cyclin-dependent kinases. Arquivo Brasileiro de Medicina Veterinária e Zootecnia. 2007;59:280-7.
[252] Bohrer RC, Duggavathi R, Bordignon V. Inhibition of histone deacetylases enhances DNA damage repair in SCNT embryos. Cell cycle (Georgetown, Tex). 2014;13:2138-48.
[253] Bohrer RC, Coutinho AR, Duggavathi R, Bordignon V. The Incidence of DNA Double-Strand Breaks Is Higher in Late-Cleaving and Less Developmentally Competent Porcine Embryos. Biol Reprod. 2015;93:59.
[254] Zhang N, Duncan FE, Que EL, O'Halloran TV, Woodruff TK. The fertilization-induced zinc spark is a novel biomarker of mouse embryo quality and early development. Sci Rep. 2016;6:22772.
[255] Que EL, Duncan FE, Bayer AR, Philips SJ, Roth EW, Bleher R, et al. Zinc sparks induce physiochemical changes in the egg zona pellucida that prevent polyspermy. Integrative biology : quantitative biosciences from nano to macro. 2017;9:135-44.
[256] Liu S, Cui K, Li HL, Sun JM, Lu XR, Shen KY, et al. Comparison of chemical, electrical, and combined activation methods for in vitro matured porcine oocytes. In Vitro Cell Dev Biol Anim. 2015;51:103-12.

[257] Lai L, Sun Q, Wu G, Murphy CN, Kuhholzer B, Park KW, et al. Development of porcine embryos and offspring after intracytoplasmic sperm injection with liposome transfected or non-transfected sperm into in vitro matured oocytes. Zygote. 2001;9:339-46.