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Gradu Amaierako Lana / Trabajo Fin de Grado
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OPTIMIZATION OF SPERM CONCENTRATION FOR *IN VITRO* PRODUCTION OF MOUSE EMBRYOS

Laboratorio di Biologia dello Sviluppo
UNIVERSITÀ DEGLI STUDI DI PAVIA

Egilea/Autor/a:
Arantza Muguruza Montero
Zuzendaria/Director/a:
Prof. Valeria Merico
Sonia Bañuelos

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Supervisor:

Prof. Valeria Merico

Experimental thesis by

Arantza Muguruza Montero

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During my little adventure in Italy, I have studied this huge world of the journey of life. I have understood that this area of research is really important for improving the world and giving a chance to couples that are not able of having children biologically. But it is also important to enlarge our wisdom to know how we are created and what we are. Because all of us have born thanks to this amazing journey.

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1. ABSTRACT

In vitro fertilization (IVF) is an important technique developed from assisted reproductive technologies (ARTs). Although it has been investigated since years, some optimizations should be done in order to determinate the ideal conditions for practicing this technique. In this work IVF was practiced using mice model. One million spermatozoa (sp.) per millilitre and two millions sp/mL were used in IVF in order to determinate an ideal concentration of spermatozoa for obtaining the highest fertilization rate, preimplantation developmental rate and the best blastocyst quality. Pronucleus were stained with DAPI to analyse the fertilization rate. Beside this, blastocyst were stained with DAPI after a hypotonic treatment was done to count the blastomeres for the determination of the blastocyst quality. Significant differences were not found so that using one or the other concentration we obtained similar results. However, using the lowest concentration may reduce the stress of the oocyte obtaining better result in a long-period time.

2. ACRONYMS

2-OHE_2	2-hydroxyestradiol-17β
ARTs	Assistive Reproductive Technologies
BMP8B	Bone morphogenetic protein-8B
BSA	Bovine serum albumin
CCs	Cumulus cells
COC	Cumulus-oocyte complex
DABCO	1,4-diazabicyclo[2.2.2]octane
DAPI	4',6-diamidino-2-phenylindole
FSH	Follicle Stimulating Hormone
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
hCG	human chorionic gonadotropin
I.U.	International units.
ICM	Inner cell mass
ICSI	Intracytoplasmatic sperm injection
IUI	Intrauterine insemination
IVF	In vitro fertilization
IVM	*In vitro* maturation
LH	Luteinizing Hormone
MII	Metaphase II
OHSS	Ovarian hyperstimulation syndrome
PB-I	First Polar body
PB-II	Second polar body
PCOS	Polycystic ovaries symdrome
PFA	Paraformaldehyde
PGC	Primordial germ cells
PGD	Pre-implantation genetic diagnosis
PGS	Pre-implantation genetic screaning
PMSG	Pregnant mare's serum gonadotropin

PN	Pronucleus
RT	Room temperature
Sp.	Spermatozoa
WT	Whittingham
ZP	*Zona pellucida*

3. INTRODUCTION

3.1. FERTILIZATION IN MICE MODEL

3.1.1. Maturation of gametes

3.1.1.1. Folliculogenesis

During early embryogenesis some cells differentiate into primordial germ cells (PGC) that will give the gametes. The oogenesis is referred to the process where the PGC is developed as a mature egg. Once the gonad is assembled from somatic and germ cells, germ cells start to differentiate and proliferate. In the female genital crest, PGCs differentiate as oocytes (Adams I. R. and McLaren A., 2002). However, some of the PGCs, called oogonia, are restricted to germ line differentiation. When first meiosis starts, with the replication of the DNA, the oogonia become primordial oocytes. These cells are arrested in the first prophase of the meiosis and are surrounded by a single layer of follicular cells. The complex forms by a primordial oocyte (10-20 µm in diameter) surrounded of a single layer of follicular cells, is called primordial follicle. When sexual maturation occurs, a group of primordial follicles periodically starts growing under the influence of hormonal stimuli. This process of growth is called folliculogenesis (**Figure 1**).

Although thousands of primordial follicles are present at the time of birth, most of them (around 99.9%) will undergo degeneration without entering into the growing phase.

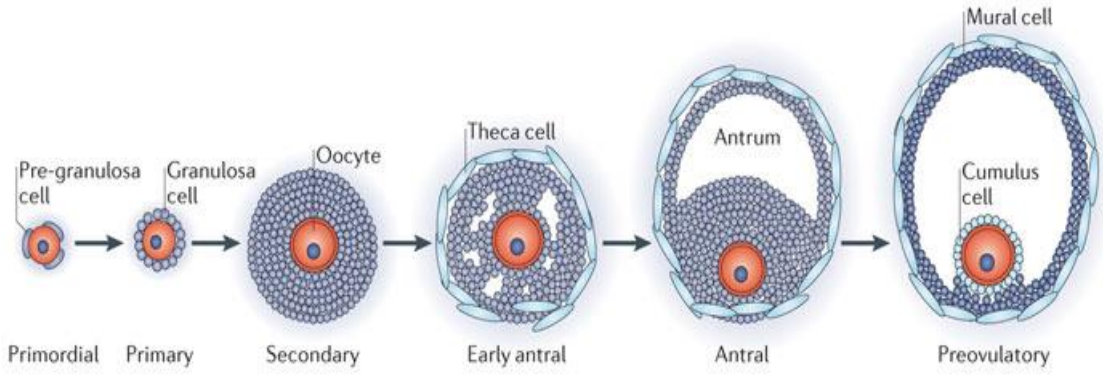

Figure 1. Folliculogenesis of mammals and the different stages of the process. Image adapted from Li R. and Albertini D. F., 2013.

When folliculogenesis oocurs a group of primordial oocytes starts growing and producing some glycoproteic components which create the *zona pellucida* (ZP), a membrane that surrounds each oocyte. Meanwhile, the monolayer of follicular cells surrounding the

oocytes become cuboidal and initiate their proliferation and specialization.

The oocyte is called secondary oocyte (pre-antral oocyte) when it is surrounded by 2-4 layers of granulosa cells (a population of follicular cells). At this stage, cavities filled with follicular fluid start to form within granulosa cells and the oocyte starts to produce mRNAs and proteins required for fertilization and early embryonic development.

Some of the follicular cells will differentiate as theca cells, located in the outer part of the follicle. Bellow the theca cells another population of follicular cells is located, called granulosa cells. A group of these granulosa cells, called cumulus cells (CCs), are directly in contact with the oocyte and they will form a complex known as cumulus-oocyte-complex (COC). The completely developed follicle is called antral follicle (Figure 2), it has an oocyte with a diameter of 70-80 µm and it is surrounded by 3-6 layers of cumulus cells (about 1500 cells). The small cavities that contains follicular fluid aggregate to form a unique follicle *antrum* determining the separation of granulosa cells into two different and specialized cell populations: mural granulosa cells, that form the follicle wall, and cumulus cells, strictly connected to the oocyte.

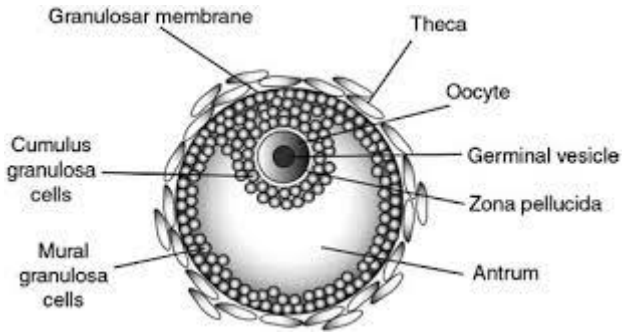

Figure 2. Schematic representation of a late mammalian follicle. Image taken from Veronina E. and Wessel G. M., 2003.

Between the mural cells layer and the theca cells layer there is a basal lamina composed by extracellular matrix, called granulosa membrane. Thanks to this basal lamina and to the theca cells, the structure of the follicle is maintained. Moreover, theca cells play an essential role in fertility by producing the androgen substrate from cholesterol required for ovarian estrogen biosynthesis (Magoffin D. A., 2005). This process of steroidogenesis is tightly regulated by hormones and involves several different signalling pathways. Theca cells will produce androgens when they are stimulated by luteinizing hormone

(LH) and the conversion of the androgens to estrogens will occur in follicle stimulating hormone (FSH)-exposed granulosa cells (Jamnongjit M., Hammes R. S., 2006).

A very important role in the maturation of the follicle is the bi-directional communication in COC between cumulus cells and oocytes. The gap junctions and adherens junctions present in the COC have a key role in the development of both CCs and oocyte. (**Figure 3a**) These junctions not only appear between CCs and oocyte, but also within CCs. On the one hand, they maintain the follicle in a functionally integrated state, that is, the structure of the follicle is maintained. On the other hand, they enable the biochemical communication between the CCs and the oocyte providing both nutritional support and developmental information for the growing gamete. A lot of processes, such as cholesterol synthesis and glycolysis, are regulated by the oocyte for controlling the synthesis of molecules that are created on the CCs and then transferred to the oocyte by

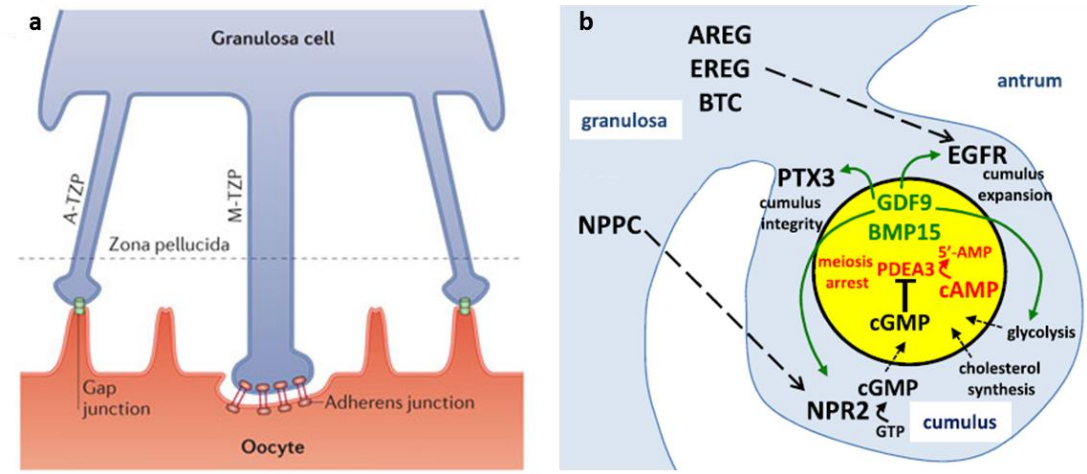

Figura 3. (a) Representation of gap and adherens junctions between cumulus cells and the oocyte. Image taken from Li R. and Albertini D. F., 2013. (b) Oocyte-cumulus cell regulatory loop in large antral and preovolatory follicles. Image taken from Monniaux D., 2016.

gap junctions (**Figure 3b**). This communication is bidirectional, so that, some processes are regulated by CCs and another by the oocyte (Monniaux D., 2016). In the mouse ovary, gap junctions form in advanced follicles (secondary follicles), but are not detected in early follicles (primordial follicles).

When the ovulation occurs a group of COCs are expelled into the oviduct. The theca cells and the mural granulosa cells rest in the ovary and they will develop the *corpus*

luteum, which is a temporary endocrine structure that produces progesterone (a part from other hormones). One of the function of progesterone is the decidualization of the endometrium, that is the process that prepares the endometrium for the implantation of the embryo. The oocyte resumes the meiosis and completes the first meiotic division, with the extrusion of the first polar body (PB-I) (see below) while cumulus cells undergo cumulus expansion. During this expansion, cells start to secrete hyaluronic acid, which becomes hydrated and forms a sticky matrix that wakens the tight junctions between cells.

During the folliculogenesis, the oocyte is blocked at prophase I, the nucleus changes and forms a structure called germinal vesicle (GV) characterized by the presence of 1-2 large nucleoli. The chromatin in the GV is mostly decondensed, dispersed, and transcriptionally active. With the initiation of maturation, transcription starts to stop, the chromatin begins to condense, the GV breaks down (germinal vesicle breakdown, GVBD), and nucleoli disperse (Masui Y. and Clarke H. J., 1979). After the extrusion of PB-I, the chromosomes remaining in the oocyte are again arranged on a meiotic spindle at metaphase II (MII). If the fertilization occurs, the oocyte undergoes to the second meiotic division, chromatids separate and the second polar body (PB-II) is formed. Finally, the chromatids remaining in the oocyte decondense and a pronucleus (PN) forms.

3.1.1.2. Spermatogenesis

Spermatogenesis is the process that produces spermatozoon from the primordial germ cells. Once the PGCs arrive at the genital ridge of a male embryo, they become incorporated into the sex cords. Sex cords hollow out to form the seminiferous tubules and the epithelium of the tubules differentiates into the Sertoli cells. These cells begin to produce anti-müllerian hormone, which induces the regression of the Müllerian ducts. The differentiation of the other somatic cells of the testis, the Leydig cells, occurs in the mesenchyme that surround the seminiferous tubules. They will produce testosterone that will induce the creation of the Wolff ducts.

The initiation of the spermatogenesis during puberty is probably regulated by the synthesis of Bone morphogenetic protein-8B (BMP-8B) by the spermatogenic germ cells, the spermatogonia (Zhao G. Q. *et al*., 1996). The spermatogenic germ cells are bound to the Sertoli cells by N-cadherin molecules on both cells surfaces and by galactosyltransferase molecules on the spermatogenic cells that bind a carbohydrate

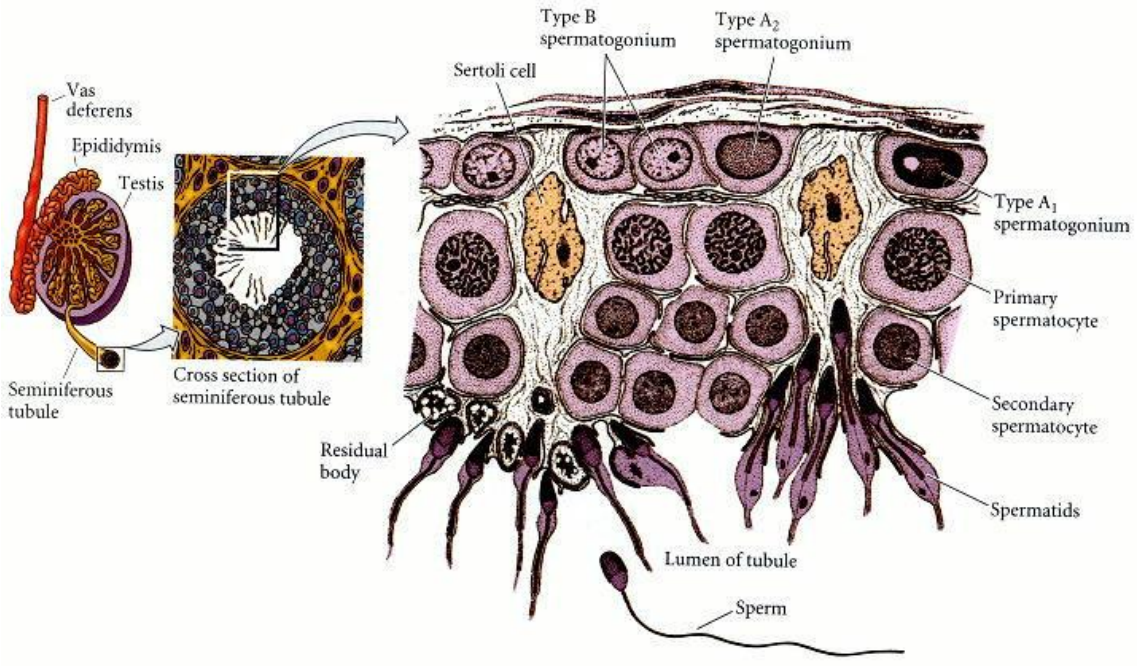

Figure 4. Drawing of a section of the seminiferous tubule, showing the relationship between Sertoli cells and the developing sperm. As cells mature, they progress toward the lumen of the seminiferous tubule. (Image taken from the book of Developmental Biology, 6th edition, Gilbert SF).

receptor on the Sertoli cells (Newton S. C., *et al.*, 1993; Pratt S. A., *et al*. 1993). The Sertoli cells nourish and protect the developing sperm cells, and the spermatogenesis occurs in the recesses of the Sertoli cells (**Figure 4**). Sertoli cells provide factors necessary for the successful progression of spermatogonia into spermatozoa. They have receptors for FSH and testosterone which are the main hormonal regulators of spermatogenesis. Hormones such as testosterone, FSH and LH are known to influence the germ cell fate (Sofikitis N. *et al*., 2008).

After reaching the gonad, the PGCs divide to form type A_1 spermatogonia. These cells are found adjacent to the outer basement membrane of the sex cords. They are stem cells, and at maturity, they are thought to divide so as to make another type A_1 spermatogonium as well as a second type of cell, A_2 spermatogonium. Each A_2 spermatogonium divide to produce two A_3 spermatogonia, that in turn, form the type A_4 spermatogonia. So there are four types of spermatogonium that are able of self-renewal (Figure 5). The A_4 spermatogonium has three options: it can form another A_4 spermatogonium (self-renewal); it can undergo apoptosis; or it can differentiate into the first committed stem cell type, the intermediate spermatogonium. Intermediate spermatogonia are committed

to becoming spermatozoa, and they divide mitotically once to form the type B spermatogonia. These cells are the precursors of the spermatocytes and are the last cells of the line that undergo mitosis. They divide once to generate the primary spermatocytes, the cells that enter meiosis (Figure 5).

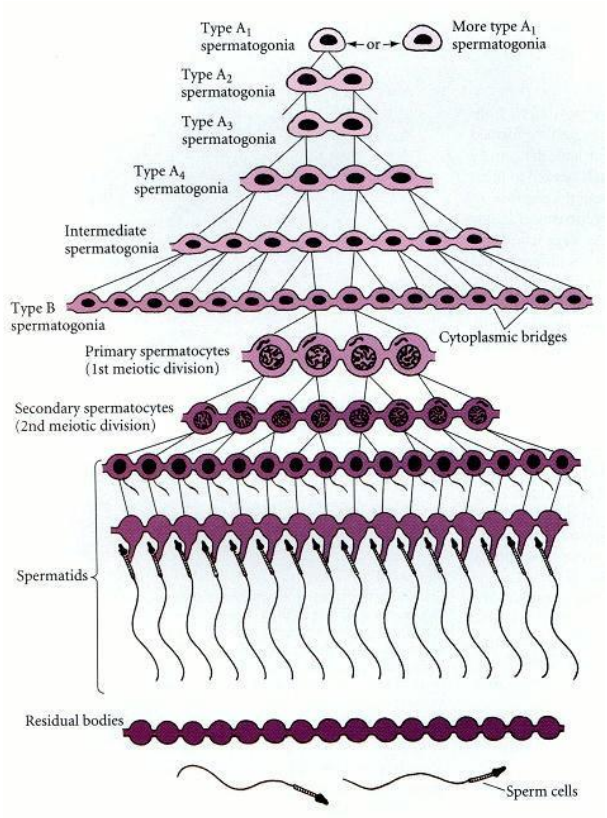

Figure 5. Spermatogenesis and formation of the syncytial clones. (Image taken from the book of Developmental Biology, 6th edition, Gilbert SF).

If we look at **Figure 4** and **Figure 5**, we find that during the spermatogonial divisions, cytokinesis is not complete. Rather, the cells form a syncytium whereby each cell communicates with the others via cytoplasmic bridges (Dym M. and Fawcett D. W., 1971). During this time, the spermatocyte nucleus often transcribes genes whose products will be used later to form the axoneme and acrosome. Each primary spermatocytes undergoes the first meiotic division to yield a pair of secondary spermatocytes. These undergo the second meiotic division forming the haploid cells called spermatids, which are still connected by cytoplasmic bridges creating a syncytium of spermatids. The spermatids that are connected in this manner have haploid nuclei, but are functionally diploid, since a gene product made in one cell can readily diffuse into the cytoplasm of its neighbours (Braun R. E. *et al*., 1989). As we can see in **Figure 4** during the divisions

from type A_1 spermatogonium to spermatid, the cells move farther and farther away from the basement membrane of the seminiferous tubule to its lumen. Thus, each type of cell can be found in a particular layer of the tubule.

Although spermatids are haploid cells, they are still round and unflagellated cells. The process of spermiohistogenesis is the production of the highly differentiated germ cells, this is, preparing the spermatozoon for the functions of motility and interaction necessary for meeting and binding the egg. This process is divided in four main steps: 1) in the Golgi's phase small vesicles of Golgi apparatus fuse, creating the acrosomic vesicle and the centrioles start to migrate to the opposite side of the acrosomic vesicle; 2) in the cap phase the flattening of the acrosomic vesicle occurs and the formation of the axoneme

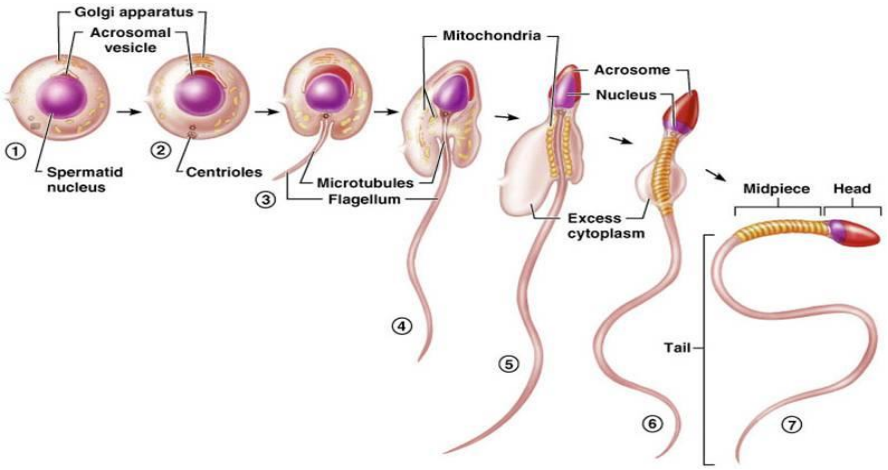

Figure 6. Process of spermiohistogenesis. Golgi's phase 1-2. Cap phase 3-4. Acrosomal phase 5-6. Adult spermatozoa 7.

starts; 3) in the acrosomal phase the nucleus of the spermatid begins to elongate, the acrosome eventually covers the majority of the anterior nucleus and the cytoplasm starts to migrate toward the developing flagellum; and 4) in the maturation phase mitochondria form a spiral assembly around the flagellum that defines the middle piece and they get rid of almost all the cytoplasm (**Figure 6**). While this process is happening, the nucleus have to get really packaged, so that, one of the major changes in the nucleus is the replacement of the histones by protamines, which are small proteins containing over 60% of arginine (Peschon J. J. *et al.*, 1987). During spermiohistogenesis, the nucleosomes dissociate, and the histones of the haploid nucleus are eventually replaced by protamines. These causes the complete shut-down of transcription in the nucleus and facilitates its assuming an almost crystalline structure. After this process of spermiohistogenesis, the resulting

spermatozoon enter the lumen of the tubule.

In the mouse, the entire development process from stem cell to spermatozoon takes 34.5 days. The spermatogonial stages last 8 days, meiosis lasts 13 days, and spermiohistogenesis takes up another 13.5 days.

3.1.2. Stages of fertilization

3.1.2.1. Crossing of spermatozoa in female's genitals vies

After ejaculation, the mammalian male gamete must undergo the capacitation process, which is a prerequisite for egg fertilization that occurs *in vivo* in the female reproductive track. The prostatic liquid confer to the spermatozoon an alkaline pH and coagulant properties, helping the spermatozoon to remain in the uterus neck. Although at the moment of ejaculation sperm cells instantly express high levels of progressive motility, they are completely incapable of recognizing the egg. During this biological journey towards the Fallopian tube, the spermatozoa avoid the maternal immune system and there is no contact with the cells in the reproductive female system. However, when they reach the isthmic region of oviduct this behaviour is suddenly reversed as the spermatozoa establish intimate contact with the endosalpingeal epithelium (Suarez S. S. and Pacey A. A., 2006). In this location, the bound cells establish a quiescent sperm reservoir and remain in this state until they receive a signal associated with ovulation. At this point, the spermatozoa suddenly break away from their epithelial resting place in a hyperactivated state and migrate rapidly toward the oocyte in a state of readiness for fertilization (Suarez S. S., 2008). By the time the spermatozoa have reached the surface of the COCs, they are completely transformed cells by the process of capacitation (**Figure 7**). This process has been shown to be correlated with changes in sperm plasma membrane fluidity, intracellular ion concentrations, metabolism, and motility. The lack of vesicles of cholesterol in the female genital ducks makes that the membrane of the spermatozoa lose the excess of cholesterol, arising the membrane fluidity. Furthermore, the membrane become more permeable to the Ca^{2+} ions, which yield more efficient and energetic the flagella's movement. The metabolism resumes because the uterus and the uterine tubes remove some inhibition factors that have repressed the activity of the spermatozoa in the male genital ducts. Beside this, after capacitation they will express various receptors for the oocyte-cumulus mass on their surface (Reid A. T. *et al*., 2011).

Once the spermatozoa arrives to the COC it will have to cross all the CCs. Therefore, hyperactivation of flagella and digestion of the hyaluronic acid by hyaluronidase are necessary. The movement of hyperactivated sperm basically involves an increase in flagellar bend amplitude thanks to the interaction of calcium ions with the axoneme (Suarez S. S. *et al*., 2003).

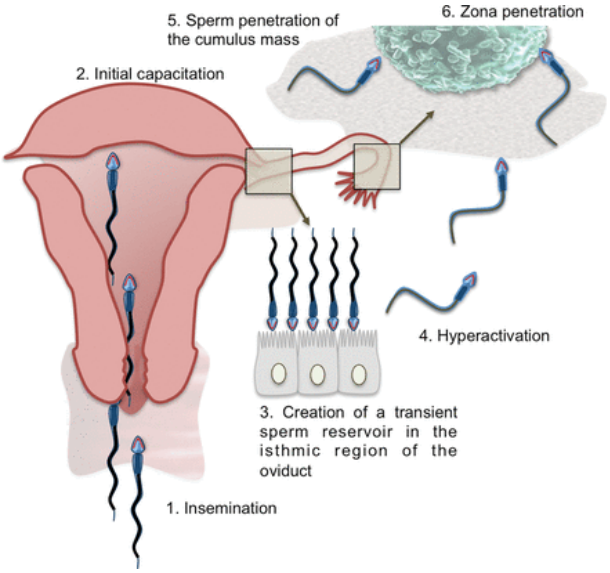

Figure 7. The stage of sperm capacitation *in vivo*. Image taken from Aitken R. J. and Nixon B., 2013.

3.1.2.2. Recognition and contact between spermatozoa and oocyte

The ZP allows the first contact between the spermatozoa and the oocyte. This glicoproteic matrix plays two important roles: 1) it binds the spermatozoa, and 2) it initiates the acrosomal reaction after the spermatozoa is bound (Saling P. M. *et al*., 1979; Florman and Storey, 1982; Cherr G. N. *et al*., 1986). The binding of sperm to ZP is relatively, but not absolutely, species-specific. As we can see in **Figure 8a**, the ZP of the mice is composed by three glycoproteins: ZP1, ZP2 and ZP3. Bleil J. D. and Wassarman P. M. (1980, 1986, 1988) found that the ZP3 is the specific glycoprotein in mouse ZP to which the spermatozoa binds. ZP3 also initiates the acrosomal reaction after sperm have bound to it. The mouse sperm can thereby concentrate its proteolytic enzymes directly at the point of attachment at the ZP.

In mammals the acrosomal reactions only occurs after the sperm has bound to the ZP. The mouse sperm acrosomal reaction is induced by the crosslinking of ZP3 with the receptors for it on the sperm membrane: SP56, kinase SP95 and β1,4-galactosil-

transferase (Leyton L. and Saling P., 1989). This crosslinking opens calcium channels to increase the concentration of calcium in the sperm (Florman H. M. *et al*., 1998). With the increase of this concentration, the acrosomic reaction will start with the fusion of both plasmatic membrane and acrosomal membrane. (**Figure 8b**). The fusion will create some enlarging slits making possible the exocytosis of all the enzymes of the acrosome, such as, acrosin. Moreover, some ionic changes, as the entrance of Na+ and the exit of H^+, cause the acidification of pH in the external medium, activating the acrosomic enzymes.

Beside to the ZP3 binding, there is a secondary binding of sperm to the ZP. In mice, it appears that this secondary binding is accomplished by proteins in the inner acrosomal membrane that bind specifically to ZP2 (Bleil *et al*., 1988).

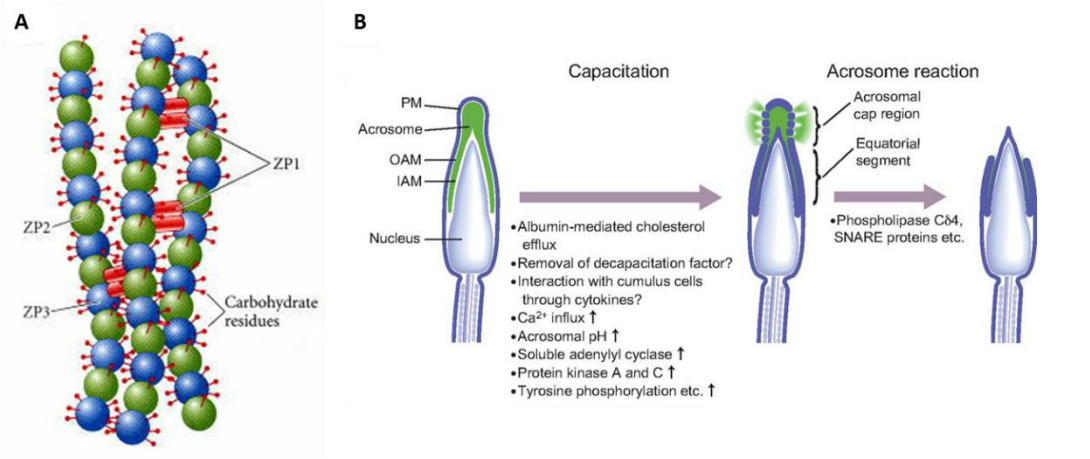

Figure 8. (a) Diagram of the fibrillar structure of the mouse ZP. The major strands of the ZP are composed of repeating dimers of proteins ZP2 and ZP3. These strands are occasionally crosslinked together by ZP1, forming a meshlike network. Image taken from the book of Developmental Biology, 6th edition, Gilbert SF. (b) Acrosomic reaction. (Image taken from Okabe M., 2013).

3.1.2.3. Penetration of spermatozoa into the oocyte

After crossing the ZP, the spermatozoa is in the perivitelline space for then fusing with the plasmatic membrane of the oocyte. The oocyte is covered by microvilli in all the surface, except the area of the PB-II expulsion. The fusion occurs between the molecules IZUMO, located in the equatorial segment of the plasmatic membrane of the spermatozoa, and CD9, located in the microvilli of the plasmatic membrane of the oocyte (**Figure 9**). However, it is thought that some other molecules take part on this process.

When the fusion occurs the membrane of the spermatozoa will stay fused with the membrane of the oocyte.

Upon fusion, spermatozoa activate the egg thereby inducing calcium oscillations and

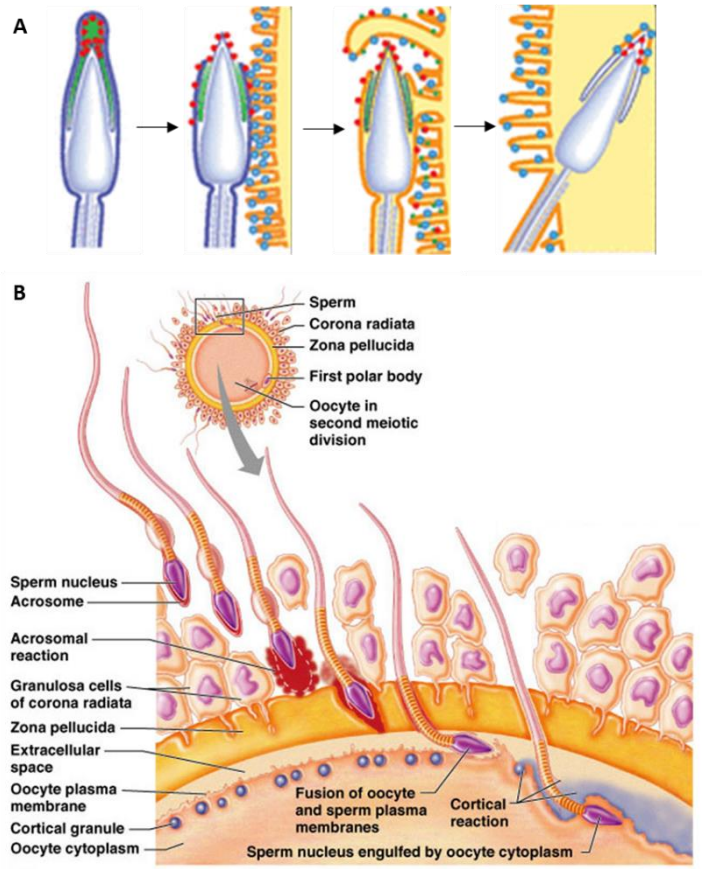

Figure 9. (a) Fusion of both gametes. Image taken from Okabe M., 2013. (b) Process of penetration of the spermatozoa to the oocyte and the cortical reaction.

completion of the second meiotic cell division. Activation of the egg lead to exocytosis from peripherally located cortical granules (Okabe M., 2013). This process is called cortical reaction, and it involves in polyspermic block. In fact, there are two reaction for avoiding the polyspermy: 1) the fast reaction is created by the inversion of the membrane potential because of the Ca^{2+} changes; and 2) the cortical reaction or expulsion of the cortical granules will destroy the ZP.

It is known that when both membranes fuse, not only the nuclear information and the centromere of the spermatozoa enter, but also the mitochondria and the axoneme (Yanagimachi R., 2005). However, they will not be part of the male gamete contribution to the embryo, because of their destruction shortly after fertilization. Sutovsky P. *et al*.

(2000) reported that the sperm mitochondria are ubiquitinated inside the oocyte cytoplasm and later subjected to proteolysis during preimplantation development. Although, the nucleus is the most important sperm component that enters the oocyte, the proximal centrosome adjacent to the sperm nucleus may become the center of the sperm aster that brings the male and female pronuclei to the centre of the zygote.

3.1.2.4. Activation of the metabolism of the oocyte and pronuclei formation

As said before, when spermatozoa fuses with the oocyte, the oocyte will resume the second meiosis by a signal transduction pathway, in which intracellular calcium takes part. In fact, it has seen that the Ca^{2+} has an important role in the oocyte activation. The initial rise of free cytoplasmic Ca^{2+} starts from the site of sperm penetration and expands as a wave through the oocyte (Jaffe, 1983; Whitakerand Swann, 1993). This wave is repeated as calcium oscillations during hours in mammals (Miyazaki *et al*., 1993; Sun *et al*., 1994; Nakada and Mizuno, 1998). Once meiosis II is completed, the oocyte's nuclear material reorganizes with the dispersion of the chromatin by the protamine removal. The pronuclear envelope forms along the periphery of the dispersed chromatin as an aggregation of vesicles which later coalesce (**Figure 10**). This process is similar to nuclear envelope formation in meiotic and mitotic cells (Chang J. P. and Gibley C. W. Jr., 1968).

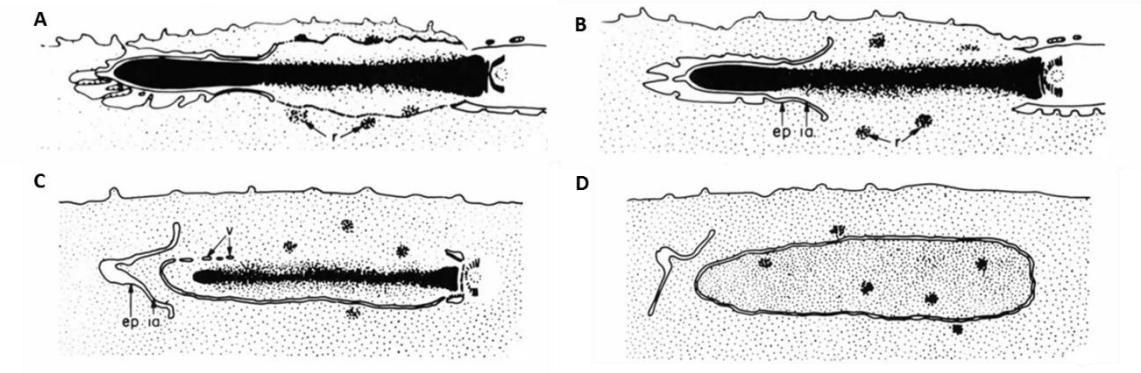

Figure 10. Process of the male pronucleus formation. (a) and (b) Penetration of the nuclear information of the spermatozoa. (c) and (d) dispersion of the chromatin and the formation of the pronuclear envelope. Imagine taken from the book of Fertilization Mechanisms in Man and Mammals, Ralph B. L. Gwatkin (1977), chapter 12, page 104.

Both pronuclei migrated through the center of the oocyte with the help of the sperm centrosome, as mentioned before. An interdigitation zone appear when both nuclear envelopes are almost in contact and the chromosomes duplicated. When all is ready for

the first mitotic division, the envelopes broke and straightaway separation of chromosomes occurs, arising the 2-cell stage embryo (**Figure 11**).

The preimplantation development starts when the spermatozoa fuses with the oocyte and this expels the PB-II. The preimplantation journey, called segmenation, will continue passing from the 2-cell stage, 4-cell stage, Morulae and arriving to the finally stage of blastocyst. In mice the blastocyst use to have between 32 cells close. The first differentiation starts when compactation occurs in Morulae stage. Once that the blastocyst is formed there will be to types of cells: an outer layer of cells forming trophectoderm

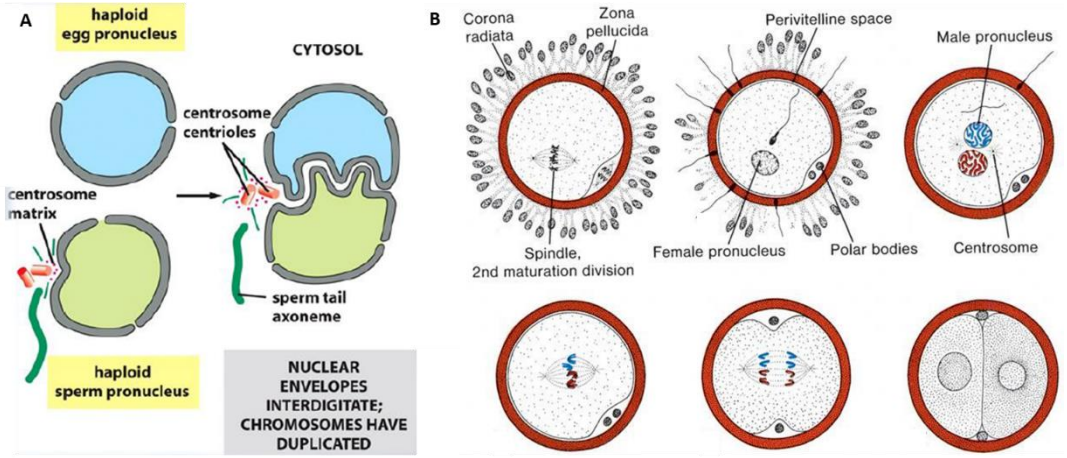

Figure 11. (a) The approaching between both PNs and the nuclear envelopes interdigitation. (b) The different stages of the embryo from the moment that the spermatozoa enters until 2-cell stage.

and the inner cell mass (ICM). When implantation occurs the ICM will develop into the three germs layers.

3.2. *IN VITRO* FERTILIZATION

Infertility is defined as the inability to conceive after one year of regular unprotected intercourse (Quaas *et al*., 2008). It can be created because of different reasons: female or male reproductive diseases or disorders, environmental toxins, smoking, adiposity, patients treated with radio- or chemio-therapy, and age among others. Many studies have shown that an increase in the woman's age leads to a significantly decrease for the ability of conceiving (Dinelli L. *et al*., 2014). Taking into account that nowadays for socio-economical reasons most of the couples are not able to establish a family when the woman is still fertile, this characteristic of age in reproduction becomes a problem.

For all these reasons, on the beginning of the ’80 decade, the Assistive Reproductive Technologies (ARTs) started to develop in order to help couples to conceive and ultimately giving birth to a healthy live baby. Between these treatments called ARTs we have a lot of choices, as intrauterine insemination (IUI), pre-implantation genetic diagnosis/screaning (PGD/PGS), *in vitro* fertilization (IVF) and embryo transfer, intracytoplasmatic sperm injection (ICSI), etc. However, the success rates of the available infertility treatments are still low.

At present, IVF is the most frequently technique used in clinical practice (Nyboe Andersen A. *et al*., 2009). In this technique, first of all, a hormonal treatment is gave to the female patient. Huge amount of exogenous gonadotropins like FSH and LH are administrated for obtaining an adequate number of mature oocytes. The mature oocytes are fertilized *in vitro* and finally implanted in the female uterus.

Although there are drawbacks in IVF, it is actually a useful technique for both research and clinic. In fact, thanks to this technique a lot of process involved in development have been understood. It is true that for clinical aspects there is still plenty of work to do, and the different stages that occurs *in vivo* should be translated to IVF. Some stages of course will never be the same *in vitro* but they can be evaluated for and ideal IVF such as, the quality of the blastocyst, the quality and the number of spermatozoa, the time of fertilization and the quality of the culture media. However, lot of new techniques are developing for helping infertile people and IVF can be the tool for understanding this complicated but amazing journey of the life.

4. AIM OF THE WORK

Preliminary experiments conducted in the Laboratory of Developmental Biology of University of Pavia (Italy) were done for evaluating the in vitro matured oocyte to be in vitro fertilized. The results highlighted that, after the in vitro fertilization, it is present a high percentage of plurifertilised oocytes. This rate of embryos reduces the efficiency of the embryo development. Optimization of IVF procedure could be a good strategy to improve the efficiency of IVF and the further embryo development.

Aim of this project is defined the best concentration of sperm to obtain a higher frequency of normally fertilized embryos (presence of 2 pronuclei) and to reduce the rate of prulifertilized embryo (presence of more than 2 pronuclei) obtained from the IVF of MII oocytes and improve the efficiency of the technique. According with the literature (Fraser and Drury, 1975) the standard concentration of the sperm is 2×10^6 sperm/mL, together this concentration I tested also 1×10^6 sperm/mL.

In order to verify the best concentration, I analyse the efficiency of insemination (analysis of number of pronuclei in the embryos) the developmental rate (blastocyst rate) and in the blastocyst quality (number of the cell forming the blastocyst).

5. MATERIALS AND METHODS

5.1. ANIMALS, REAGENTS AND HORMONAL TREATMENTS

Four-five week old female and 6 month-old male CD1 mice bought from Charles River Laboratories (Como, Italy) were used. Animals were kept in the animal house of the University of Pavia, under controlled conditions of 22ºC, 60% air humidity and a light/dark cycle of 12:12 hours. Researches were carried out in accordance with the guiding principles of European (n. 86/609/CEE) and Italian (n. 116/92, 8/94) lows for animal protection used for scientific research. All chemicals used were purchased from Sigma-Aldrich (St. Luois, MO, USA), unless otherwise stated.

Three days before the sacrifice of animals for *in vitro* fertilization (IVF) experiments, from 2 to 3 females were injected with 10 I.U. of pregnant mare's serum gonadotropin (PMSG) (Folligon, Intervet Srl, Italy), in order to synchronize the maturation of the follicles into the ovary. Then, 15 h before experiments, females injected with PMSG were re-injected with human chorionic gonadotropin (hCG) (Corulon, Intervet Srl, Ital) for causing the superovulation of *in vivo* matured oocytes.

5.2. *IN VITRO* FERTILIZATION

Two different media were used for the IVF: Whittingham (WT) and M16 (**Table 1**). At least 8 hr before the use, WT medium was supplemented with 3% of BSA (bovine serum albumin) and 0.5 M of NaOH was added until the optimum pH was reached. Instead, M16 medium was supplemented with 0.4% of BSA, 2 mM of glutamine (Life Technologies, Monza, Italy), 5 mM of taurine and 25.3 mg/mL of sodium pyruvate. Moreover, WT and

Table 1. Components of the Whittingham and M16 media.

Whittingham components (1 L)		M16 components (1 L)	
NaCl	5.803 g	NaCl	5.698 g
KCl	0.201 g	KCl	0.356 g
$NaHCO_3$	2.106 g	$CaCl_2 \cdot 2H_2O$	0.251 g
$Na_2HPO_4 \cdot 12H_2O$	0.056 g	KH_2PO_4	0.136 g
$MgCl_2 \cdot 6H_2O$	0.102 g	$MgSO_4$	0.143 g
$CaCl_2 \cdot 2H_2O$	0.264 g	Glucose	0.036 g
Glucose	1 g	$NaHCO_3$	2.101 g
Sodium pyruvate	0.055 g	Sodium lactate (60%)	5.53 mL
Sodium lactate (60%)	3.5 mL	Phenol red Na	0.01 g
Phenol red	0.01 g	Pen/Strep (50 U/mL)	5 mL
Pen/Strep (50 U/mL)	5 mL	EDTA (0.1 mM)	1 mL
EDTA	1 mL		

M16 were equilibrated in humidified incubator at 37ºC, 5% CO_2 and maintained in these conditions until use.

5.2.1. Isolation and preparation of spermatozoa

The epididymis from the male mice were isolated and the swim-up method was used in order to get spermatozoa. This method consists in puncturing the epididymis with the help of a needle, placing them on the bottom of a 3 mL sterile tube with 1.5 mL of WT and incubating for 20 minutes in humidified incubator at 37º in 5% CO_2. As Mahadevan M. M. *et al*., (1984) described, after following the swim-up method the best mobile spermatozoa are able to separate from non-motile sperm by swimming from the bottom to the top part of the tube. A capacitation drop of 200-500 µL was done taking from the top part of the tube, covered with mineral oil and incubated for 40 minutes in humidified incubator at 37ºC in 5% CO_2. From the capacitation drop 15 µL of WT containing spermatozoa were taken and mixed with 15 µL of MilliQ water for slowing down and counting the spermatozoa in the Burker's chamber (**Figure 1**). The insemination drop was made (100 µL of final volume) and covered with mineral oil in a 35 mm x 11 mm Petri dish (VWR International), with one or two millions spermatozoa/mL basing of experimental conditions (see below).

Figure 1. Burker's chamber.

5.2.2. Isolation and preparation of oocytes

MII oocytes surrounded by cumulus cells (CCs) were isolated from the oviducts. In order to remove the CCs a treatment with 1 mL of hyaluronidase (500 U/mL) was used. After 5 minutes of treatment the naked MII oocytes were collected with a Pasteur mouth-controlled micropipette and washed in WT drops for eliminating the enzyme.

5.2.3. Insemination

The washed oocytes were transferred in the insemination drops ([Sp.] = 1 million and [Sp.] = 2 millions) for two hours in the humidified incubator at 37 ºC and 5% CO_2 and then for another hour into drops of WT (2 µL/oocyte) covered with mineral oil in the

humidified incubator at 37ºC and 5% CO_2 in order to eliminate the amount of spermatozoa. Finally, after the insemination, the presumptive zygotes were transferred into drops of M16 medium (2 µL/oocyte) covered with mineral oil in the humidified incubator at 37ºC and 5% CO_2, for allowing the development until the analysis of the pronuclei or the analysis of the blastocyst.

5.3. ANALYSIS OF PRONUCLEI

Six hours after the IVF, when oocytes were placed in the insemination drop, the presumptive zygotes were recuperated and washed in drops of 1 × PBS + 0.01 % Tween for eliminating the excess of M16 medium. They were fixed in 4 % paraformaldehyde (PFA) diluted with 1 × PBS for 20 minutes at room temperature (RT). After the fixation the presumptive zygotes were maintained in drops of 1 × PBS + 0.01 % Tween at 4ºC until the experimental processing. The staining was done with 0.2 µg/mL of 4',6-diamidino-2-phenylindole (DAPI) for 10 minutes in the darkness at RT. Before the staining, three wash steps were done with DAPI drops in order to obtain the final staining concentration. After staining, the presumptive embryos were transferred into microscope

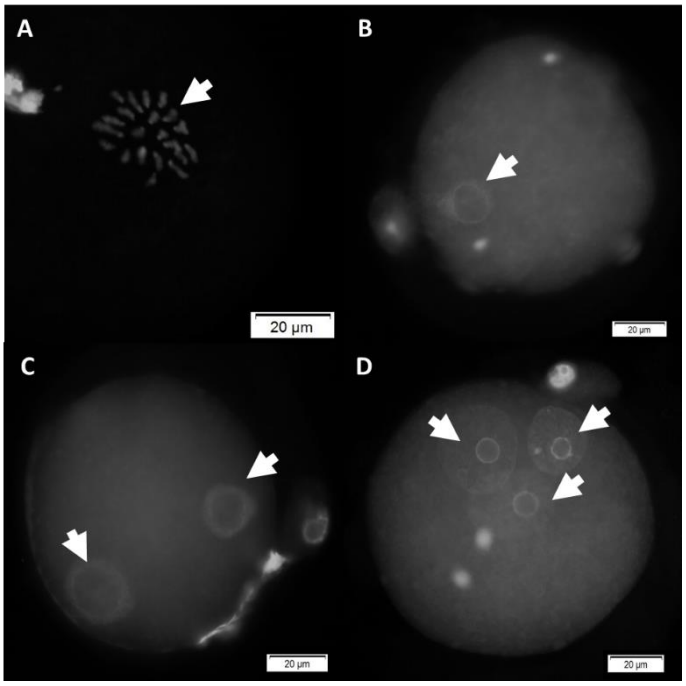

Figure 12. Photos of the nuclei taken with fluorescent microscopy. (A) Oocytes arrested at MII, no pronucleus is present (arrow) but metaphasic chromosomes are seen. (B) Auto-activated oocyte, one pronucleus is present (arrow). (C) Normally fertilized oocyte, two pronuclei are present (arrows). (D) Plurifertilized oocyte, more than two pronuclei are present (arrows), in this case three pronuclei are present.

slide glasses, taking special care on maintaining the different groups separated, the excess of liquid in the slides was eliminated, immediately mounted in 1,4-diazabicyclo[2.2.2]octane (DABCO) and covered with the cover slide. The embryos were maintained in the darkness at 4ºC until the observation.

The analysis was done under a fluorescent Olympus BX60 microscope equipped with a DAPI filter and with a mechanic control that allows acquiring in z-stack. The oocytes/embryos were classified in four groups: 1) stopped oocytes: the oocytes were blocked at MII stage (presence of metaphasic chromosomes) and no pronuclei were present (**Figure 12a**); 2) auto-activated oocytes: presence of one pronucleus was sign of auto-activation of the egg (**Figure 12b**); 3) normally fertilized oocyte; presence of two pronuclei after 6 hours post-insemination (**Figure 12c**); and 4) plurifertilized oocyte: presence of more than two pronuclei (**Figure 12d**).

5.4. ANALYSIS OF BLASTOCYST

For calculating the rate of development, the embryos were checked at different times after insemination, controlling and counting the two-cell-stage, four-cell-stage, Morulae stage (16-32 cells) and blastocyst stage (32-64 cells) (**Figure 13**). Once that the blastocyst stage was reached, the counting of the blastomeres was done. Therefore, blastocysts were putted in a hypotonic solution (0.56 % KCl) for 5 minutes at 37ºC until the swelling of the embryos was seen. Each blastocyst was transferred into one of the squares prepared (5 × 5 mm) with the diamond point pen on the microscope slides and the excess of hypotonic solution was eliminated. Two microliters of Tween 20®/HCl (0.01 M HCl, 0.1 % Tween 20®) (1st solution) were added, and after the evaporation of this first solution, some drops of the fixative Methyl:Acetic (3:1, 2nd solution) were added. The microscope slides were left in the heater at 37ºC for at least 12 hours for allowing the second fixative to evaporate completely.

The samples were rehydrated in a solution of 1 × PBS for 5 minutes at RT. The excess of PBS was eliminated, 10 µL of 0.2 µg/mL of DAPI was added and after covering the slides with plastic paraffin, they were left in the darkness for 10 minutes at RT. To eliminate the excess of DAPI, the microscope slides were rinsed into 1 × PBS solution for some seconds. Finally, the slides were mounted with DABCO and covered with a cover slide.

All the samples were conserved in the darkness at 4ºC until the analysis. The analysis was done under a fluorescent Olympus BX60 microscope equipped with a DAPI filter and with a mechanic control that allows acquiring in z-stack.

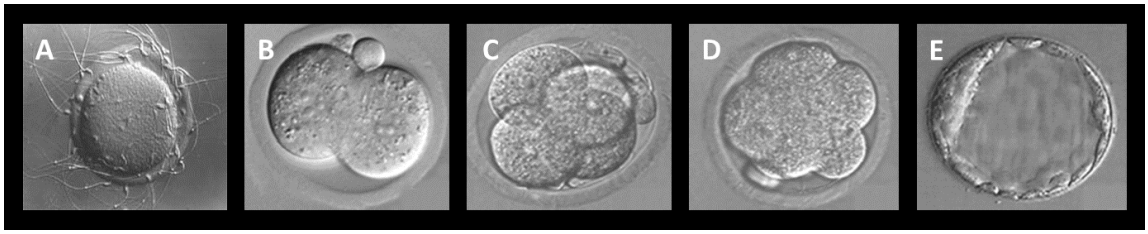

Figure 13. Pre-implantation embryo developmental stages. (A) *In vitro* fertilization or insemination. (B) 2-cell stage. (C) 4-cell stage. (D) Morulae stage. (E) Blastocyst stage.

5.5. STATISTICAL ANALYSIS

The statistical analysis was performed using SigmaStat 3.5 software. Values are expressed as mean ± SD. Student's t test was performed for paired observations between results obtained using 1 or 2 million/mL spermatozoa. A value of $p \leq 0.05$ was considered statistically significant.

6. RESULTS

During this period in Italy, I have done one semester of training period for learning well how to do all the experiments in an accurate way. After learning, I have done five IVF experiments in order to obtain data of the fertilization rate using 2 millions of sperm/mL. Six IVF experiments were done also using 2 millions of sperm/mL (the standard concentration) for taking data about the developmental rate. Beside this, I have done three indipendent experiments using 1 million of sperm/mL for recording data of the fertilization rate and another three experiments using the same concentration for the developmental rate. Eighteen blastocysts, fertilized with 2×10^6 sperm/mL, and N. 6 blastocysts, fertilized with 1×10^6 sperm/mL, were used to analyse the blastocyst quality (**Table 2**).

Table 2. Number of realized experiments.

	1×10^6 sperm/mL	2×10^6 sperm/mL
Nº of experiments for the determination of fertilization rate.	3	5
Nº of experiments for the determination of the developmental rate.	3	6
Nº of blastocysts use for the determination of blastocyst quality.	6	18

The comparison between both concentrations in the fertilization rate and the developmental rate is resumed in **Table 3** and **Table 4** respectively. Moreover, in **Table 5** we can see the resumed data of the analysis of blastocyst quality.

Fertilization rate was determinate depending on the number of pronuclei present in the zygote at six hours post-insemination. The putative zygotes were observed after 6 h.p.i. and the presence of the pronuclei was analyzed and classified in: 1) not fertilized egg (0 pronucleus); 2) auto-activated egg (1 pronucleus); normally fertilized zygote (2 pronuclei) and 4) abnormally or plurifertilized egg (>2 pronuclei) (Figure 12). As observed in Table 3 and **Image 14** with 2×10^6 sperm/mL we obtain a higher number of non-fertilized zygotes and normally fertilized zygotes. When a lower concentration was use (1×10^6 sperm/mL), autoactivated zygotes and plurifertilizated zygotes show a higher number. However, these data are not significant because the high variability of the results (high value of standard deviation, s.d.).

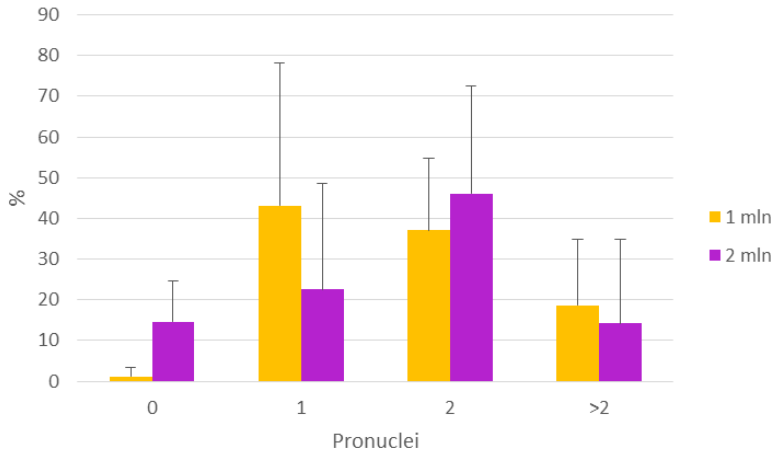

Image 14. Graphic of fertilization rate. Percentage of zygotes is presented depending their number of pronuclei for both concentrations.

If we compare all the stages during pre-implantation development, during 2-cell-stage and 4-cell-stage there is no difference between the two concentrations. In the last two stages instead, it can see that using 2×10^6 sperm/mL we obtain a higher number of morulae and blastocyst, although it is not significant ($p > 0.05$) (Table 4 and **Image 15**).

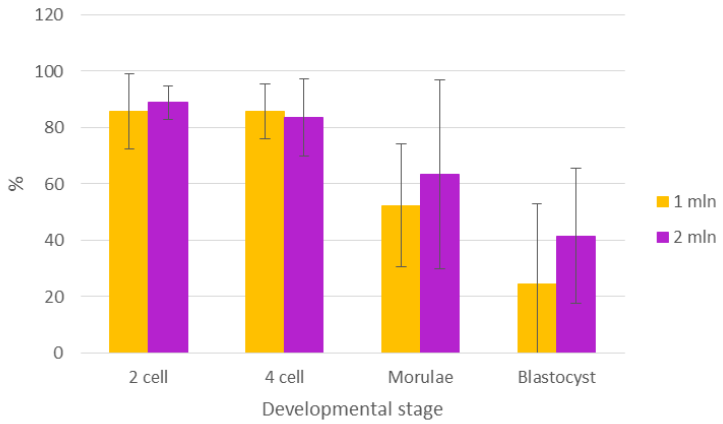

Image 15. Graphic of pre-implantation developmental rate. Percentages of embryos in each pre-implantation stage are presented in respect to both concentrations.

In order to analyse the blastocyst quality, the blastomeres were counted after a long procedure of fixation and staining with the DAPI fluorochrome (for technical details see materials and methods) (**Figure 16**). The quality of blastocysts is independent by the

sperm concentration used. These data were obtained when the number of blastomeres was calculated (Table 5 and **Figure 17**).

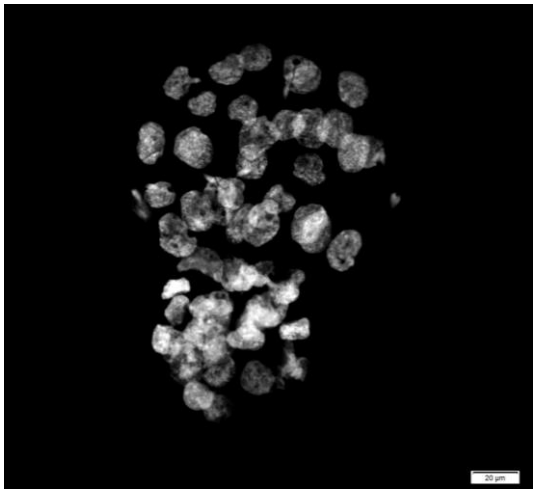

Figure 16. Blastocyst stained with DAPI.

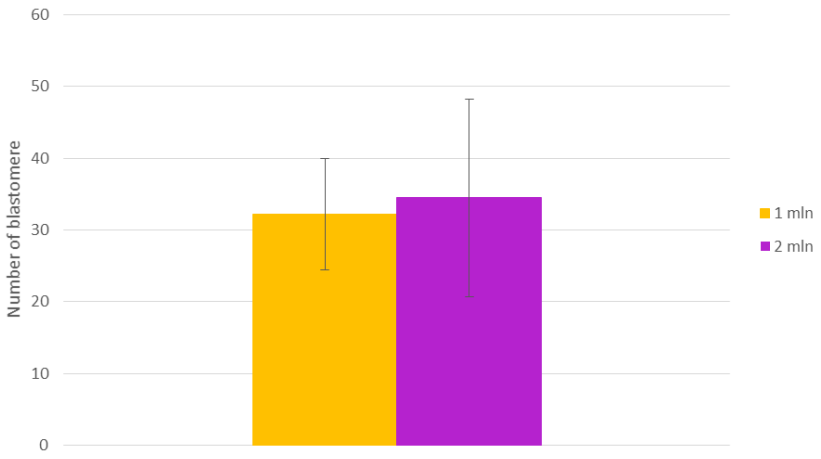

Figure 17. Graphic of the quality of the blastocysts for both concentrations. The quality of blastocysts is represented by the number of blastomeres.

Table 3. Fertilization rate.

Sperm/mL	Experiment N.	MII	N. of proculei (%)			
			0	1	2	>2
1×10^6	1	10	0 (0)	2 (20)	5 (50)	3 (30)
	2	6	0 (0)	5 (83.33)	1 (16.67)	0 (0)
	3	27	1 (3.7)	7 (25.92)	12 (44.44)	7 (25.92)
	Total	14.33 ± 11.15	0.33 ± 0.58 (1.23 ± 2.14)	4.67 ± 2.52 (43.08 ± 34.98)	6 ± 5.57 (37.04 ± 17.86)	3.33 ± 3.51 (18.64 ± 16.27)
2×10^6	1	16	1 (6.25)	7 (43.75)	4 (25)	2 (12.5)
	2	8	2 (25)	0 (0)	6 (75)	0 (0)
	3	8	1 (12.5)	1 (12.5)	6 (75)	0 (0)
	4	8	2 (25)	0 (0)	2 (25)	4 (50)
	5	23	1 (4.34)	13 (56.52)	7 (30.43)	2 (8.69)
	Total	12.6 ± 6.77	1.4 ± 0.55 (14.62 ± 9.95)	4.2 ± 5.72 (22.55 ± 26.08)	5 ± 2 (46.09 ± 26.49)	1.6 ± 1.67 (14.24 ± 20.73)

Table 4. Preimplantation developmental rate.

Sperm/mL	Experiment N.	MII	N. of embryos (%)			
			2-cell	4-cell*	Morulae*	Blastocyst*
1×10^6	1	14	14 (100)	12 (85.71)	4 (28.57)	1 (7.14)
	2	30	22 (73.33)	21 (95.45)	12 (57.14)	2 (9.52)
	3	25	21 (84)	16 (76.19)	15 (71.43)	12 (57.14)
	Total	23 ± 8.18	19 ± 4.36 (85.78 ± 13.42)	16.33 ± 4.51 (85.78 ± 9.63)	10.33 ± 5.69 (52.38 ± 21.82)	5 ± 6.08 (24.60 ± 28.20)
2×10^6	1	24	20 (83.33)	17 (85)	15 (75)	14 (70)
	2	9	8 (88.88)	8 (100)	8 (100)	4 (50)
	3	10	9 (90)	8 (88.88)	4 (44.44)	3 (33.33)
	4	13	11 (84.6)	9 (81.81)	8 (72.72)	6 (54.54)
	5	39	34 (87.17)	20 (58.82)	2 (5.88)	0 (0)
	6	24	24 (100)	21 (87.5)	20 (83.33)	10 (41.66)
	Total	19.83 ± 11.51	17.67 ± 10.25 (88.99 ± 5.95)	13.83 ± 6.18 (83.67 ± 13.65)	9.5 ± 63.56 (63.56 ± 33.54)	6.17 ± 5.08 (41.59 ± 23.85)

*Calculated in respect to 2-cell embryos.

Table 5. Blastocyst quality.

Number of blatomere ± SD	
1×10^6 sperm/mL	2×10^6 sperm/mL
32.17 ± 7.78	34.44 ± 13.70

7. DISCUSSION

Previous experiments in my same laboratory of Developmental Biology in the University of Pavia (Italy) demonstrated that 1.8×10^6 sperm/mL is the ideal concentration in order to perform IVF (Nabi Dalileh, master thesis, 2014/15). The commonly used sperm concentration for IVF experiments vary from 1 to 2 million of sperm per millilitre. (Ge L. *et al.*, 2008; Mori C. *et al.*, 1988; Li M. W. *et al.*, 2016). However, it would be interesting to reduce this concentration in order to improve IVF, reducing like this the number of plurifertilized oocytes. Because of all of this reasons and in order to establish an ideal concentration of sperm I have done all this setting during my short period in Italy with the Erasmus grant. Therefore, I have observed what happens if the concentrations decreases from 2×10^6 sperm/mL to 1×10^6 sperm/mL.

Although in the results we can see visually some differences, there is not significant through this two concentrations. However, as it can be seen at the **Image 14**, the number of oocytes that were not fertilized decreases when 1×10^6 sperm/mL was used. Nevertheless, the rates of plurifertilized oocytes and autoactivated oocytes increase using less sperm concentration. Although Lu K. H. and Seidel Jr. G. E. explain that when they used a higher concentration of sperm the plurifertilization rate was higher, I obtain the opposite. Beside this, during the preimplantation development we can see that when 2×10^6 sperm/mL was used a mayor rate of blastocyst was obtained (**Image 15**). I want to emphasize that all this differences are just visuals and not significant, so the main conclusion of my project is that there is no difference between this two concentrations in the preimplantation development and in the fertilization rate.

I would like to mention that reducing the usual concentration used in our laboratory (1.8×10^6 sperm/mL) to 1×10^6 sperm/mL does not affect the IVF outcome. Nevertheless, the oocytes suffer less stress with decreasing number of spermatozoa.

In order to support these results and this conclusion in the future some other experimental settings should be done. Reducing drastically the concentration (10^5 sperm/mL or 10^4 sperm/mL), IVF could be improved because the sperm would not stress so much the oocytes. Another reason for explaining that the oocytes can be stressed in our experimental protocol is that we let them with the sperm for two hours (2 hr in the insemination drop + 1 hr in WT drop) (see materials and methods,). New experiments could describe if there is any difference in the fertilization rate if we let the oocytes for 1 hr in the insemination drop and then two hours in the WT drop (1 hr +2 hr).

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