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Master of Science Thesis

Study of Porcine *in vitro* Fertilization: Effect of Sperm Selection and Concentration on the Blastocyst rate

Full-Time Master of Science

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Abbreviations

AI: Artificial Insemination

AID: Acrosome Intact Dead cells

AIL: Acrosome Intact Live cells

ARD: Acrosome Reacted Dead cells

ARL: Acrosome Reacted Live cells

CASA: Computer Assisted Sperm Analyzer

COC(s): Cumulus-Oocyte Complex(es)

EGF: Epidermal Growth Factor

IVC: *in vitro* Culture

IVEP: *in vitro* Embryo Production

IVF: *in vitro* Fertilization

IVM: *in vitro* oocyte Maturation

PXM: Porcine X Media

RT: Room Temperature

ZP: Zona Pellucida

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Abstract

Porcine reproductive biology has gained more interest recently due to increase in pig meat requests; demand to enhance and preserve the good genetic material of the animal, along with improvement of animal genetic transportation biosecurity made the reproductive biotechnology create more suitable ways to achieve this destination, *in vitro* embryo production (IVEP) is one of this ways. Norsvin SA is a research and development-based company looking for optimization of the IVEP process. Porcine IVEP is more challenging than most domestic animals, due to gamete sensitivity. On the other hand, for the research purposes, due to the utilization of oocytes from the slaughterhouse, assessment, and control of the oocyte quality is even more out of reach, therefore, in this study, the main focus was on sperm cell quality (motility criteria, viability, acrosome reactivity) assessment by CASA and flocytometry. This study tried to find standardization for sperm selection by examining two different percoll batches and storage time. Furthermore, it was proven by prior studies that sperm concentration in IVF plays a crucial role in the fertilization and blastocyst rate; therefore after sperm selection by percoll discontinuous gradient, and oocyte selection and maturation two different sperm concentrations per COC were applied: 1'000:1 ratio (normal) and 2'000:1 ratio (double). The data analysis after culturing potential zygote revealed no significant difference between the two treatments at cleavage and blastocyst rate (day 6 and 7) (n= 935; P>0.05). However, in polyspermy assessment of a total of 120 cells, 0.0%, and 13.4% polyspermy were observed in normal and double sperm concentrations respectively. In a conclusion, it was proven that the 1'000:1 ratio of sperm cell/COC was seemed to be a more suitable ratio for IVF.

Keywords: *in vitro* embryo production (IVEP), *in vitro* fertilization (IVF), sperm selection, cleavage and blastocyst rate, polyspermy

1. Introduction

1.1 Importance of Porcine *In Vitro* Embryo Production (IVEP)

The Porcine in vitro embryo production (IVEP) importance can be discussed in two different aspects: agriculture, and biomedicine and researche; however if we want to combine these two fields, it is beneficial to observe usefulness of these techniques in the field of biotechnology as well.

1.1.1 Agriculture

A human being needs carbon and nitrogen source which comes from the foods for producing energy, and for metabolism. One of the most important food sources after crops are animal meats which can be considered as a very good source of nitrogen for metabolism; the two most commonly used meats worldwide are poultry and swine meat whose demand is rising yearly, from 75 million tonnes in 1994 the pork meat production reached approximately 125 million tonnes in 2018 (figure 1.1); Europe possesses 25.9% of this production (UN Food and Agriculture Organization (FAO), 2022). This demand and importance of pig meat might be due to its adoption characteristic to different environments and weathers types, high fertility which comes from a short maturation and gestation time, and plurality of the offspring ("ROTHSCHILD, M. F., RUVINSKY, A. (Eds)," 2001; Whittemore et al., 2006).

Recent pig production can be divided into four main areas: breed to wean, which specialized for transferring gilts to sows, breeding and lactation; gilts development units; boar stud, which is semen resource; and finally farrow to finish, which focuses on the farrowing and growing the piglets (Knox, 2014). Use of proper genetic selection system, improvement in pigs fertility, and most importantly reproductive management (in two last areas mentioned above) are the most common reasons for improvement of farrowing rate in this ten years (Knox, 2014). As a result of several years efforts to conserve, propagate, and improve

genetic and phenotypic properties of boars and gilts, the yield of swine production is going higher to fulfil the demands; one of the technologies, that is used since 1990s, is *in vitro* embryo production, usually, by means of IVF; this technology helped to conserve and improve (good) genetic traits (by using frozen semen of good reproductive boars (Knox, 2014), and oocytes of genetically/phenotypically selected sows), in addition to yield up production from ovarian pools by utilizing immature oocytes (Grupen, 2014).

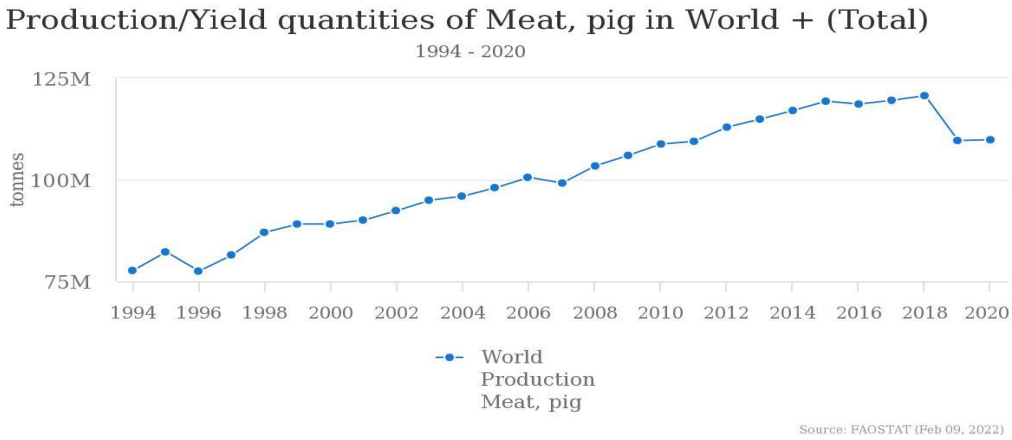

Figure 1.1 *United Nation Food and Agriculture (FAO) diagram about total pig meat production, in tonnes, in the world corresponding to the years.*

What is called domestic pig is scientifically classified as: *Sus* (Genus), *scrofa* (Species), *domestica* (subspecies). Domestic swine birth weight can be 1-2 kg; they may reach up to 100-110 kg in commercial farms, depending on the nutritional situation, as early as less than one year age (according to the Norsvin SA, 5.5 months Norwegian Landrace can weigh up to 120 kg); the life span of swine in a natural situation is 15-25 years, however, in commercial farms they usually live less than six months for meat production and about five years for breeding purpose (Smith & Swindle, 2016). Swine are organisms with 38 chromosomes; they are polytocous and it takes approximately 114 days to complete gestation, in a bicornuate womb (figure 1.2). Estrous cycle can vary between 18 to 24 days (average 21 days) with an estrus duration of two days, usually after 30-36 hours ovulation occurs. At parturition typically 4 to 20 piglets are born, and each sow is able to have two litters per a year for almost 5-6 years (Smith & Swindle, 2016).

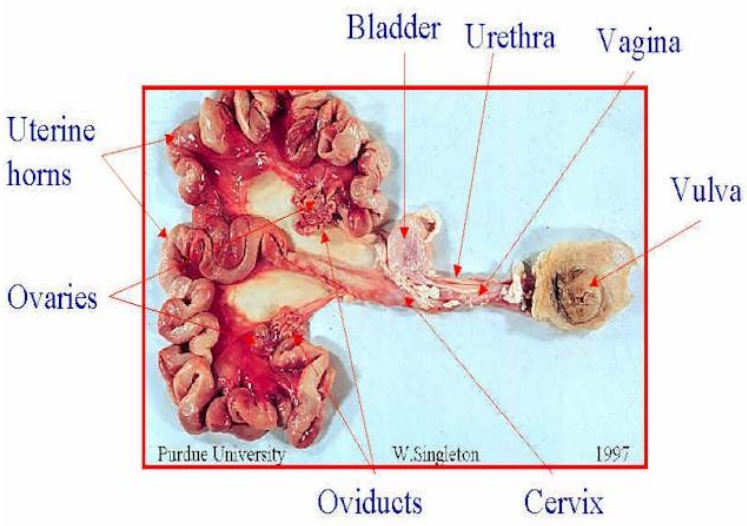

Figure 1.2 *An anatomical picture of the bicornuate uterine of a female pig; notice the ovaries location within the uterine horns. (Singleton & Diekman, n.d.)*

1.1.2 Biomedicine and Research

As opposed to the wide range of rodents utilization, specifically mouse, in biomedical research as a model and representative for human cells. There are some differences between mouse and human such as cell's reaction to mutagens, physiology, protein interactions, anatomy, and size which make it not very ideal as a model for human medical research (Flisikowska et al., 2016). For instance, in one study the affectivity of mouse model in breast cancer was investigated and the result supported the deficiency of mouse as human model for research (Vargo-Gogola & Rosen, 2007). On the other hand, pigs are sharing many similarities with human (body size, anatomy, physiology and pathophysiology), in addition they can live long enough to study some effects and side-effects of diseases or treatments; therefore, as in mouse, genetically modified swines are needed. This modification can be done either in somatic or germinal cells. Hence, optimization of IVF and *in vitro* embryo production (IVEP) is important if the pig germinal cells undergo manipulation (Flisikowska et al., 2013, 2016). The other field of porcine cells usage is in reproductive toxicology, for example for study of environmental toxicity in oocyte *in vitro* maturation (IVM), by

utilization of slaughterhouses material (ovaries), many animals life can be saved (such as mice) (Santos et al., 2014). In addition to the reasons mentioned above about similarities between human and pigs. Thus porcine IVF/IVEP study might be a potential method for rising up quantity and quality of produced embryos in the laboratory.

1.1.3 Biotechnology and Porcine Reproduction

Porcine reproductive biotechnology concentrates to increase the yield of porcine reproduction and, simultaneously, enrich the genetic pool; the other goal is to maintain diversity and sanity of animal production and live stock trading by utilizing technologies such as gamete and embryo collection and preservation (e.g. cryopreservation), IVEP, culturing techniques, embryo modification (gene transfer, chimerization, and etc.), and transferring the embryos (Naqvi, 2007; Okere & Nelson, 2005). In the current swine industry the main genetic resource is the live animal, shipping and transferring them may not only cause disease transmission, but is also uneconomical (Okere & Nelson, 2005). One of the technologies that is used for gene transfer is “Artificial Insemination (AI)”, however, as opposed to its effectiveness, high risk of disease transmission (e.g. porcine reproductive and respiratory syndrome (PRRS) which is spread by a mutative, low temperature resistant virus, potentially transmitted by semen) (Knox, 2014), make the industries to find some safer and more cost-effective ways to transfer genetic material. IVEP is one of this ways, although the efficiency of this method is not high enough to use as a primary method currently, due to porcine gamete and embryo sensitivity to manipulations (Martinez et al., 2005; Okere & Nelson, 2005).

1.2 Oocyte selection, and *in vitro* Maturation (IVM)

Oocyte is the name for developing egg cell (ovum), i.e. a matured cell, under a process called oogenesis; during fetal stages of the female pig oogonia (the ovum stem cell) undergoes division and produces certain amount of precursor, some of the precursors will experience first meiosis, these cells called primary oocytes (Gilbert, 2000). The main

difference in mammalian ovum and other cells is during oocyte development meiosis arrests in two different points, first before birth to puberty in the prophase I and second is after puberty until ovulation, that stops in the metaphase II of meiosis; the end product of this process is secondary oocyte with the first polar body, and the ovum (after fertilization) including the second polar body respectively for the first and second oocyte dictyate (Mira, 1998). As a result of uneven distribution of cytoplasm in the meiosis cytokinesis of oocytes, ovum is the largest cell in animals, and possesses all the necessary organelles, components, and nutrition for developing to an embryo (Gilbert, 2000). As stated before, estrous cycle of the pigs varies between 18-24 days, the follicular and luteal phase take 5-7 and 13-15 days respectively; within follicular phase normally 15-30 oocytes will be released; the process is controlled by positive and negative feedback of several hormones such as gonadotrophin-releasing hormone (GnRH) from the hypothalamus, follicle-stimulating hormone (FSH), luteinizing hormone (LH) oxytocin and prolactin from the pituitary (hypophysis), progesterone (P4), 17β-estradiol (E2), inhibins and relaxin from the ovaries, and prostaglandin F2α (PGF2α), from the uterus (Soede et al., 2011).

Hence for IVF (mainly for research purposes) the oocytes are normally captured from ovaries of the dead pigs, from slaughterhouses, within a complex with surrounding cells called “cumulus oophorus” the complex is also called “cumulus-oocyte complex (COCs)”. Since oocytes within the ovarian follicles are not completely matured, they need to be matured *in vitro*. Cumulus cells are responsible for sterol biosynthesis, regulating gene transcription, helping for transporting amino acids and providing energy by glycolysis promotion, and finally a good physical protection for oocytes, thus, they are necessary for the oocyte *in vitro* maturation (The Evian Annual Reproduction (EVAR) Workshop Group 2010 et al., 2011). Pig IVM was not as successful as for other species; *in vitro* maturation duration takes about 40-44 h. Several media has been tested for IVM, for instance Tissue Culture Medium (TCM)-199, Waymouth MB 752/1, North Carolina State University (NCSU23), and modified Tyrode’s solution (mTLP-PVA) in addition to porcine follicular fluid and cysteine to promote sperm pronuclear establishment (Redel et al., 2019). In the porcine IVEP the oocyte maturation is a crucial step since embryo development is directly depending on the quality of oocyte nuclear and cytoplasmic maturation (Redel et al., 2019); according to two early investigations, this effect is due to disruption of pronuclear formation, and permission of multiple sperm to enter zona pellucida (polyspermy) (Naito et al., 1988; Niwa, 1993). Different applications have been practiced to overcome this bottleneck

including changing or adding some substances to the maturation media for example supporting the medium with epidermal growth factor (EGF) or FLI (Fibroblast growth factor 2 (FGF2), leukemia inhibitory factor (LIF), and insulin-like growth factor 1 (IGF1)), application of different sperm concentration and reducing time of coincubation in IVF procedure to lower the polyspermy rate which sometimes can be as high as 50% (Redel et al., 2019).

1.3 Role of Sperm cells in Fertilization

Anton van Leeuwenhoek (1678) first discovered sperm cells, however, he did not know about the role of these cells in reproduction, therefore, he Hypothesized these were parasitic animals contaminating semen (the name spermatozoa, indicates this fact, means "sperm animals"). The role of sperm cells were not recognized until 1824 when J. L. Prevost and J. B. Dumas confirmed the importance of these cells in fertilization (Gilbert, 2000). Sperm cells are very small (ca. 53 µm length) and motile, due to the tail, without any normal organelles and ribosomes and contains three main parts: head, midpiece, and tail. The head is a place of genetic material (nucleus) and an modified organelle accommodating hydrolyzing enzyme for penetration of sperm cell into ZP called "acrosome". The midpiece holds mitochondria for tail ATP production; the tail duty is to motor the sperm movement by hydrolyzing ATP (protein called Dynein). Sperm cells are paternal cells which carry the genetic material (haploid) to the haploid oocyte and fertilize it by penetrating into the zona pellucida (ZP) of the MII oocyte in mammals; (Alberts, 2002; Darszon et al., 2005). According to Levitas et al., 2007; and Silva & Gadella, (2006), for mammalian successful fertilization, in spermatozoa aspect, several factors are necessary such as: motility status (total motility, progressive motility, and hyperactive motility are important examples), viability, acrosome integrity, sperm concentration, etc.; different instruments are developed for measurement of these criteria; computer assisted sperm analyzer (CASA) and flowcytometry are for motility, and viability-acrosome integrity assessment respectively (Ugur et al., 2019).

1.3.1 Sperm Selection for IVF

For IVF procedure sperm cells need to be selected and prepared, meaning to separate motile and progressive sperm from non-motile sperms, in addition to remove interfering agents like diluent media, and cryoprotectant, and also capacitate sperm cells for IVF. For selecting sperms there are various methods, dilution and wash method: sperm migration, density gradient method, and adherence method are so far implemented (Gadea et al., 2020). Many studies support density gradient method rather than others (Berger & Horton, 1988; Grant et al., 1994; Mortimer, 2000; Matás et al., 2011).

1.3.2 Density Gradient by Colloidal silica

Application of colloidal silica goes back to 1959 where Mateyko and Kopac used this method to separate cells, however, it was discovered that the pure silica is toxic to cells, hence, some polymers also added to the silica to maintain the pH at the neutral area in addition to be iso-osmatic; in 1977 a silica base colloid were introduced named "percoll", the average molecular weight of percoll is 6×10^6 g/mol, and less than 20 mOsm osmolality at a concentration of 1.13 g/ml; the percoll is covered by polyvinylpyrrolidone (PVP) (Pertoft, 2000).

1.4 *In Vitro* Fertilization (IVF) and *In Vitro* Embryo Production (IVEP):

In vitro literally means "in glass" which came from a Latin root; fertilization is also, generally, a term that describes fusion of male and female gametes together to form a diploid cell called zygote for further development to reach embryotic stages; Therefore, *in vitro* fertilization is pointing to the fertilization out of normal reproductive organs, in a laboratory environment (tubes or petri dishes). The earliest experiments are related to the end of 1800s by Walter Heap in rabbits (Zhao et al., 2011), after that many successful experiments were done by various scientists in different species; the beginning of human IVF was started

somewhere around 1950s by fascinating experiments of Robert Edwards and lead to the first human (Louise Brown) whose birth was assisted artificially on 1978 (Edwards, 2001), since then many children were born by IVF procedures.

The history of swine IVF relates to 1986 where Cheng et al. successfully used IVF technique to make embryos and transfer them to the surrogate pig, as a result, 19 offspring were born (Gadea et al., 2020). In the past days there were some problems that needed to be overcome such as low quality and quantity matured oocytes as well as defective sperm penetration, whereas today the main problem in porcine IVEP is polyspermy; as opposed to numerous studies to solve this problem or optimizing the procedure, to date there is almost no decent protocol which guarantees good outcome, high rate of blastocyst with potential to progress to the further embryotic stages (monospermic) and the success rate in most of the systems is around 40% (Gadea et al., 2020). The culture media usually contain inorganic salts, nutrients, vitamins, and growth factors. The other factors which play an important role are pH, gases, and humidity (Gadea et al., 2020); in contrast with the physiological pH in the porcine oviduct (pH: 8), in IVF procedure usually neutral pH is used (7.4) (Rodriguez-Martinez, 2007); oxygen is, on the other hand, important normally it is around 20% in the incubator in companion with 5% CO_2, the temperature should also be maintained at 38.5ºC, the pigs physiological temperature, although, there are some studies, due to normal temperature and oxygen pressure of oviduct, suggesting to use less oxygen (~ 7%) and temperature (37ºC) for higher success rate (Gadea et al., 2020; García-Martínez et al., 2018).

2. Material and Methods

The entire experiment was performed at Høgskolen i Innlandet (Inland Norway University of Applied Sciences), Biohuset (Faculty of Applied Ecology, Agricultural Sciences and Biotechnology), Cell biology and IVF laboratories. All the reagents and chemicals used in this study were produced by the Sigma-Aldrich chemical company (Germany) unless otherwise stated.

2.1 Experimental Design

In this experiment first it was tried to find the standard for sperm selection by using percoll® gradient; Therefore, in case of assurance the motility (total, progressive, and hyperactive) of the sperms were assessed and were compared between two different batches. Although unopened percoll batches can be stored for as long as two years, and opened one should whether be kept in the < 8 ºC (Sigma-Aldrich official website https://www.sigmaaldrich.com/NO/en/product/sigma/p1644), there is no specific study regarding the quality of sperm cells and the storage time of 90% percoll used to sperm preparation gradient (45%/90%); thus the effect of different storage times of 90% percoll, fresh, and old (more than two weeks) on sperm motility were evaluated. The proper percoll (in case of batch and storage time) was used to assess the four different boars sperm samples (fresh and frozen), in case of motility, viability, and acrosome integrity to find the most suitable boar to utilize the sperm for the IVF experiment. The main focus of study, however, was on applying two different concentrations of progressive motility adjusted sperms in relation to the collected and matured oocyte, normal (1’000 sperm cells per each oocyte) and double (2’000 sperm cells per each oocyte), in *in vitro* fertilization process in order to study the cleavage and blastocyst rate of embryos after culturing. Alternatively, polyspermy rate of the fertilized eggs was inspected; figure 2.1 is showing the whole process schematically.

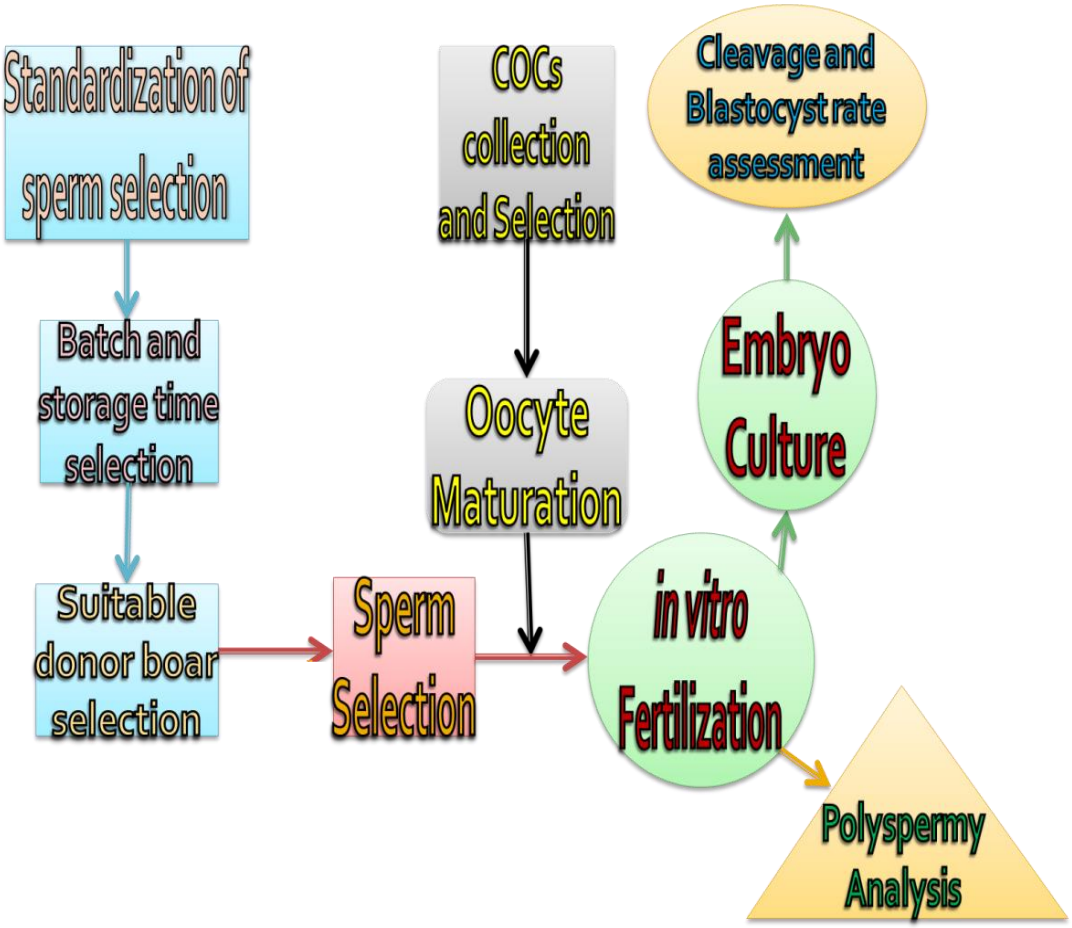

Figure 2.1 *Flow diagram of the current study. After sperm standardization, sperm underwent percoll gradient to select the potential proper sperms (motility criteria), COCs were also collected and matured (incubated in the maturation media for 44 h in total) and fertilized by the selected sperm; after incubation and co-incubation, potential zygotes were transferred to the culture media and assessed after three, five, six, and seven days. Additionally, polyspermy analysis was performed around 10-11*

2.2 Washing, Maturation, IVF, and culture media

In this part the component of the five media used in this experiment has depicted

2.2.1 Wash media, Porcine X Media (PXM)

Wash media (PXM) is consisted of Base* (10x stock), HEPES (25.0 mM) and bicarbonate (5.0 mM) as buffering agents, Calcium L-lactate (2.0 mM), and Gentamicin (0.01 mg/ml); after filter sterilization (0.22 µm diameter) under laminar flow cabinet; pH was maintaind at 7.2 – 7.4 with NaOH. For washing the oocyte in order to reduce surface adsorption of tubes, IVF dishes, and petri dishes Bovine Serum Albumin (BSA) (0.004 g/ml) was used. Also sodium pyruvate (0.2 mM) was added to the freshly prepared PXM before washing the oocytes/zygotes.

2.2.2 *In vitro* Oocyte Maturation (IVM) Media

For the IVM preparation, the Base* was used from the 10x stock in addition to Bicarbonate (25.0 mM), Glucose (4.0 mM), sodium pyruvate (0.2 mM), Calcium L-lactate (2.0 mM), L-glutamine (2.0 mM), Hypotaurine (5.0 mM), Basal Medium Eagle 50x (BME) (UK) (20 ml/l), Minimum Essential Medium Eagle 100x (MEM) (UK) (10 ml/l), L-cysteine (0.58 mM), Gentamicin (0.01 mg/ml), BSA (0.004 g/ml), Epidermal Growth Factor (EGF) (10 ng/ml), and β- mercoptoethanol (50 µM); the medium osmolality was adjusted to the 286 mOsm/kg, and filter sterilized (0.22 µm diameter).

2.2.3 *In vitro* Fertilization (IVF) Media

The IVF media which also called Porcine Gamete Media (PGM) was consist of: Base* (10x stock), Bicarbonate (25.0 mM), Glucose (4.0 mM), Sodium Pyruvate (0.2 mM), Calcium L-lactate (2.0 mM), TALC (15x) [Theophylline (2.5 mM), Adenosine (1.0 µM), L-Cysteine (0.25 µM), Gentamicin (0.01 mg/ml), and BSA (0.004 g/ml); the osmolality was adjusted to 280 mOsm/kg, and filter sterilized (0.22 µm diameter).

* The ingredients is presented in the Appendix section

2.2.4 *In vitro* Embryo Culture (IVC) Media

This media is called PZM as well, and is the media for culturing zygotes to reach hatching and also embryo stages (e.g. morula, blastocyst stages); the media is composed of: Base (10x stock) Bicarbonate (25.0 mM), Sodium Pyruvate (0.2 mM), Calcium L-lactate (2.0 mM), L-glutamine (2.0 mM), Hypotaurine (5.0 mM), Basal Medium Eagle 50x (BME) (UK) (20 ml/l), Minimum Essential Medium Eagle 100x (MEM) (UK) (10 ml/l), L-cysteine (0.58 mM), Gentamicin (0.01 mg/ml), and BSA (0.004 g/ml); the osmolality was adjusted to 284 mOsm/kg, and filter sterilized (0.22 µm diameter).

2.3 Standardization of Sperm Selection

Sperm selection for IVF was carried out by percoll discontinuous gradient concentration (45%, and 90%) in companion with two rounds of centrifugation. One millilitres of 90% (for the ingredients refer to the Appendix) percoll was added, 1:1, to the IVF media (prepared without BSA) to prepare two millilitres 45% percoll. In the 15 ml falcon tube 2 ml of 90%, 2 ml of 45% percoll, and 1ml of thawed sperm cells in the extender were added respectively to make a discontinuous gradient (figure 2.2); the tube was centrifuged (700 × g, 20 min; Thermo Scientific) and the supernatant was removed in the way which only ca. 200 µl of solution remains at the end, then 4 ml of the IVF media without BSA was included; the tube underwent the second centrifugation (500 × g, 5 min) and the same way removal of supernatant was done to achieve potential high motile sperm cells for IVF.

2.3.1 Percoll from two Different Batches

The sperm motility (total motility, progressive motility, and hyperactive motility) was assessed by CASA (microscope: Nikon, Japan; digital camera: Basler Vision Technologies, Ahrensburg, Germany; software: Sperm Class Analyzer® version 6.1, Spain; instrument setup is in the Appendix) before and after making gradient concentration for sperm selection by two different percoll batches (batch 1, Lot# SLCH4415; and batch 2, Lot# SLCK0826).

The sperm samples were used for these sets of experiments were frozen thawed in 50ºC water bath for 50 sec; the sample straws were from two random boars, for each boar two experiment replicates and for every replicate two technical replicates were analyzed; final analyses were done by taking mean of the two technical replicates, and calculating the average of the two experiments for both boars in addition to combining both data to reach a meaningful total average; standard deviation shows the dispersion of the data from the calculated mean.

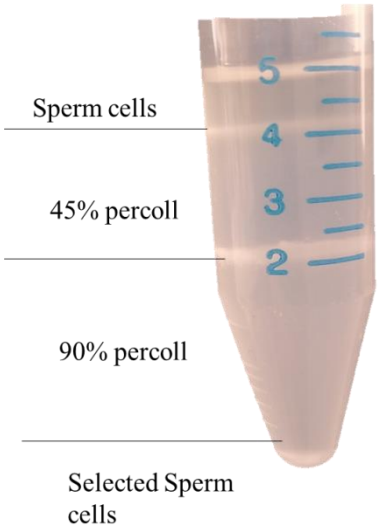

Figure 2.2 *Percoll discontinuous gradient concentration tube after second centrifugation; the sperm cells in the top are post thawed/fresh sperms with extender, and the sperm cells in the bottom are selected sperms, expected to have higher motility criteria for the further IVF.*

2.3.2 Storage Standard for 90% Percoll

Alike different batches examination, sperm motility was assessed by CASA for two different 90% percoll storage times, i.e. the old percoll, which indicates 90% percoll stayed more than 10 days at 4 ºC, and the fresh percoll, indicates freshly made, prior to the sperm selection,

90% percoll. The experiment set-up and the thawing condition were similar to the section 2.3.1.

2.4 Suitable Sperm donor boar Selection

In this part of the study we tried to find the most suitable sperm donor for our study (and the other parallel study). Our collaborator, Norsvin SA, gave us four different potentially suitable boar sperm sample reservoirs for IVEP; all the four boar (A: Duroc; B, C, and D: landrace) samples were once analyzed fresh, to compare effect of cryopreservation of semen from the boars liquid fresh semen (before cryopreservation) in days 0, 2, and 7 after arrival (incubated at 16 ºC); mixed with the extender (Androstar® Plus; minitube), and analyzed by CASA and Flowcytometry (Beckman coulter, Cytoflex S, USA; software: CytoExpert 2.4). The four frozen boar samples were also analyzed once 30 min after thawing in the TRIXcell+ extender (IMV technologies, France), after implementation of the percoll sperm selection, and after four hours staying in the room temperature; the thawing condition was the same as the section 2.3.1. Viability assessment and acrosome integrity was done by 10 min incubation of the sperm cells with the two fluorochromes, Peanut agglutinin (PNA)-Alexa Fluor® 488 (Invitrogen™) and Propidium iodide (PI) from LIVE/DEAD® kit (Invitrogen™), Syto™ 60 (Invitrogen™) was added to determine cells from particles (for more information about the filters used refer to Appendix). For each instrument, two experiments were designed, and for each experiment two technical replicates were practiced. Finally the most suitable boar was assessed based on possessing the highest average progressive motility and viability, also the lowest average hyperactivity and acrosome reactivity in compare to the others.

2.5 Cumulus-Oocyte Complex (COCs) Collection and Maturation

Ovaries were collected at the local slaughterhouse from the random pigs (sows and gilts) right after slaughtering; the ovaries were transferred to the laboratory in a thermoflask

containing saline (0.9% w/v), temperature set at 36-39 ºC; at the laboratory, ovaries were washed twice with the same warm media supplemented with the kanamycin (0.25% v/v), and kept in the water bath for 4 hours before start of oocyte aspiration. COCs were aspirated from 3-8 mm follicles by 18 gauge needle and 5 ml syringe, then rested 10 min at 38 ºC, and selected by their appearance (compacted cumulus cells, and homogeneous dark oocytes). After washing the COCs in the PXM twice, COCs were transferred in the groups of 20-30 to the 4-well plate with the 500 µl of IVM media, containing cAMP (10 µl), and porcine FSH/LH (10 µl). The plates were incubated (6% CO_2, normal air pressure oxygen, and 38.5 ºC) for 20 h, then after changing media with only 500 µl IVM media a second incubation was performed under the same condition as the first incubation, lasting for 24 h. All the media used were pre-equilibrated for 2 h at the incubator. In this study in total 935 COCs were collected, matured, and fertilized (December 2021-April 2022).

2.6 Sperm Selection

The sperm straw from the selected much suitable boar, for the IVEP, was thawed (50 ºC, 50 sec) in the water bath and diluted into the 40 ml TRIX-Cell+ extender. After 30 min incubation at room temperature (RT), sperm cells were analyzed by CASA once before and after implementation of percoll discontinuous gradient. Sperm concentration was adjusted based on CASA motility result and also if the progressive motility was below 60%, concentration of the sperm cells was increased to compensate motility.

2.7 *In vitro* Fertilization (IVF) and *in vitro* Embryo Culture (IVEP)

COCs were washed first in the IVF medium and transferred to the pre-equilibrated IVF medium (20 µl, each 20-30 COCs groups, per every well) as sperm cells concentration was adjusted according to the experimental setup (normal: 1000 sperm cells per COC, double: 2000 sperm cells per COC) in order that the final volume of each well become 500 µl. The first incubation (6% CO_2, normal air pressure oxygen, and 38.5 ºC) was done right after mixing sperm cells with the COCs for 2 h; the second incubation was performed (2 h), under the same condition, after transferring potential zygotes to the new 500 µl IVF media plates.

The cells in each well was transferred to the 15 ml falcon tube with 2 ml of pre-warmed (38 ºC) PXM; the tubes were vortexed vigorously to remove cumulus cells and washed three times in pre-warmed PXM, in case of some un-removed cumulus cells EZ-grip special (125 µm) pipet were used. Zygotes washed once in the IVC media and transferred to the 500 µl IVC media (pre-equilibrated) culture 4-well plates, covered with mineral oil and lid open, incubated at 38.5 ºC, 6% CO_2, and 7% O_2. The cleavage was evaluated objectively by 10x-20x magnification, phase contrast inverted microscope (Leica); at day 3 and, media was changed with the pre-equilibrated IVC media at day 4 (without mineral oil at the top and lid close). Evaluation continues at days 5, 6, and 7.

2.8 Polyspermy Analysis

Polyspermy rate were observed after 10-11 h after onset of fertilization by fixing zygotes in 4% paraformaldehyde and PBS solution and staining zygotes with the Hoechst 33342 (10 µg/ml) and 6 µl of mounting solution for fluorescence (Dako, Glostrup, Denmark) over a slide and covered with the coverslip. Observation was performed by confocal microscope (Lica TCS SP 8; software: LasX 3.5.7; blue filter (DAPI), excitation: 405 nm, emission: 410-480 nm). Since polyspermy analysis reduced the amount of the final data for blastocyst analysis, and the study had no control over amount of the ovaries (oocytes) which was taken from the slaughterhouse, it was decided to analyze polyspermy for only two rounds of IVF. Therefore 120 zygotes (60 for the normal sperms concentration and 60 for the double sperms concentration) were observed for the pronuclei formation of both sperm cells and the ovum in addition to two polar body formation as normal fertilized zygotes, more than two pronuclei formation also considered polyspermy.

3. Results

3.1 Comparison between two different Batches of Percoll

As mentioned before, the goal for this analysis was to determine if there is a difference between post thawing sperm cells and sperm motility after percoll centrifugation, also between different batches of percoll. In figure 3.1 the final average of all the analyses was depicted; the average value for the three criteria showed no particular differences, neither in total motility, progressive motility or hyperactive motility, between sperm analysis of the two used percoll batches for sperm selection. However, there were a significant difference between post thawed sperm cells hyperactivity and sperm cells hyperactivity after percoll centrifugation.

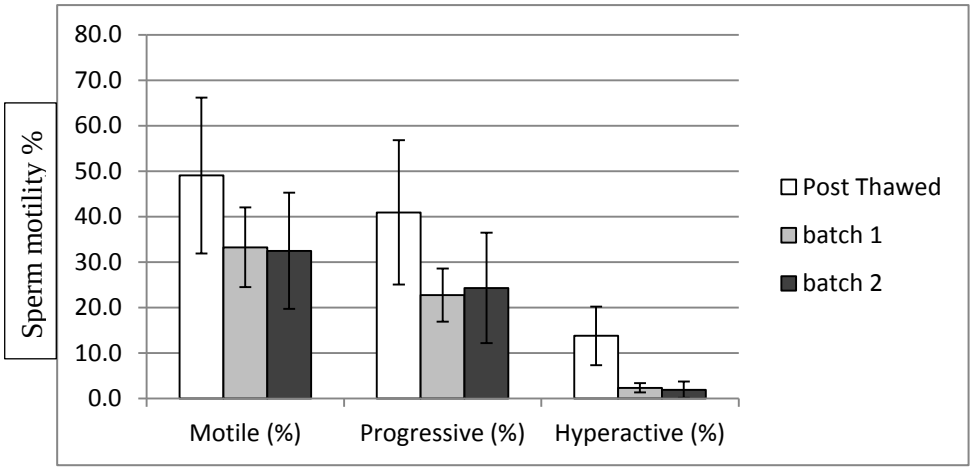

Figure 3.1 *Bar diagram of the average value of the sperm cells total, progressive, and hyperactive motility after performing discontinuous percoll gradient made from two different batches. The diagram is the final average of two biological replicates from random boars, for each boar two experimental replicates and for every experiment, two technical replicates were analyzed; the error bars show the standard deviation between the final data averages. Post thawed analysis was performed to compare sperm motility before and after percoll application.*

3.2 Storage Standard for 90% Percoll

Observation and the final result in this section helped to optimize the sperm selection process; The average value comparison and the standard deviation of the data was illustrated as figure 3.2; according to the figure sperm motility after application of the gradient with a fresh made 90% percoll was considered equal (no significant difference was seen) to the post thawed motility, whereas sperm motility after making gradient with a 90% percoll which was kept at 4 ºC for less than 10 days was significantly lower in comparison to the fresh one. Therefore, the data support using freshly made 90% percoll for the sperm selection process.

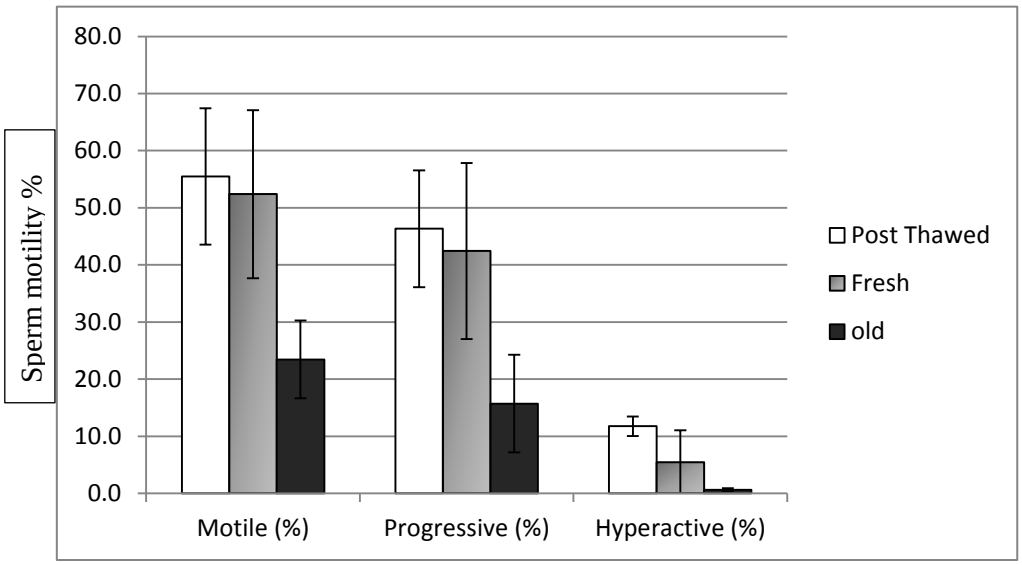

Figure 3.2 *Bar diagram of sperm motility percentage after thawing (post thawed), gradient concentration made with 90% fresh percoll (fresh), and gradient concentration made with, approximately, two weeks kept (4 ºC) 90% percoll (old). The error bar shows the standard deviation of the data. There is a significant difference obvious between the fresh and the old percoll.*

3.3 Sperm donor boar determination

Determination of the most suitable boar for IVF sperm donation was done by assessing a frozen sample of each boar by CASA and flowcytometry. The sperm motility total average value for each boar, and acrosome reactivity is shown in figure 3.4, notice for better comparison total motility, progressive motility, and hyperactive motility have been displayed in different diagrams. Also acrosome reactivity of the four boars was assessed by flowcytometry in days 0 and 7 (figure 3.3). As figure 3.4 section I indicates there is no particular difference in the total motility of the four samples, especially after percoll implementation. Since standard deviation of the bars in the figure 3.4 section II covered each other, there is no meaningful difference in the progressive motility. In the section III of the same figure boar B showed higher hyperactivity in PT, and boar A had slightly higher hyperactivity after percoll, and lower after incubation for 4 h. As summary of all the data, boar A was sensitive to the RT or any prolonged incubation, and boar B also presented more hyperactivity after thawing (figure 3.4 section III) in addition to slightly higher acrosome reactivity and lower live cells after percoll (figure 3.4 section IV, and V), therefore, these boars were excluded from the experiment to increase the potential outcome of the IVEP; under a sentence, boar C, and D were used for IVF experiment of parallel Ph.D. and the current studies respectively. Alternatively it is noticeable that the percoll reduced the percentage of acrosome reaction of the live cells.

3.4 *In vitro* Embryo Production and different sperm concentrations

In this experiment six rounds of IVF has been practiced, however, one of the IVF rounds had some issue in day six (cells were collapsed due to incubation problem), therefore, the final cleavage and blastocyst rate were acquired by calculating the average of each five IVF rounds cleavage (in day 3) and blastocysts rate in day six of the embryo culturing; the final rate was captured based on calculating the mean of the entire five IVF rounds averages. Figure 3.5 demonstrate the percentage rate of cleavage and all blastocysts stages (young,

normal, expanded, and hatched) the average values show no particular difference between the two experimental variations (normal sperm concentration, and double sperm concentration); in spite of the fact that the control demonstrated almost 50% cleavage in average, the average blastocyst rate was very low (0.6%) which was a sign of almost no parthenogenesis.

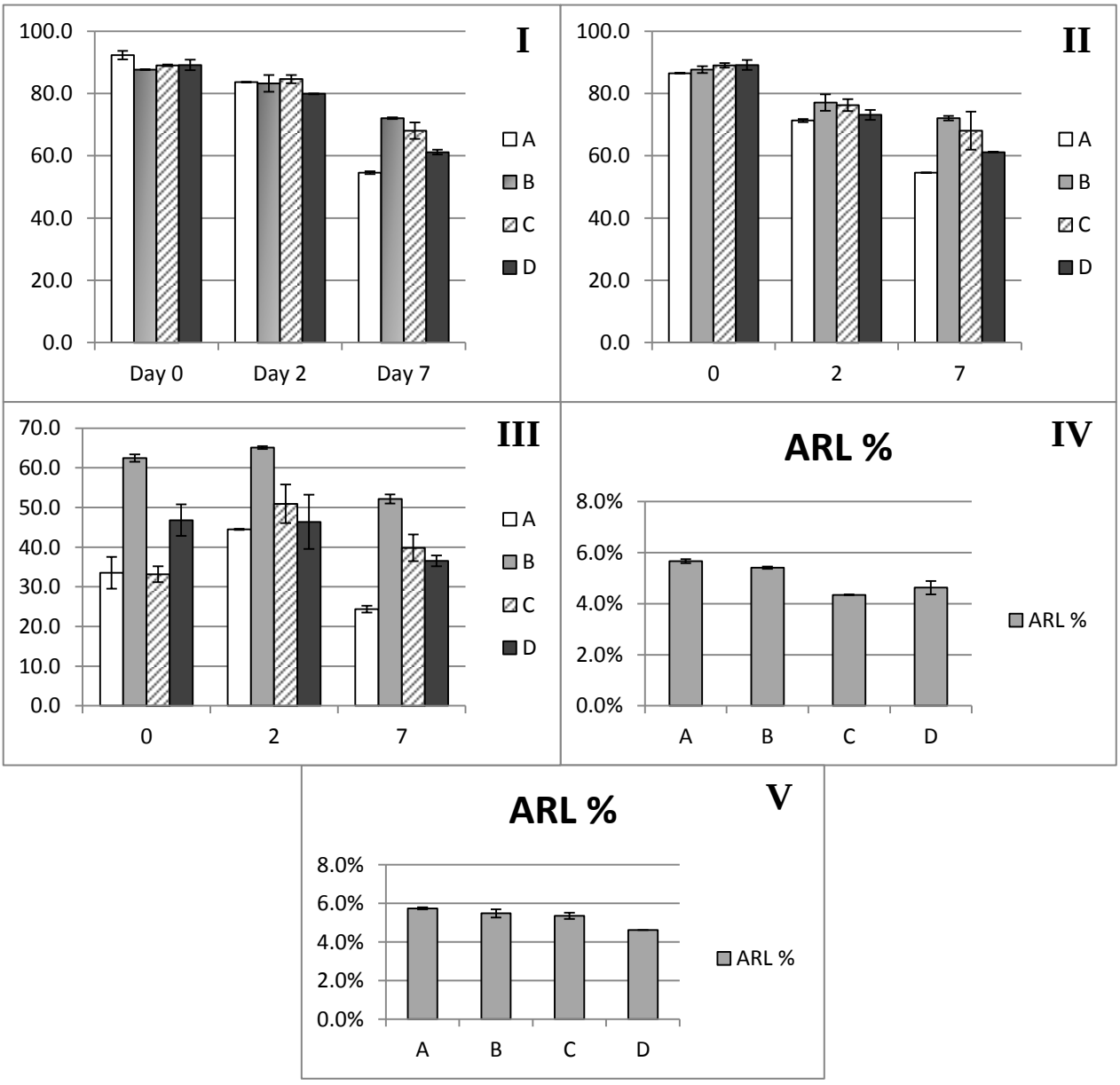

Figure 3.3 *Fresh sample analyses of the four candidate boars (A, B, C, and D). I, II, and III) The total average value of the total motility, progressive motility, and hyperactive motility (respectively) the Y axis shows percentage of the motility and the X axis indicates the days which analyses have been done; as data for day 7 shows, the boar A was more sensitive to the prolong preservation and incubation. The data indicates at the day 0 boar C and D had less hyperactivity, however, hyperactivity of the boar C was increased during incubation (day 7). IV, and V) Bar diagram related to the flowcytometry data of the acrosome reacted live sperm cells percentage at the day 0 (IV) and the day 7 (V).*

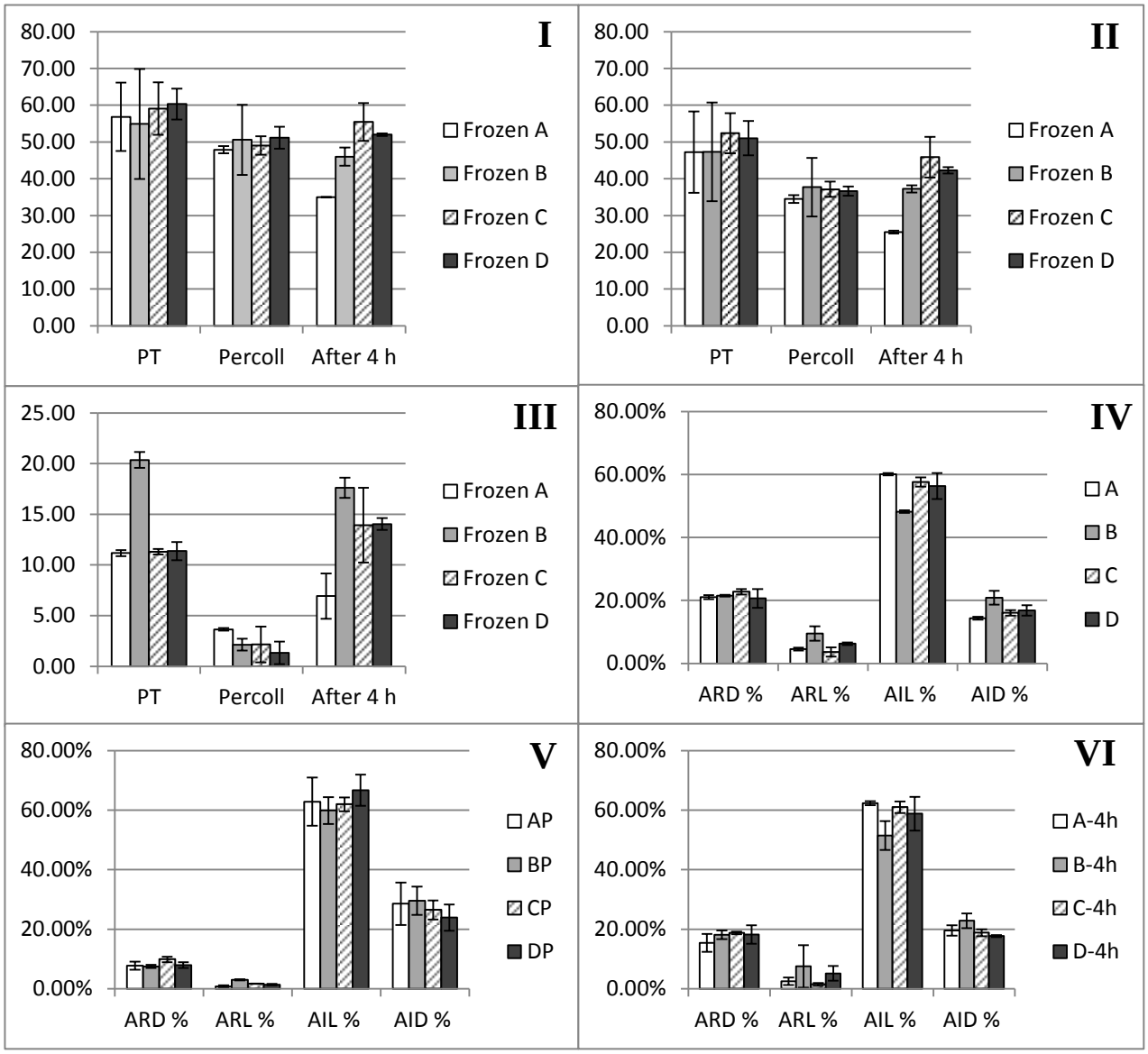

Figure 3.4 *I-III bar diagram indicating the average value of total, progressive, and hyperactive motility of each boar (frozen A, frozen B, frozen C, and frozen D) sperm cells (I, II, and III respectively) that were analyzed by CASA; the Y axis shows motility percentage and the X axis represents post thawed sperms (PT), sperms which selected by the percoll concentration gradient, and sperm cells motility after incubation at RT for 4 h. IV, V, and VI) the average data of acrosome reactivity and viability of PT sperm cells (IV), after percoll implementation (V), and after 4 h incubation at RT of the four mentioned boars.*

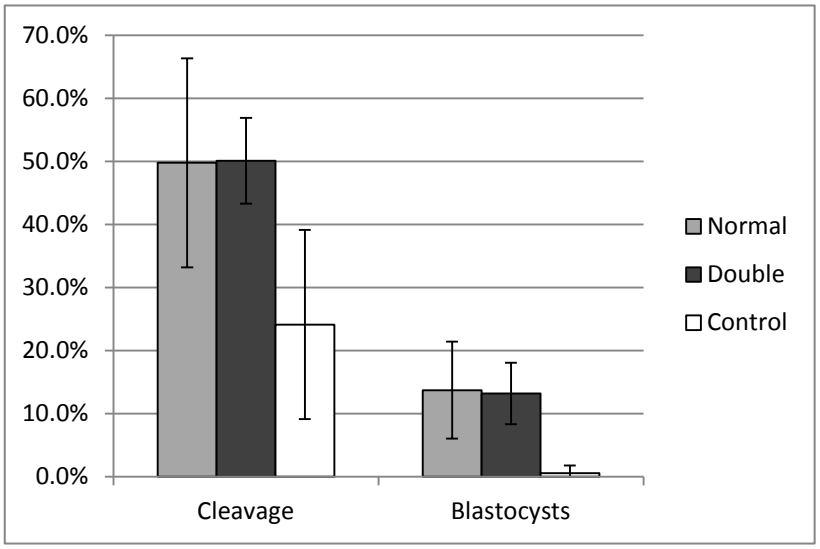

Figure 3.5 *The bar diagram related to the cleavage (in day 3) and blastocysts (in day 6) average rate percentage of the total observed embryo cultured cells (X axis) from the two experiment variations, the two sperm concentration applied to the IVF culture wells (normal and double) with the control (without adding sperm cells to the COCs). The mean value and standard deviation coverage (the error bars) of the normal and double concentrations are very close together, i.e. no evidence were observed in this experiment to support experimental hypothesis, the more sperm concentration could lead to more cleavage and blastocysts rate.*

3.5 Polyspermy analysis

Potential zygotes analyzed for polyspermy assessment after 10-11 h of the fertilization onset; from 120 analyzed samples (60 per each treatment) only 35.8% were fertilized at the time of observation which 26.7% and 31.7% of the normal sperm concentration (1000:1 COC) and double sperm concentration (2000:1 COC) treatments was fertilized monospermic respectively, with no sign of polyspermy for the normal sperm concentration and 13.4% polyspermy for the double sperm concentration. Therefore, there is a significant difference between the two groups in either case of monospermy or polyspermy.

4. Disscusion

The purpose of the current study was to optimize IVEP by addressing sperm criteria (selection quality, sperm quality, and sperm concentration for IVF). In this section, mentioned parameters and other factors which might be important in the blastocyst rate will be discussed.

Selecting proper sperms, in case of motility and acrosome integrity, for the IVF experiment is undoubtedly one of the important ways to improve the blastocyst rate after IVF and embryo culture (Grant et al., 1994). Therefore, in this study it was tried to find the proper sperms by examination of percoll and by finding the most suitable boar; although even when you find the best individual boar for the IVF there are some differences between each frozen straw (Jochems et al., 2022); thus sperm selection make a standard for lowering the differences, still this race, individual (Xu et al., 1996), and even ejaculation or frozen straw differences should be considered, these make it even harder to predict or have control over the outcomes. Some study also believed the batches of percoll has different effect on spermatozoa, due to toxicity (Mortimer, 2000b); therefore, by experiment of different random percoll batches it was proven that the effect was not significant enough to make a difference, at least in the aspect of motility criteria. Also it was found that using percoll might reduce the hyperactivity of the sperm cells; thus, percoll slightly reduced all of the motility criteria, it is difficult to say the above statement firmly. As opposed to existence of many studies about affectability of the percoll gradient, to date there is no study about the storage time of 90% percoll. Findings in this study revealed, storage of 90% percoll more than two weeks would influence sperms motilities, thus, influence ZP penetration and IVEP outcome. Although in some articles using high ratio of sperm cells to the oocyte were reported to have a better cleavage and blastocyst rate, such as (Gil et al., 2004; Xu et al., 1996), according to them, the polyspermy rate was also higher. For optimizing the concentration of the progressive sperm cells in IVEP, it should be considered to keep penetration level up for more cleavage rate with the two pronuclei formation simultaneously hold the polyspermy rate back to the lowest possible level. Previously, the higher fertilization and blastocyst rate at 500:1 ratio of sperm cells/COC to 250:1 ratio in day 6 was proven by Jochems et al. (2022), however, they stated no significant difference between the two ratios was observed in total polyspermy rate; this study depicted, even though the outcome of 2’000:1 ratio was expected to be higher than 1’000:1 ratio, and also Gil et al.

(2004) used it as a standard and control, there was no significant difference between (at cleavage and blastocyst rate) the two mentioned ratio (P>0.05). Even after comparison of the day 7 data for blastocyst formation (total mean: 13.6± 7.3% for the normal concentration (1’000:1); and 9.7± 8.5% for the double concentration (2’000:1)), despite of a slight decrease in the double concentration due to apoptotic nucleic formation or simply cell collapse, there was no significant difference found in the two ratio as well (P>0.05). Polyspermy analysis, on the other hand, showed higher percentage of polyspermy for the double sperm concentration; this is not easy to conclude if the ratio is higher the polyspermy incidence is also higher as shown in Gil et al. (2004) and Jochems et al. (2022) the polyspermy rate increase by increasing the ratio more than certain level (e.g. 8’000:1 for Gil et al. (2004) study).

5. Conclusion

As conclusion, although both of the sperm concentrations (1’000:1 ratio, and 2’000:1) showed the similar fertilization and blastocyst rate, still due to higher chance of polyspermy in the double concentration, the normal sperm concentration is assumed to be a better option for adjusting sperm ratio to the oocyte. Alternatively, the effect of different percoll batches and 90% percoll storage on the sperm cells showed no difference in the two various batches, and for better result in sperm motility the 90% percoll should be prepared freshly before sperm centrifugation.

6. References

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Appendix

Base Stock : NaCl 1080 mM, KCl 100 mM, KH_2PO_4 3.5 mM, $MgSO_4$.$7H_2O$ 4.0 mM

CASA properties:

Frame rate: 45 frames per second

Filed captured: 8 filed

Total cell analyzed per each filed: 800

Total motility: (VCL) > 10 μm/s

Progressive motility: (STR) > 45 %.

Flowcytometry CytoFlex filters:

PI filter used: ECD-A

(PNA)-Alexa Fluor® 488 filter used: FITC-A

Syto 60 filter used: APC-A

Table of figure 3.1

Samples	Motile (%)	Progressive (%)	Hyperactive (%)
Post Thawed	49.03±17.13	40.91±15.88	13.78±6.44
batch 1	33.23±8.77	22.75±5.86	2.33±1.04
batch 2	32.48±12.77	24.30±12.14	1.90±1.79

Table of figure 3.2

Samples	Motile (%)	Progressive (%)	Hyperactive (%)
Post Thawed	55.49±11.94	46.34±10.23	11.75±1.73
Fresh percoll	52.38±14.69	42.42±15.40	5.42±5.65
Old percoll	23.45±6.81	15.72±8.45	0.63±0.25

Table of figure 3.3

I)

	A	B	C	D
Day 0	92.3± 1.4	87.6±0.1	89.0±0.3	89.2±1.7
Dy 2	83.6±0.1	83.2±2.7	84.6±1.4	79.9±0.1
Day 7	54.5±0.4	72.1±0.3	68.1±2.6	61.1±0.8

II)

	A	B	C	D
Day 0	86.5±0.0	87.6±1.1	89.0±0.7	89.2±1.6
Dy 2	71.3±0.5	77.1±2.6	76.3±1.9	73.1±1.6
Day 7	54.5±0.1	72.1±0.7	68.1±6.1	61.1±0.0

III)

	A	**B**	**C**	**D**
Day 0	33.5±0.4	62.455±0.9	33.16±2.0	46.81±4.0
Dy 2	44.5±0.1	65.075±0.4	50.92±4.9	46.37±6.9
Day 7	24.4±0.9	52.14±1.1	39.855±3.4	36.53±1.4

IV)

		ARL %
	A	5.67%± 0.08%
	B	5.41%±0.04%
	C	4.35%±0.01%
	D	4.63%±0.26%

V)

		ARL %
	A	5.74%±0.05%
	B	5.48%±0.21%
	C	5.36%±0.16%
	D	4.62%±0.0%

Table of figure 3.4

I)

	Frozen A	Frozen B	Frozen C	Frozen D
PT	56.8±9.3	54.9±0.9	59.1±0.1	60.3±4.2
Percoll	47.9±15.0	50.6±9.5	49.0±2.5	51.2±3.0
After 4 h	35.0±.1	46.0±2.5	55.4±5.2	52.0±0.4

II)

	Frozen A	Frozen B	Frozen C	Frozen D
PT	47.3±11.1	47.3±1.1	52.4±0.4	51.0±4.7
Percoll	34.5±13.4	37.7±7.9	37.1±1.0	36.6±1.2
After 4 h	25.5±5.5	37.3±2.1	45.9±5.5	42.3±0.9

III)

	Frozen A	Frozen B	Frozen C	Frozen D
PT	11.2±0.3	20.4±0.1	11.3±2.2	11.4±0.9
Percoll	3.6±0.8	2.1±0.6	2.2±1.0	1.3±1.1
After 4 h	6.9±0.3	17.6±1.8	13.9±3.7	14.0±0.6

IV)

	ARD %	ARL %	AIL %	AID %
A	21.0%±0.6%	4.5%±0.5%	60.1%±0.4%	14.4%±0.4%
B	21.5%±0.2%	9.5%±2.3%	48.2%±0.4%	20.9%±2.2%
C	22.7%±0.9%	3.6%±1.5%	57.6%±1.5%	16.0%±0.8%
D	20.6%±3.0%	6.2%±0.4%	56.3%±4.1%	16.8%±1.6%

V)

	ARD %	ARL %	AIL %	AID %
A	7.8%±1.3%	0.8%±0.4%	62.8%±8.1%	28.6%±7.1%
B	7.5%±0.5%	3.1%±0.2%	59.9%±4.5%	29.6%±4.8%
C	9.9%±0.9%	1.7%±0.1%	62.0%±2.3%	26.5%±3.2%
D	8.0%±1.0%	1.4%±0.2%	66.7%±5.2%	23.9%±4.4%

VI)

	ARD %	ARL %	AIL %	AID %
A	15.5%±3.0%	2.6%±1.2%	62.4%±0.7%	19.6%±1.7%
B	18.1%±1.4%	7.6%±7.1%	51.4%±4.9%	22.9%±2.5%
C	18.7%±0.5%	1.5%±0.5%	61.0%±1.9%	18.8%±1.2%
D	18.2%±3.1%	5.2%±2.5%	58.8%±5.7%	17.7%±0.3%

Table of figure 3.5

	Cleavage	Blastocysts
Normal	49.8%±16.6%	13.7%±7.7%
Double	50.1%±6.8%	13.2%±4.9%
Control	24.1%±15.0%	0.6%±1.3%

IVF Day 7

	blastocyst day 7
Normal	13.6%±7.3%
Double	9.7%±8.5%
Control	1.6%±3.3%