IR-PERPUSTAKAAN UNIVERSITAS AIRLANGGA

THESIS

INFLUENCE OF SWIM-UP TIME ON THE PLASMA MEMBRANE INTEGRITY OF RAM'S (*Ovis aries*) SPERMATOZOA FOLLOWING CENTRIFUGATION

By :

ALDINO YANUAR EFENDI

SIM 060911130

FACULTY OF VETERINARY MEDICINE
AIRLANGGA UNIVERSITY
SURABAYA

2012

IR-PERPUSTAKAAN UNIVERSITAS AIRLANGGA

INFLUENCE OF SWIM-UP TIME ON THE PLASMA MEMBRANE INTEGRITY OF RAM'S (*Ovis aries*) SPERMATOZOA FOLLOWING CENTRIFUGATION

Thesis

Submitted in partial fulfillment of the requirement for the degree of

Bachelor of Veterinary Medicine

at

Veterinary Medicine Faculty of Airlangga University

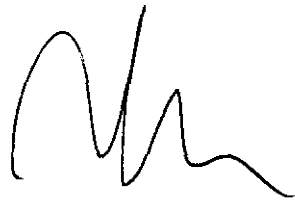
By

ALDINO YANUAR EFENDI

060911130

Approved

Supervisors Commitee,



(Dr. Bambang Poernomo S., MS.drh.)
Major Supervisor

(Budiarto, MP., drh.)
Secondary Supervisor

SKRIPSI INFLUENCE OF SWIM-UP ... ALDINO YANUAR EFENDI

IR-PERPUSTAKAAN UNIVERSITAS AIRLANGGA

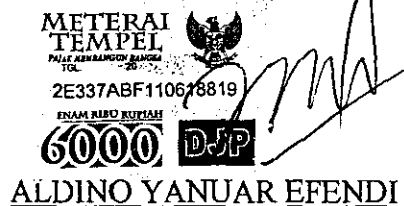
DECLARATION

I hereby declare that in this thesis entitled:

INFLUENCE OF SWIM-UP TIME ON THE PLASMA MEMBRANE INTEGRITY OF RAM'S (*Ovis aries*) SPERMATOZOA FOLLOWING CENTRIFUGATION

is my own account of researched and there are no works that have been proposed to obtain Bachelor's degree at any college and best of my knowledge there is no such works or opinions ever written or published by other, except those in writing referred to in this paper and are mentioned in the references.

Surabaya, 23 July 2012



ALDINO YANUAR EFENDI

060911130

Assessed on

Date: July 23th, 2012

ASSESSMENT COMMITTEE

Chairman	: Suzanita Utama, M.Phil., drh
Secretary	: Dr. Suherni Susilawati, M.Kes., drh.
Member	: Dr. Rimayanti, M.Kes., drh.
Major Supervisor	: Dr. Bambang Poernomo S., M.S., drh
Secondary Supervisor	: Budiarto, M.P., drh.

Surabaya, 18th july 2012
Faculty of Veterinary Medicine
Airlangga University
Dean,

Prof. Hj. Romziah Sidik, drh., Ph.D
NIP. 195312161978062001

IR-PERPUSTAKAAN UNIVERSITAS AIRLANGGA

INFLUENCE OF SWIM-UP TIME ON THE PLASMA MEMBRANE INTEGRITY OF RAM'S (*Ovis aries*) SPERMATOZOA FOLLOWING CENTRIFUGATION

Aldino Yanuar Efendi

ABSTRACT

The aim of this research was to determine the influence of swim-up time on plasma membrane integrity of ram's spermatozoa following centrifugation. The experimental design was completely randomized design with three treatments. The treatments were semen swim-up for 15, 30, and 45 minutes respectively for groups T1, T2, and T3. The spermatozoa were assessed for their plasma membrane integrity. The results of this research showed that the T1 have the highest percentage of plasma membrane integrity of ram spermatozoa, the modest was T2, and the lowest was T3. In conclusions, swim-up time influenced the plasma membrane integrity of ram's (*Ovis aries*) spermatozoa following centrifugation.

Key words: spermatozoa, centrifugation, swim-up, plasma membrane integrity

ACKNOWLEDGEMENTS

Gratitude to Allah SWT has bestowed so author can conduct research and complete the thesis with the title:

INFLUENCE OF SWIM-UP TIME ON THE PLASMA MEMBRANE INTEGRITY OF RAM'S (*Ovis aries*) SPERMATOZOA FOLLOWING CENTRIFUGATION

On this occasion the authors wish to thank:

1. Prof. Romziah Sidik, Ph.D., drh. as Dean of Faculty of Veterinary Medicine, Airlangga University for the opportunity to take part in education in the Faculty of Veterinary Medicine Airlangga University.
2. Dr. Suherni Susilowati, M. Kes., drh. as a research supervisor, Dr. Bambang Poernomo S., M.S., drh. as a major supervisor and Budiarto, M.P., drh. as a secondary supervisor who has given advice and guidance for the completion of this thesis.
3. Suzanita Utama, M.Phil., drh. as chairman of examiners, Dr.. Suherni Susilowati. M. Kes.. drh as secretary and Dr. Rimayanti. M. Kes.. drh. as member.
4. Dr. Mirni Lamid, MP., drh. as the guardian lecturers who have given support so the author can finish this research
5. The entire staff of the Laboratory of Artificial Insemination (AI) Faculty of Veterinary Medicine University for all for helping this research process.
6. Both authors parents Mr. Purwadi and Mrs. Sulastri thank you for motivation and pray.
7. To author's family, all lecturers, class of 2008, SC of 2011 Veterinary

Medicine, Airlangga University, and people who could not be mentioned one by one who has helped in many things until the completion of this thesis.

The author realizes that this paper is far from perfect, for that the author expects criticism and suggestions from readers. Finally, the author hope this paper can be particularly useful for writers and for readers generally, and can provide a positive contribution in the field of Veterinary education and as a source for further researches.

Surabaya, July 2012

Author

TABLE OF CONTENTS

APPROVAL ii

DECLARATION PAGE iii

IDENTITY PAGE iv

ABSTRACT vi

ACKNOWLEDGEMENTS vii

TABLE OF CONTENTS ix

LIST OF TABLES xi

LIST OF FIGURES xii

LIST OF APPENDICES xiii

ABBREVIATIONS AND SYMBOLS xiv

CHAPTER 1 INTRODUCTION 1
- 1.1 Background 1
- 1.2 Problem Statement 4
- 1.3 Theoretical Base 5
- 1.4 The Aim of Research 6
- 1.5 Benefit of Research 6
- 1.6 Hypothesis 6

CHAPTER 2 LITERATURE REVIEW 7
- 2.1 Fat Tailed Sheep 7
- 2.2 Anatomy of Ram Genital Organ 8
- 2.3 Ram Semen and Spermatozoa 10
  - 2.3.1 Semen 10
  - 2.3.2 Spermatozoa 11
- 2.4 Centrifugation 13
- 2.5 Swim-up 14
- 2.6 Spermatozoa Plasma Membrane Integrity 15

CHAPTER 3 MATERIALS AND METHODS 18
- 3.1 Research Location and Time 18
- 3.2 Research Materials and Equipments 18
  - 3.2.1 Research Materials 18
  - 3.2.2 Research Equipments 18
- 3.3 Research Methods 18
  - 3.3.1 Semen Collection 18
  - 3.3.2 Semen Observation 19
  - 3.3.3 Sperm Swim-up 20
- 3.4 Observed Variable 20

3.4.1 Independence Variable 20
3.4.2 Dependence Variable 20
3.4.3 Controlled Variable 20
3.5 Research Design and Data Analysis 21
3.6 Researched Frameworks 22
CHAPTER 4 RESULTS 23
4.1 Semen Observation before Treatment 23
4.2 Semen Observation after Treatment 25
4.2.1 Spermatozoa Plasma Membrane Integrity 25
CHAPTER 5 DISCUSSION 28
CHAPTER 6 CONCLUSION 31
6.1 Conclusion 31
6.2 Suggestion 31
SUMMARY 32
REFERENCES 33
APPENDICES 38

IR-PERPUSTAKAAN UNIVERSITAS AIRLANGGA

LIST OF TABLES

Table 1. Per capita consumption of meat by region, 1980 and 2005 2

Table 4.1 Macroscopic observation results of ram's semen before treatments......... 23

Table 4.2 Microscopic observation results of ram's semen before treatments.......... 24

Table 4.3 Spermatozoa plasma membrane integrity after treatments (Means ± SD) 25

LIST OF FIGURES

Figure 2.1 Ram spermatozoa morphology 12

Figure 2.2 Schematic representation of typical morphological changes in human spermatozoa subjected to hypo-osmotic stress. (a) damaged plasma membrane integrity (straight tail). (b)-(g) plasma membrane intact (swelling in tail is indicated by the gray area) 17

Figure 3.1 Research Frameworks 22

Figure 4.1 Spermatozoa with HOS test and eosin negrosin staining (400 times magnification). A. Damaged plasma membrane (the straight tail), B. The plasma membrane intact (coiled tail). 26

Figure 4.2 Spermatozoa plasma membrane integrity after swim-up for 15, 30 and 45 minutes. 27

LIST OF APPENDICES

Appendix 1. Composition of Ram's Semen 38

Appendix 2. Composition of *Brackett and Oliphant* (BO) Medium 39

Appendix 3. Hypo Osmotic Solution... 40

Appendix 4.The Percentage of Spermatozoa Plasma Membrane Integrity after Treatments 41

Appendix 5. Data Analysis of Spermatozoa Plasma Membrane Integrity 42

IR-PERPUSTAKAAN UNIVERSITAS AIRLANGGA

ABBREVIATIONS AND SYMBOLS

°C	= Celcius
AI	= Artificial Insemination
ANOVA	= Analysis of Variance
AR	= Acrosome Reaction
ART	= Assisted Reproductive Technology
ATP	= Adenosine Triphosphate
BO	= Brackett – Oliphant
Ca^{2+}	= Calcium (ion Calcium)
CRD	= Completely Random Design
DNA	= Deoxyribonucleic Acid
Et al	= Et alia
FSH	= Follicle Stimulating Hormone
g	= gram
HOS	= Hypo Osmotic Swelling
IVF	= In Vitro Fertilization
NaCl	= Sodium Chloride
O_2	= Oxygen
pH	= power of Hydrogen
ROS	= Reactive Oxygen Species
rpm	= rotation per minute

IR-PERPUSTAKAAN UNIVERSITAS AIRLANGGA

CHAPTER 1

INTRODUCTION

CHAPTER 1 INTRODUCTION

1.1 Background

Up to now government's efforts to meet the need of food from livestock origin is still largely revolves around the improvement of livestock products quantitatively. In fact, to achieve meat self-sufficiency in 2014 it is not only increasing livestock production quantitatively, but also the quality improvement of livestock products is equally important (Ditjennak, 2008). More and less 30% of food needed and agricultural fulfilled by livestock, so livestock become strategic in life of people.

One among the livestock with high reproduction rate is the sheep. Population of sheep in Indonesia of 2008 is 10,915,000, and number of slaughtering is 1,597,000. (Ditjennak, 2012). Sheep is one of the producers of animal's protein, not only that the farmers chose to maintain sheep because sheep do not require vast land, sheep's feed more easily available and do not compete with people demand, not only that sheep farming can be done traditionally but also tend to be more easily maintain compared to other livestock. Prices of sheep meat and fur relatively more stable (Heriyadi, 2010). Market's demand of sheep increased as increased number of birth each year, besides of income increased, public education about nutrition of animal, and also government policy to maximized market chance in Indonesia, especially for Iedul Adha and moslem ritual like aqiqah, demand of sheep's meat slightly increased. Based on fishery and agricultural department of Jakarta the numbers of sheep slaughtered in Jakarta

at Idul Adha 2006 reach 1,721. In 2011 demand of sheep and goat due to Idul Adha increased by 600% from daily's demand (Nurbiajanti, 2011). Based on Table 1 per capita consumption of meat increased quadruple in Southeast Asia from 1980 to 2005.

Table 1. Per capita consumption of meat by region, 1980 and 2005 (FAO, 2009).

REGION/COUNTRY GROUP/ COUNTRY	MEAT	
	1980	2005
	(kg/capita/year)	
DEVELOPED COUNTRIES	76.3	82.1
Former centrally planned economies	63.1	51.5
Other developed countries	82.4	95.8
DEVELOPING COUNTRIES	14.1	30.9
East and Southeast Asia	12.8	48.2
China	13.7	59.5
Rest of East and Southeast Asia	10.7	24.1
Latin America and the Caribbean	41.1	61.9
Brazil	41.0	80.8
Rest of Latin America and the Caribbean	41.1	52.4
South Asia	4.2	5.8
India	3.7	5.1
Rest of South Asia	5.7	8.0
Near East and North Africa	17.9	27.3
Sub-Saharan Africa	14.4	13.3
WORLD	30.0	41.2

IR-PERPUSTAKAAN UNIVERSITAS AIRLANGGA

In case to increase sheep production and reproduction, researchers develop many technologies. Among reproductive technology which frequently used In Vitro Fertilization (IVF) and Artificial Insemination (AI). The maximum achievement results of AI or IVF should be free from disease, high-quality, high power fertilization. IVF is becoming one of the most exciting and progressive procedures available in today's producers. Although the major emphasis in the development of reproductive technologies has been on cattle, particularly dairy cattle, technologies such as AI and IVF have also played a part in improving the biological and economic efficiency of sheep and goats. In vitro embryo production in small ruminants may also provide low-cost source of embryos for research and for commercial applications in the emerging of cloning and the production of transgenic animals (Gordon, 2004). IVF offers progressive producers fascinating genetic and management opportunities using assisted reproductive technology (ART) to advance superior genetics. IVF is often done in sheep's reproduction technology (Sujoko *et al.*, 2009).

IVF is way to produce embryo outside the body, spermatozoa directly taken from epididymis from waste product in slaughter house or collect by using artificial vagina. Success rate of IVF depends on matured ovum and capacitated spermatozoa. Minimum concentration of spermatozoa for IVF are one million cells/ml, progressive motility 40%, live spermatozoa 40% and abnormalities of less than 14% (Klinc *et al.*, 2006). IVF is a complex procedure involving oocyte maturation, sperm separation and sperm capacitation. Sperm capacitation is the biochemical modification sperm must undergo within the female tract before the

IR-PERPUSTAKAAN UNIVERSITAS AIRLANGGA

cell can bind to the zona pellucida and undergo the acrosome reaction (AR) (Gordon, 2004). One of the factors that increase the success of IVF is the motility of spermatozoa. High motility of spermatozoa is needed for penetration into the zona pellucida (Hunter, 1995). The higher level of membrane plasma integrity means also higher level of spermatozoa motility (Sardjito, 2003). Plasma membrane components have a very unique function such as attachment of spermatozoa with an ovum, substrate transport and metabolism. Spermatozoa for IVF should firstly be separated from the semen plasma and materials which are toxic to spermatozoa with a particular method (Fitri, 2002).

Washing spermatozoa by centrifugation in fresh medium is generally held to be quickest, most effective method of removing seminal plasma. Centrifugation is a method used IVF process, for selection spermatozoa from seminal plasma and toxic materials in semen such as lactic acid, ammoniac, hydrogen peroxide (Yovich, 1995). Centrifugation with added medium can eliminate decapacitating factors from seminal plasma (Vandervoort, 2004). After centrifugation, spermatozoa needed to incubate for swim-up to select the highest motile spermatozoa, motile spermatozoa moving up from lower layers to the top layer of the medium

1.2 Problem Statement

Based on the background of the explanation above the problem statement can be formulated as follow: Does the swim-up time influence the plasma membrane integrity of ram's (*Ovis aries*) spermatozoa following centrifugation?

1.3 Theoretical Base

Semen consists of two parts, namely the seminal plasma and spermatozoa. Spermatozoa are produced in the seminiferous tubules through spermatogenesis. After spermatozoa produced seminiferous tubule, spermatozoa will through two stages of maturation before occurred into fertilization. The first maturation occur inside epydidimys and the second maturation occur inside female reproduction tract, called capacitation. Capacitation process includes ability to motile, ability to fertile and eliminate cytoplasmic droplet, without capacitation, spermatozoa unable to done fertilization process. Capacitation of spermatozoa *in vitro* is factor that effected to successful rate of IVF technologies. Seminal plasma contains materials or factors that can damage the sperm fertilizing capacity. Seminal plasma may also contain microorganisms that could contaminate the growth media and can produce toxic substances that can inhibit the fertilization process. Separation of spermatozoa and seminal plasma can be done by centrifugation.

Centrifugation will cause a centrifugal force which causes the separation of the semen between solids (pellets) and the liquid (plasma). Washing sperm with centrifugation is important to produce sediment that containing high motile spermatozoa, in addition to washing the spermatozoa with the addition-sediment is to remove decapacitation factor from seminal plasma (Vandervoort, 2004). After centrifugation, remove supernatant and re-suspended with medium, spermatozoa needed to incubate for swim-up to select the highest motile spermatozoa, higher level of membrane plasma integrity means also higher level of spermatozoa motility (Sardjito, 2003). Motile spermatozoa moving up from

lower layers to the top layer of the medium. In order to harvest highest number of plasma membrane integrity on ram's spermatozoa, this research conduct to determine the optimum swim-up time.

1.4 Aim of Research

This research aims to determine the influence of swim-up time on plasma membrane integrity of ram's (*Ovis aries*) spermatozoa following centrifugation.

1.5 Benefit of Research

This research was useful to give information the influence of swim-up time on plasma membrane integrity of ram's (*Ovis aries*) spermatozoa following centrifugation which can be considered in application of IVF program.

1.6 Hypothesis

The hypothesis that can be proposed in this current research is: Swim-up time influence the plasma membrane integrity of ram's (*Ovis aries*) spermatozoa following centrifugation.

IR-PERPUSTAKAAN UNIVERSITAS AIRLANGGA

CHAPTER 2

LITERATURE REVIEW

CHAPTER 2 LITERATURE REVIEWS

2.1 Fat Tailed Sheep

Sheep is a common farm animal in Indonesia for their meat, milk and also wool. Sheep's meat is good sources of protein and animal fat, the demand of their milk also increased in community because of the nutrition values, another benefit of raising sheep is wool that can be used for textile industries. In good conditions a sheep can gave birth every 8 months with interval less than two years. Sheep is the result of human domestication whose its history is derived from three types of sheep that were wild sheep, the Mouflon sheep (*Ovis musimon*) originating from Southern Europe and Asia Minor, Argali sheep (*Ovis amon*) comes from Southeast Asia, Urial sheep (*Ovis vignei*) originating from Asia (Tiyo, 2010). There are at least four different types of sheep in Indonesia namely Priangan sheep, Fat tailed sheep, Garut and *Teksel* sheep.

In Indonesia, fat tailed sheep has been domesticated well in Madura Island, East Java, Lombok, and Gorontalo (Mulyono, 2004). Based on tail shape, Fat tailed sheep divided into *gibas bajing* (straight tail), *gibas peh* (straight and large tail), and *gibas merino* (tail more than 24 cm length, 20 cm wide, and point of tail shaped '*S*'. The differences of the tail can be determine since lamb, and more clearly when reach puberty (Mulyono, 2004). Ram has weight around 40 – 60 kg and height around 60 – 65 cm, while ewe has weight around 25 – 35 kg and height around 52 – 60 cm (Ridwan, 2010). Both male and female sheep generally do not have horns.

IR-PERPUSTAKAAN UNIVERSITAS AIRLANGGA

Sheep are usually reaches puberty age of 4-12 months, depend on climate conditions, diet, heredity and hormonal release. Ram reached puberty after his weight reached 40-60% of adult ram body weight (Hardijanto *et al.*, 2010).

2.2 Anatomy of Ram Genital Organ

Ram reproduction divided into 3 major parts: main genital organ are testis; genital tract consists of epididymis, vas deferens, ampulla, and urethra, accessories gland are seminal vesicle, prostate, and bulbourethral; and external genitals are penis, prepuce, and scrotum (Ismudiono *et al.*., 2010). The testes have two functions as reproductive and endocrinology. Reproductive function produces cells that form spermatozoa in the testis (seminiferous tubules) through the process of spermatogenesis. The endocrinology of the testes produces variety of steroid hormones (androgens / testosterone and estrogen) and non-steroidal hormone (inhibin). Testes are wrapped by scrotum, where the scrotum contains two testicle lobes each containing one testis (Ismudiono *et al.*, 2010). The function of scrotum are for protect testes from physical trauma and to maintain testes temperature below body temperature for spermatogenesis. In normal conditions, scrotum maintain testes temperature by constrict or loosen the scrotum's wall.

Epididymis is divided into three parts, namely, caput (head), corpus (body), and cauda (tail) epididymis (Ismudiono *et al.*, 2010). The epididymis functions are 1. Transportation, the epididymis first function means of transport for spermatozoa. Travelling time of spermatozoa in the epididymis in sheep, cattle and pigs are varied, 2. Concentration, the concentration of spermatozoa, which

when spermatozoa enter the epididymis together with liquid from the testes are in a state of relatively dilute, estimated 100 million per millimeter in cattle, sheep and pigs. In the epididymis the spermatozoa were concentrated to approximately four billions spermatozoa per millimeter, 3. Depotition. The third function is as a place of deposition (storage) of spermatozoa. Most are stored in the cauda, where spermatozoa are concentrated in the part that has a large lumen, 4. Maturation,It can be proved that the spermatozoa had just entered the caput epididymis from vasa efferentia has no fertility, and also lack of motility. Spermatozoa after passing through the epididymis, they will have fertility and motility (Keiko, 2009). Vas deferens is the tract for spermatozoa to move from caudal epididymis into urethra, Part of the vas deferens is enlarged near the urethra, called the ampulla. The vas deferens has a thick smooth muscle on the walls and has a single function that means for transport of spermatozoa. Vas defferens together with blood vessel, lymph, and nerves form funiculus spermaticus. Urethra is a single channel that runs from the junction with the ampulla up to the base of the penis. The function of the urethra is the urinary tract and semen (Ismudiono *et al.*, 2010).

Bull and ram during ejaculation occurs a complex mixing between dense spermatozoa from the epididymis and vas deferens with liquid secretion from accessory glands on the urethra into semen (Keiko, 2009). Accessory gland is a complement of the genitals. The liquid produced the biggest part of the semen, and contain lots of carbohydrates, proteins, amino acids, enzymes, vitamins that are soluble in water, minerals, citric acid and materials - other organic materials.

These accessory glands consist of additional vasicular gland, prostate gland and bulbourethral glands or Cowper's glands (Ismudiono *et al.*, 2010).

Cattle, sheep, goats, and pigs have a penis shaped like the letter "S" (sigmoid flexure) so that the penis can be pulled and be totally in the body (Ismudiono *et al.*, 2010). In ram size of penis around 5 – 7.5 cm and diameter 1.5 – 2 cm. function of penis are for urination and to provide semen penetrating into the female reproductive tract. At the time of holding the semen for artificial insemination programs, is important to shave the hair around prepuce, to keep the semen from bacterial contamination (Hardijanto *et al.*, 2010).

2.3 Ram Semen and Spermatozoa

2.3.1 Semen

Semen is fluid that contains male gamet (spermatozoa) and secretion from accessory gland of male genital organs. Secretion of accessory gland formed at ejaculation and called seminal plasma. Seminal plasma has important role in male reproduction system because contains nutrition for feeding spermatozoa. Volume per ejaculate in sheep around 0.5 to 2.0 cc with spermatozoa concentration of 800-4000 million / cc. Ram's semen consists of 1/3 spermatozoa cells and the least is accessory fluid that contain of fructose and citric acid, both came from seminal vesicule gland. Ram's semen also contain Fe, Zn, Cu and Plasmalogen (Hardijanto *et al.*, 2010).

Quality and quantity of semen depend on some factors, such as: ages, health conditions, frequency of semen collection. Ram semen characteristics

based on macroscopic examination are follows: Volume range between 0.8 - 2.0 ml; Thick concentrated; Dark white color; pH range 6.4 – 7 (Hardijanto *et al.*, 2010). Meanwhile, microscopically spermatozoa has the characteristic as follows: concentrations of 800-900 million per milliliter; more than 80% motility; the percentage of dead spermatozoa not more than 50%; abnormalities not more than 20% to (Evan and Maxwell, 1987).

2.3.2 Spermatozoa

Spermatozoa are male genital cells produced in seminiferous tubule by the influence of Folicle Stimulating Hormone (FSH) via spermatogenesis process (Ismudiono *et al.*, 2010). Spermatozoa consist of three parts, namely the head, neck and tail (Darusman, 2005). Tail itself divided into mid-piece, main-piece, and end-piece. Bull and ram spermatozoa have heads 8 - 10μ long, width from 4 to 4.5 μ and 1.5 μ thick. In the middle part of spermatozoa body has a length of 1.5 - 2 times the length of the head with a diameter of 1μ. While the length of spermatozoa 35 - 45μ in diameter of 0.4 to 0.8 μ, overall length of 50-70μ.

Spermatozoa consist of some chemicals compound such as, dexyribonucleoprotein mostly found in the nucleus from head of spermatozoa. Mucopolysacarides bound to protein molecules in acrosome consist of fructose, manose, galactose, and hexose. Protein covers all spermatozoa body, these protein can be found in membrane cells and fibrils. Enzyme and coenzyme used by spermatozoa for hydrolysis and oxidation.

The process of spermatozoa formation is called spermatogenesis. Spermatogenesis process divided into two stages; 1. Spermatogenesis stage, in

this phase development of spermatogenic tissue occurs by simple cell division and reduction division followed, which ended with the formation of spermatids. In reduction division, going by the number of mitotic chromosomes of diploid (2n) to haploid (n); 2. Spermiogenesis stage, in this stage spermatids metamorphosis into spermatozoa, it will perfect changes. These changes include the formation of the acrosome, head, body, and tail of spermatozoa. During growth to adulthood, spermatozoa attached to sertoli cells and get the food to be able to escape into the hole of the seminiferous tubules.

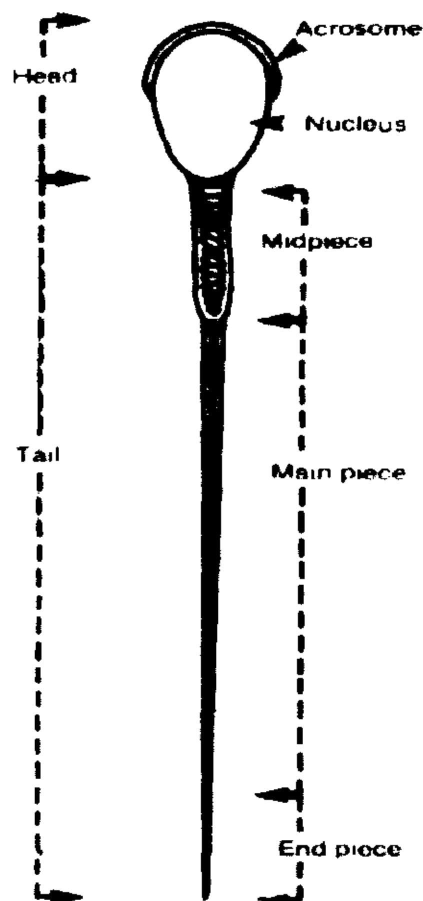


Figure 2.1 Ram spermatozoa morphology (Evan and Maxwell, 1998)

2.4 Centrifugation

Success rate of IVF entirely depend on washed spermatozoa intact with oocytes. Seminal plasma contains materials or factors that can damage the sperm fertilizing capacity. Seminal plasma may also contain microorganisms that could contaminate the growth media and can produce toxic substances that can inhibit the fertilization process.

The removal of sperm from the seminal fluid is a very important preparation step in semen processing for assisted reproductive procedures. Most sperm processing techniques involved centrifugation to separate motile sperm from non-motile or dead sperm as well as other contaminating debris. However, sperm processing techniques that employ centrifugation have their own disadvantages, studies have shown that sperm preparation, processing and handling lead to increased free radicals derived from oxygen called reactive oxygen species (ROS); examples include superoxide anion, and hydroxyl radicals (Lampiao, 2010).

Washing semen by centrifugation method as standard procedure IVF program consisted of 0.5 ml of semen dilution with 5 ml of physiological media. Suspense in the form of semen and the physiological medium was centrifuged at 1800 rpm for 10 minutes at room temperature. After the centrifugation process is completed is usually followed by a swim-up process. Swim-up is a technique to obtain highest motile spermatozoa as well as to stimulate the capacitation of spermatozoa. Motile spermatozoa will move or swim-up from the bottom layer to top layer of the medium (Hinting, 1989). One of isotonic spermatozoa washing

media is Brackett and Oliphant (BO). BO medium contains calcium, magnesium, and sodium chloride, in addition to glucose, pyruvate as spermatozoa nutrition as well as ammonia acid required for the survival of spermatozoa (Rimayanti *et al.*, 1998).

2.5 Swim-up

Sperm washing is a method for preparing samples of semen to use for IVF. Semen used for IVF should be washed to remove debris, white blood cells, and prostaglandins that can cause uterine contraction. This process can also throw off the dead spermatozoa, this method can also increase the fertility rate of spermatozoa so it can be used to overcome fertility problems (Bollendorf *et al.*, 1994).

Spermatozoa may be selected by their ability to swim out of seminal plasma and into culture medium. This is known as the "swim-up" method. Swim-up is one of the sperm washing technique and is one of the best techniques. Swim-up is a technique to obtain motile spermatozoa as well as to stimulate the capacitation of spermatozoa. Motile spermatozoa moving up from lower layers towards the top layer of the medium. During capacitation process, acrosomal changes occur to prepare for penetration of spermatozoa in to the ovum (Austin and Shorts 1972).

Swim-up method is an excellent method for measuring the motility of spermatozoa (Suttiyotin and Thwaites, 1993). Swim-up method does not cause DNA damage to spermatozoa (Younglai *et al.*, 2001). Swim-up method procedure

to select nonapoptotic spermatozoa, in addition to viable and noncapacitated sperm, compared with other sperm preparation methods (Marti *et al.*, 2006). For sheep IVF Swim-up can be routinely used with good fertilization result (Batista *et al.*, 2006). This method is useful for IVF when the percentage of motile spermatozoa is low (Cooper, 2010)

One medium that is isotonic washing spermatozoa was Brackett-Oliphant (BO) medium containing calcium, magnesium, and sodium chloride in addition to glucose and pyruvate as nutritional ingredient as well as acid ammonia spermatozoa indispensable to the survival of spermatozoa. Natrium sodium pyruvate and glucose in BO medium as an energy source. Glucose in BO medium serves as a source of energy through glycolysis process that converts glucose into glucose 6 phosphate. Furthermore, glucose 6 phosphate change to fruktosa 6 phosphate. Then through enzyme difosphophiridin nucleotide (DPN) help, fructose 6 phosphate is converted into glycerin acid monophosphat act as energy source that is used for sperm motility and cell metabolism and biosynthesis. (Rimayanti *et al.*, 1998)

2.6 Spermatozoa Plasma Membrane Integrity

Integrity of the sperm plasma membrane is essential for cell survival and fertilizing ability (Mehmood, 2009). The sperm plasma membrane is an outer cell structure that acts as a physiological barrier and its integrity is required for their normal activities. Spermatozoa plasma membrane is responsible for managing ions flow, especially when fertilization occurs. Spermatozoa membrane is

composed of 43% lipid (phospholipids bilayer), 48% protein and 9% carbohydrate. The composition of the lipid membrane are phospholipids, sterols, fatty acids, and poly unsaturated fatty acids they are susceptible to damage, cholesterol is also known to regulate cell membrane fluidity and permeability (Hardijanto *et al.*, 2010).

Plasma membrane components have a very unique function such as attachment of spermatozoa with an ovum, substrate transport and metabolism. Seminal plasma damage can cause damage to fatty acids, especially poly unsaturated fatty acids which are the essential components of membrane phospholipids constituent of spermatozoa, inactivation of glycolytic enzymes, DNA chain termination, then cause a decrease in motility and death of spermatozoa (Suherni, 2008).

Examination of the plasma membrane using the HOS (Hypo Osmotic Swelling) where hypo osmotic solution composition are 0.9 g fructose added 0.49 g of sodium citrate dissolved in aquabides until it reaches a volume of 100 ml (Souhoka *et al.*, 2009). Hypo osmotic solution is then mixed with sperm, incubated for 30 minutes (Cooper, 2010) and then made thin preparations. Spermatozoa that have intact plasma membrane are characterized by coiled or distended tail, while the spermatozoa with broken plasma membrane are characterized by straight tail (Souhoka *et al.*, 2009).

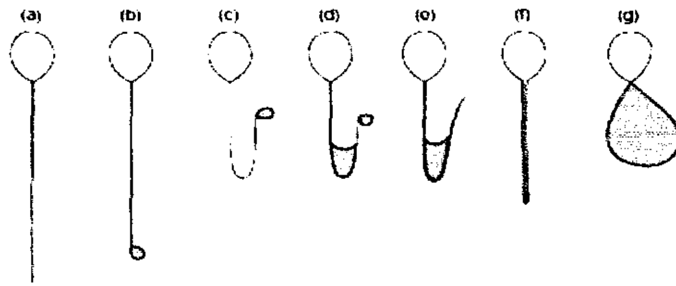


Figure 2.2 Schematic representation of typical morphological changes in human spermatozoa subjected to hypo-osmotic stress. (a) damaged plasma membrane integrity (straight tail). (b)-(g) plasma membrane intact (swelling in tail is indicated by the gray area) (Cooper, 2010).

IR-PERPUSTAKAAN UNIVERSITAS AIRLANGGA

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 3 MATERIALS AND METHODS

3.1 Research Location and Time

The research was conducted at Artificial Insemination (AI) Laboratory of Reproduction Department of Faculty of Veterinary Medicine, Airlangga University. The research was performed from November 2011 to January 2012.

3.2 Research Materials and Equipments

3.2.1 Research Materials

Materials used in this research are ram semen, Brackett – Oliphant (BO) medium, Hypo Osmotic Swelling (HOS) medium.

3.2.2 Research Equipments

Equipment used in the current research consisted of an artificial vagina, pipettes, test tubes, test tube with scale, object-glass, cover glass, centrifuge, cotton, microscopy, sperm counter, litmus paper, Bunsen burners, and digital cameras.

3.3 Research Methods

3.3.1 Semen Collection

Prior to collection, the water jacket part of artificial vagina was filled with warm water (42-45°C) and the inner liner was lubricated with vaseline. The ram was prepared with the prepuce area cleaned from hair to prevent both

contaminations of dirt and microorganisms. Ram was walked around the teaser ewe so that the libido will increase and a large volume of semen would be ejaculated. Ram will mount the ewes with the semen collector manually diverting the ram's penis into the artificial vagina. Semen was collected in scaled conical tube attached to the artificial vagina, exposure of semen to sunlight was avoided by covering the tube with tissue or aluminum foil.

3.3.2 Semen Observation

Semen which has been collected is observed macroscopically and microscopically. Macroscopic observation such as semen color, odor, pH, consistency and concentration, while the microscopic Observation includes motility, mass movements, individual movements, or living dead sperm viability.

To observe the motility of spermatozoa, the movement of the masses by means one drop of semen plus one drop of physiological saline and then cover with a glass lid on top of a glass object and then viewed under a microscope magnification of 400 times. Then make an assessment based on actively moving spermatozoa on glass objects.

To observe the live spermatozoa with making preparations with one drop of semen and one drop of eosin negrosin on object glass and swipe with another object, after that make fixation on the bunsen burner. View under a microscope with a magnification of 400 times. Live spermatozoa does not absorb dye so the head of is colored transparent and the dead will absorb dye so the head of spermatozoa is colored purple. The color of the spermatozoa tail can't be determine the living or dead spermatozoa.

3.3.3 Sperm Swim-up

BO medium was taken out of refrigerator and warmed to room temperature. Then spermatozoa that have been observed and have good quality prepared and eligible are incorporated into three different centrifuge tubes. Each tubes consists of semen as much as 0.25 ml and 0.5 ml of medium BO then centrifuged at 1800 rpm for 5 minutes. BO medium replaced with fresh one, half ml of BO medium was then layered gently on top of sperm pellet and sperm was allowed to swim-up for 15, 30, and 45 minutes respectively for T1, T2, and T3.

3.4 Observed Variable

In this research the observed variable is the plasma membrane integrity of spermatozoa. This research investigated the effect of swim-up time after being centrifugation given to the observed variables. Plasma membrane was observed by using HOS test after 30 minutes incubation (Cooper, 2010). Spermatozoa that have intact plasma membrane characterized by coiled or distended tail, while the spermatozoa with broken plasma membrane characterized by straight tail (Souhoka *et al.*, 2009).

3.4.1. Independent Variable : Swim-up time

3.4.2. Dependent Variable : Plasma membrane integrity of ram spermatozoa.

3.4.3. Controlled Variable : Ram, room temperature, feeding, rearing system, and environment condition.

3.5. Research Design and Data Analysis

Research design use Accidental Sampling (AS) consisting of three treatments and six replications. Obtained data were processed using Analysis of Variance (ANOVA) and to determine difference between the treatment given, if there are significant different then proceed with a test distance of Double Duncan's (Duncan's Multiple Range Test) with a level of significant 5% to determine the influence of treatments (Kusriningrum, 2008).

3.6 Research Frameworks

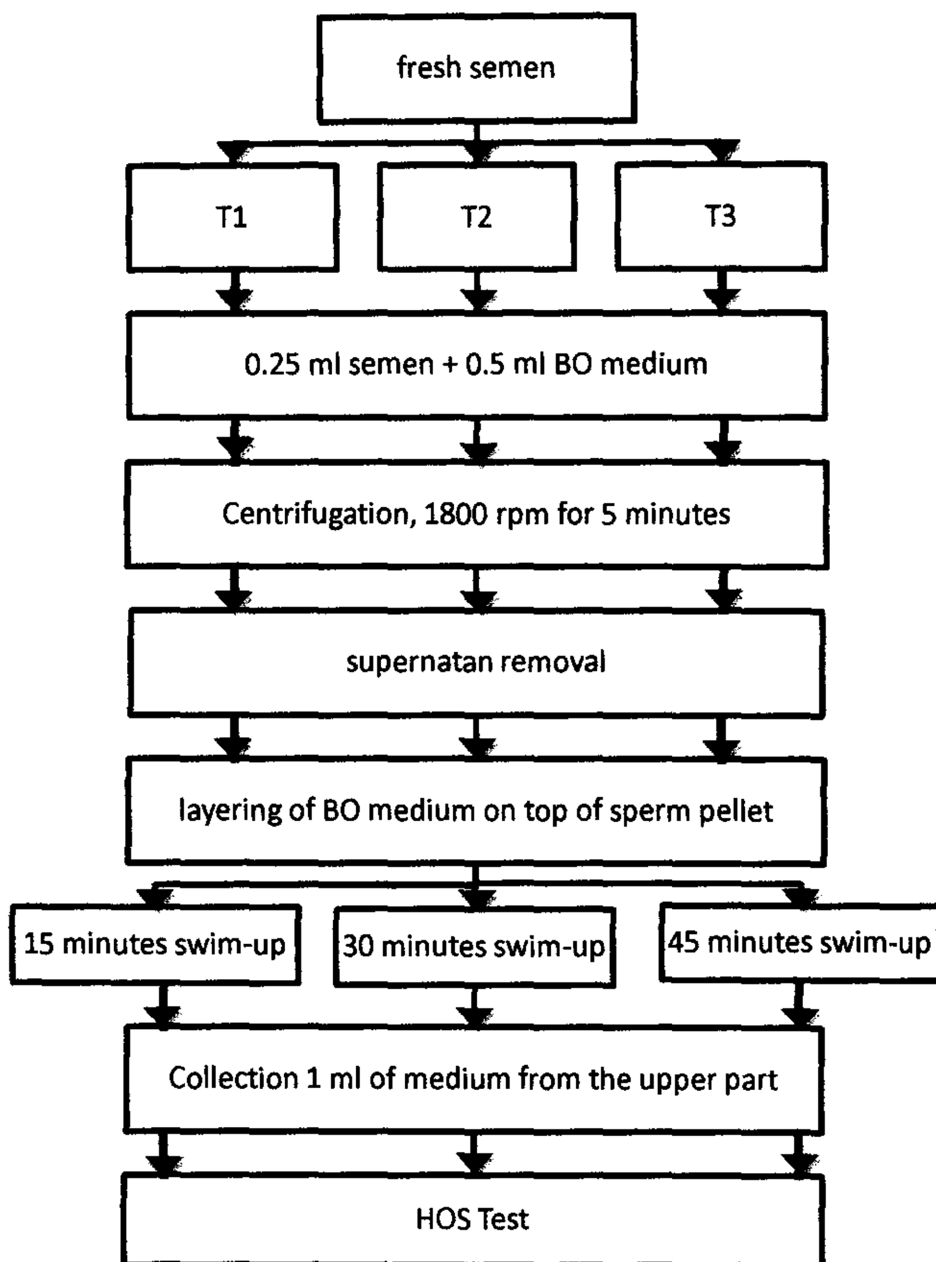


Figure 3.1 Research Frameworks

CHAPTER 4

RESEARCH RESULTS

CHAPTER 4 RESULTS

4.1 Semen Observation before Treatment

Before further treatment semen should first be observed macroscopically and microscopically. Macroscopic observation includes volume, color, odor, pH, and consistency. While microscopic examination involves the observation of mass movement, individual movement, motility, viability, and plasma membrane integrity. The results of preliminary observation prior treatment can be seen in Table 4.1.

Table 4.1 Macroscopic observation results of ram's semen before treatments.

Observation	n1	n2	n3	n4	n5	n6
Volume	1,2 ml	1 ml	1 ml	1,1 ml	1 ml	1 ml
Consistency	Thick	Thick	Thick	Thick	Thick	Thick
Odor	Specific	Specific	Specific	Specific	Specific	Specific
Color	Creamy white	Creamy white	Creamy white	Creamy white	Creamy white	Creamy white
pH	6-7	6-7	6-7	6-7	6-7	6-7

n = replication

Ram semen characteristics based on macroscopic observation as follows: volume range between 0.8 - 2.0 ml, thick concentrated, the dark white color or creamy white, pH range 6.4 – 7 (Evan and Maxwell, 1987).

Table 4.2 Microscopic observation results of ram's semen before treatments.

Observation	n1	n2	n3	n4	n5	n6
Mass movement	+++	+++	+++	+++	+++	+++
Individual movement	Prog 4	Prog 4	Prog 4	Prog 4	Prog 4	Prog 4
Concentration	Densum	Densum	Densum	Densum	Densum	Densum
Percentage of motility	94%	95%	93%	95%	95%	88%
Percentage of viability	95%	96%	90%	93%	95%	90%
Percentage of membrane plasma integrity	92%	90%	88%	95%	88%	87%

n = replication

Microscopically spermatozoa have the characteristic as follows: concentrations of 800 – 900 million/milliliter, more than 80% motility, the percentage of dead spermatozoa less than 50%, abnormalities less than 20% (Evan and Maxwell, 1987).

Three (+++) positive assessment means that the mass movement of spermatozoa formed large waves with fast movement which means that spermatozoa is in good quality (Hardijanto *et al*, 2009). Observation of individual movement was done under microscope with magnification of 400 times, the result

is P (progressive) for all samples, this means that each spermatozoon moves forward. Fresh semen motility and viability of the results of each were 88% - 95% and 90% - 96% for live spermatozoa. As can be seen in Table 4.2 the plasma membrane integrity was 87% - 95%. According to Cooper (2011) the samples used have met the eligibility requirements for the purpose of this study.

4.2 Semen Observation after Treatment

4.2.1 Spermatozoa Plasma Membrane Integrity

Spermatozoa plasma membrane is responsible for managing ions flow, especially when fertilization occurs. Plasma membrane integrity was observed using HOS test, Hypo osmotic solution is then mixed with sperm, incubated for 30 minutes (Cooper, 2010). Plasma membrane integrity percentage was counted by coiled or distended tail spermatozoa which is means that spermatozoa have intact plasma membrane (Figure 4.1). The result of spermatozoa plasma membrane integrity is presented in Table 4.3.

Table 4.3 Spermatozoa plasma membrane integrity after treatments (Means $\pm$ SD)

Treatment	Spermatozoa plasma membrane integrity ($X \pm SD$)
T1 (swim-up 15 minutes)	63.55 ± 3.256^{a}
T2 (swim-up 30 minutes)	44.03 ± 5.484^{b}
T3 (swim-up 45 minutes)	39.65 ± 5.210^{b}

Different superscript in the same column indicate significant differences ($p < 0.05$)

Based on the Analysis of Variance (ANOVA) in Table 4.3 followed by Duncan's Multiple range test with a significancy level of 5% it was found that the 15 minutes swim-up after centrifugation (T1) showed the highest yield, namely 63.55 $\pm$ 3.256 and there was significant difference between T1 to T2 and T3 (P <0.05). Meanwhile there was no significant difference between T2 to T3.

From Figure 4.2. it can be seen that swim-up time influence the spermatozoa plasma membrane integrity. On 15 minutes swim-up after centrifugation (T1) the percentage of spermatozoa plasma membrane integrity is the higest followed by T2 and T3. On treatment with 45 minutes swim-up (T3) showed the lowest percentage of spermatozoa plasma membrane integity.

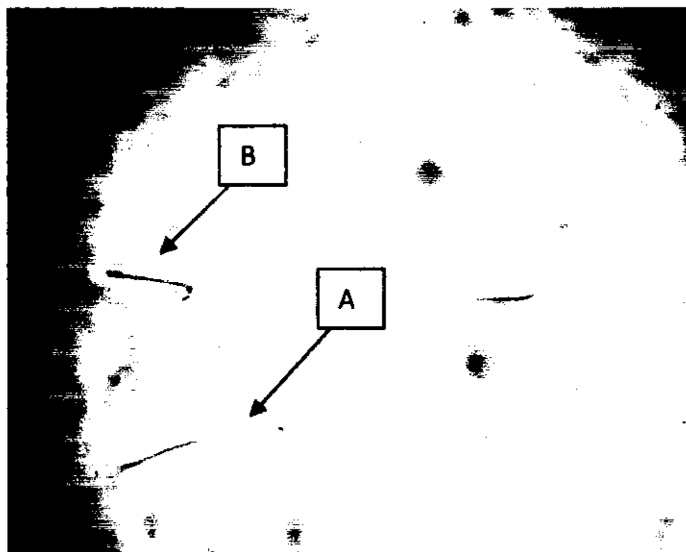


Figure 4.1 Spermatozoa with HOS test and eosin negrosin staining (400 times magnification). A. Damaged plasma membrane (the straight tail), B. The plasma membrane intact (coiled tail).

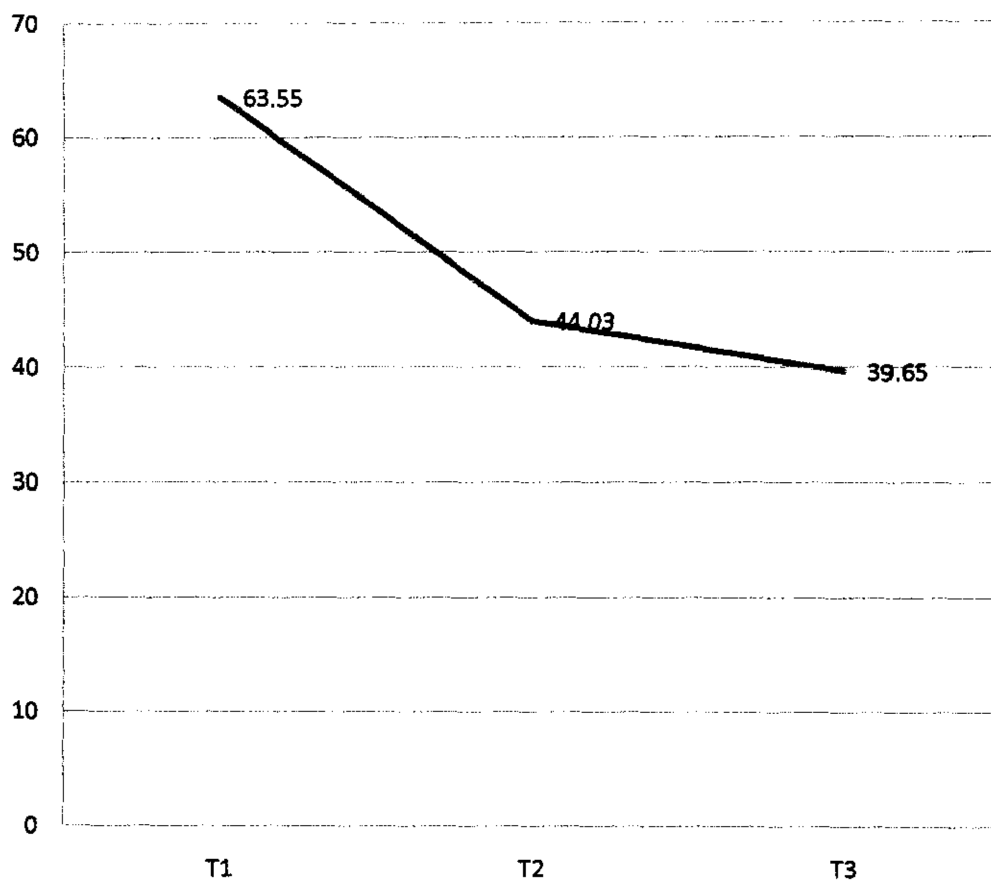


Figure 4.2 Spermatozoa plasma membrane integrity after swim-up for 15, 30 and 45 minutes.

IR-PERPUSTAKAAN UNIVERSITAS AIRLANGGA

CHAPTER 5

DISCUSSION

CHAPTER 5 DISCUSSION

Spermatozoa must be washed free from seminal plasma before they are capable of undergoing capacitation, acrosome reaction, and penetration of the zona pellucida. Under normal physiological conditions, seminal plasma is removed as the spermatozoa traverse the cervical mucus, and capacitation occurs as they are transported across the cervix, uterus, and fallopian tubes. Centrifugation is one of the common sperm preparation techniques for both experimental and practical program. Centrifugation facilitates the removal of seminal plasma. Centrifugation force determined membrane integrity of spermatozoa, centrifugation with 700 g caused damage to the plasma membrane of spermatozoa, thus decreased membrane integrity of spermatozoa. Plasma membrane also protect cells from mechanical damage, the plasma membrane also acts as a filter for the exchange of substances intra and extra cellular maintained in metabolic processes (Garner and Hafez, 2000). Spermatozoa plasma membrane that remains intact will give a positive effect on motility and the viability of spermatozoa (Firda *et al.*, 2012). Motility of spermatozoa is depending on the supply of energy in form of ATP from metabolism, that normal metabolism will take place properly if the plasma membrane intact. This is because the plasma membranes regulating the exchange of the substrate and the electrolyte that is needed in the process of metabolism (Souhoka *et al.*, 2009).

In this current research the highest mean of plasma membrane integrity was obtained from the 15 minutes (T1), which were significantly different those were allowed to swim-up for 30 minutes (T2) and swim-up for 45 minutes (T3).

While there were no significant difference between T2 and T3 treatment. This results is in accordance with results observed by Mardini *et al.* (2012) for swim-up without centrifugation shows decreased sperm plasma membrane integrity during longer incubation time. This is possibly due to sperm ageing, low survival rate of spermatozoa in room temperature (Valcarcel *et al.,* 1994), mechanical damage cause by centrifugation (Alvarez *et al,* 1993), BO medium damage, and the effect of the recurrent pippetting to which sperm are subjected during process (Marti *et a.l,* 2006). Compositions of seminal plasma are important to maintain the stability of membrane plasma especially cholesterol (Aurich *et al.*, 1996).

Damage to the plasma membrane integrity of spermatozoa also can cause by ROS (Suherni, 2008). ROS is a strong oxidant and can lead to impaired cell integrity, ROS can also damage the cells or molecules. ROS generated by sperm in response to centrifugation should be able to initiate lipid peroxidation and, as a consequence of the resulting loss of membrane fluidity and integrity, bring about the observed decline in sperm function (Agarwal *et al.,* 1994). Damage to the cell membrane will increase the permeability of cell membranes, so the material that should not be able to pass through cell membranes pass freely in and out the cells and eventually disrupted the integrity of sperm cells (Argawal *et al.*, 2003). Sperm are particularly susceptible to peroxidative damage because they contain an extremely high concentration of polysaturated fatty acids, exhibit no capacity for membrane repair, and possess a significant ability to generate ROS, chiefly superoxide anion and hydrogen peroxide (Agarwal *et al.,* 1994). The levels of ROS increase by many factor such as radiation, chemicals, hyperoxia, and normal

cell metabolism (Da Silva, 2010). Accumulation of the ROS in sperm leads to ATP depletion (Dokmeci, 2005). During the incubation process there still metabolism it lead the increase of ROS. This metabolism exist as long as incubation process, on the other hand in this study there were no antioxidant added, where antioxidants were then subjected of extensive research as they protect cells against the damaging effects of reactive oxygen species (ROS) (Da Silva, 2010). Actually there is a natural antioxidant contained in semen plasma act by neutralizing excessive ROS, and prevent it from damaging the cellular structure but over time during the incubation process, centrifugation and resuspension, the antioxidants were damaged (Agarwal *et al.*, 2003).

In Conclusion, the longer incubation time will produce more ROS and lead the decrease of plasma membrane integrity. This research recommend incubation time for swim-up following centrifugation is 15 minutes to minimize plasma membrane integrity damage.

CHAPTER 6

CONCLUSION AND RECOMMENDATION

IR-PERPUSTAKAAN UNIVERSITAS AIRLANGGA

CHAPTER 6 CONCLUSION

6.1 Conclusion

The results of this study can be concluded as follow: Swim up time influenced the plasma membrane integrity of ram's (*Ovis aries*) spermatozoa following centrifugation.

6.2 Suggestion

Suggested in the process of swim-up it is better with incubation for 15 minutes following centrifugation in order to obtain the highest level of plasma membrane integrity for IVF.

IR-PERPUSTAKAAN UNIVERSITAS AIRLANGGA

SUMMARY

IR-PERPUSTAKAAN UNIVERSITAS AIRLANGGA

SUMMARY

Aldino Yanuar Efendi. The study entitled "Influence of Swim-Up Time on The Plasma Membrane Integrity of Ram's (*Ovis aries*) Spermatozoa Following Centrifugation" under the guidance of Dr.. Suherni Susilawati, M.Kes., drh., as the Research Supervisor, Dr. Bambang Poernomo S., M.S., drh. as the Major Supervisor and Budiarto, M.P., drh. as the Secondary Supervisor.

Washing sperm by centrifugation method was a procedure that must be performed in-vitro fertilization program, this procedure was performed to obtain spermatozoa without semen plasma, freezing media and other polluting substances, with a motility rate were eligible for in-vitro fertilization.

This study was conducted to assess the influence of swim-up time on plasma membrane integrity of ram's spermatozoa following centrifugation for 15 minutes (T1), 30 minutes (T2), and 45 minutes (T3). Every each groups were repeated six times. The research was conducted on November 29, 2011 to January 10, 2012. The procedure of this study took the semen of fat tailed sheep every two times a week. Semen obtained then was taken to the laboratory Artificial Insemination Section Reproduction, Faculty of Veterinary Medicine, University of Airlangga for microscopic observation, macroscopic observation, and treatments.

The results showed that the swim up time affect the plasma membrane integrity of ram spermatozoa. Suggested that incubation for 15 minutes following centrifugation gives lowest damage to plasma membrane integrity of ram spermatozoa.

IR-PERPUSTAKAAN UNIVERSITAS AIRLANGGA

REFERENCES

REFERENCES

Agarwal A., I. Ikemoto, and K.R. Loughlin. 1994. Effect of Sperm Washing on Levels of Reactive Oxygen Species in Semen. Archieves of Andrology. Cleveland. 33:157-162.

Agarwal A., Shaleh R.A., Bedalwy M.A., 2003. Role of Reactive Oxygen Species in the Patophysiology of Human Reproduction. Fertil Steril. 79: 829 – 843.

Alvarez JG, Lasso JL, Blasco L, Nunez RC, Heyner S, Caballero P, Storey BT. 1993. Centrifugation of human spermatozoa from induces sublethal damage. Hum Reprod. 83: 1087-1092.

Austin C.R., and R.V. Short. 1972. Reproduction in Mammals book 1. University Press. Cambridge. Pp 54 – 56.

Batista, M., MC Baptista, CC Marques, JP Barbas, MI Vasques, AEM Horta, M Silva Pereira and RM Pereira.2006. Effect of Ram Sperm Capacitation by Swim-Up or Percoll on *In Vitro Fertilization*. Jurnal Reproduction in Domestic Animal 41 (4): 342. http://docs.google.com/ /Repr_ Dom_Anim_ Batista. pdf. [March 7th, 2012]

Bearden, H.J., J.W. Fuquay and S.T. Willard. 2004. Applied Animal Reproduction. 6th ed. Mississipi State University. Mississipi.

Bollendorf A., J.H Check, D. Katsoff, and D. Laurie. 1994. Comparison of Direct Swim Up, Mini Percoll, and Sephadex G10 Separation Procedures. Archives of Andrology. 32:157 – 162.

Cooper, T. G. 2010. WHO Laboratory Manual for the Examination and Processing of Human Semen 5th Ed. WHO Press. Geneva.

Darusman. 2005. *Pengaruh Sentrifugasi dan Swim Up terhadap Motilitas dan Presentase Hiup Spermatozoa Domba pada Media Brackett and Oliphant (BO) dan Bovine Serum Albumin (BSA)* [Skripsi]. Fakultas Kedokteran Hewan. Universitas Airlangga. Surabaya.

Da Silva, F. Moreira, A. Marques, and A. Chaveiro. 2010. Reactive Oxygen Species: A Double Edged Sword in Reproduction. The Open Veterinary Science Journal. 4:127-133.

Ditjennak. 2008. *Roadmap Pembibitan Ternak. Direktorat Jendral Peternakan Departemen Pertanian.* www.ditjennak.go.id/*regulasi%5CRoadmap_ Bab1_2. pdf [March 7th, 2012]*

Ditjennak.2012.StatistikaPeternakan.www.datainfonak.22web.net/index.php?page =statistikpeternakan&action=info. [March 7th, 2012]

Dokmeci, D. 2005. Oxidative Stress, Male Infertility and The Role of Canitines. Folia Med. 47 ; 26-30.

Evans, G. and WMC Maxwell. 1998. Salamon's Artificial Insemination of Sheep and Goat. Butterworths.

FAO. 2009. FAOSTAT statistical database. www.faostat.fao.org . [July 2nd, 2012]

Firda, N.W, Suherni S, Chairul A, and Herman S. 2012. The Influence of Length of Centrifugation Time on Motility, Viability and Plasma Membrane Integrity of Ram Spermatozoa. Thesis. Fakultas Kedokteran Hewan Universitas Airlangga.

Fitri, T.A, 2002. *Pengaruh Kecepatan dan Sentrifugasi Terhadap Keutuhan Membran Plasma dan Tudung Akrosom Spermatozoa Sapi Madura.* Skripsi. Fakultas Kedokteran Hewan Universitas Airlangga.

Garner, D.L and E.S.E. Hafez. 2000. Spermatozoa and seminal plasma In: Reproduction in farm animals. Ed-7. Edited by E.S.E. Hafez and B. Hafez. Lippincot Williams and Wilkins. Philadelphia

Gordon, I. 2004. Reproductive Technologies In Farm Animals. Cromwell Press. Trowbridge. 110-112.

Hardijanto, T. Sardjito, T. Hernawati, S. Susilowati, and T.W. Suprayogi. 2010. Buku Ajar Ilmu Inseminasi Buatan. Fakultas Kedokteran Hewan Universitas Airlangga.

Harrison, R.A.P and White, J.G. 1972. Some Method For Washing Spermatozoa From Bull, Boar and Ram: A Comparrison Using Biochemical and Other Criteria. Journal Reproduction Fertility(29).271.

Heriyadi, Deni. 2010. *Domba dan Kambing di Indonesia: Potensi, Masalah dan Solusi.* http://www.saungdomba.com/artikel-domba-garut/95-domba-dan-kambing-di-indonesia-potensi-masalah-dan-solusi. [7 March 2012]

Hinting, A. 1989. Assesment of Human Sperm Fertilizing Ability. Teses Sumiterted in Fulfillment of the Requirement for the Degree of Special Doctor in Reproduction Medicine. Rijksuniversiteit GENT. Belgium. 22 – 29.

Hunter, R.H.F. 1995. *Fisiologi dan Teknologi Reproduksi Hewan Betina Domestik* (physicology and Technology of Reproduction in Female Domestic Animal). Diterjemahkan oleh D.K. Haryaputra. Edisi Pertama Penerbit Institut Teknologi Bandung. Bandung.

Ismudiono. H. A., P. Srianto, S.P. Madyawati, A. Samik, and E. Savitri. 2010. *Fisiologi Reproduksi Pada Ternak.* Fakultas Kedokteran Hewan Universitas Airlangga. Surabaya.

Juliana L. 2004. *Pengaruh Penambahan Vitamin C dalam Diluter Terhadap Motilitas, Presentase Hidup Mati dan Keutuhan Membran Spermatozoa Domba.* [Skripsi]. Fakultas Kedokteran Hewan Universitas Airlangga. Surabaya.

Keiko.2009. *Anatomi Organ Reproduksi Jantan Ternak.* http://changestheworld.blogspot.com/2009/05/anatomi-organ-reproduksi-jantan.html. [7 March 2012]

Klinc, P., D. Rath, D.Frese, B. Sieg. 2006. Method for preservation for gender specific selection of mammalian spermatozoa. World Intellectual Property Organization.http://books.google.com/books/about/Method_for_preservation_for_gender_speci.html. [7 March 2012]

Kusriningrum. 2008. *Perancangan Percobaan.* Airlangga University Press. Surabaya.

Lampiao, F., Strijdom, H. and Du Plessis, SS. 2010. Effects of Sperm Processing Techniques Involving Centrifugation on Nitric Oxide, Reactive Oxygen Species Generation and Sperm Function. The Open Andrology Journal. 2 : 1-5.

Mardini K, Suherni S, Ismudiono, and Dady S. Nazar. 2012. Influence of Swim-up Time on the Motility, Viability, and Plasma Membrane Integrity of Ram Spermatozoa. Thesis. Fakultas Kedokteran Hewan Universitas Airlangga.

Marti E., R. Pȩrez-Pȩ , T. Muino-Blanco, and J.A. Cebrian′ N-Perȩz. Comparative Study of Four Different Sperm Washing Methods Using Apoptotic Markers in Ram Spermatozoa. Journal of Andrology, Vol. 27, No. 6. Spain. http://www.ncbi.nlm.nih.gov/pubmed/16809277. [7 March 2012].

Mehmood. A., Anwar, M. and Saqlan Naqvi, S.M. 2009. Motility, Acrosome Integrity, Membrane Integrity and Oocyte Cleavage Rate of Sperm Separated by Swim-up or Percoll gradient Method from Frozen-thawed Buffalo Semen. Animal Reproduction Science. 111: 141-148.

Mulyono, S. 2004. *Berternak Domba Profilik.* Cetakan I. Penebar Swadaya. Jakarta.

Nurbiajanti, S. 2011. *Permintaan Kambing dan Domba Naik 600 Persen - KOMPAS.com.* http://bisniskeuangan.kompas.com/read/2011/10/31/1838585 8/Permintaan.Kambing.dan.Domba.Naik.600.Persen. [14 March 2011]

Ridwan.2010.*BudidayaTernakDomba.*http://www.kambingakikah.com/tag/ternak-domba/. [March 7th, 2012].

Rimayanti. Susilowati S, H. Agoes, and Hernawati T. 1998. *Pengaruh Pengenceran Media EBSS dan BO pada Semen Beku Kambing Etawa terhadap Kejadian Kebuntingan Kambing Lokal.* Fakultas Kedokteran Hewan. Universitas Airlangga. Surabaya.

Sardjito, T. 2003. *Pengaruh Sentrifugasi Spermatozoa Sapi Terhadap Integritas Membran, Resistensi dan Kelayakan Kondisi Pada Proses Kapasitasi In-Vitro. Thesis. Penelitian Eksperimental Laboratorik.* Program Pasca Sarjana. Universitas Airlangga Surabaya. 2-4,20-22.

Souhuka, D. F., Michael Johan Matatula, Wilmientje Marlene Mesang-Nelly, and Muhammad Rizal. 2009. *Laktosa Mempertahankan Daya Hidup Spermatozoa Kambing Peranakan Etawa yang Dipreservasi dengan Plasma Semen Domba* Priangan. Jurnal Veteriner September 2009 Vol. 10 No.3:135-145.

Suherni, S. 2008. *Kompleks Insulin Like Growth Factor-I Mempengaruhi Presentase Membran Plasma Utuh dan Kadar Malondialdehid Spermatozoa.* Jurnal Veteriner Desember 2008 Vol 9 N04: 168-175

Suttiyotin, P., and C.J Thwaites.1993. Evaluation of Ram Semen Motility by *Swim up method.* Journal of Reproduction and Fertility (1993) 97: 339 – 345. Departement of Animal Science University of New England. New South Wales.

Sujoko H, M.A. Setiadi, and A. Boediono. 2009. *Seleksi Spermatozoa Domba Garut Dengan Metode Sentrifugasi Gradien Densitas Percoll.* Jurnal Veteriner 10(3).

Taylor, R.E., 1992. Scientific Farm Animal Production. 4th ed. William Wilkins. New York.

Tiyo. 2010. *Budidaya Ternak Domba (Bovidae).* http://tyo-web.faa.im/budidaya-ternak-domba-bovidae.xhtml. [March 7th, 2012].

Valcalcel, A., M.A. De Las heras, L. Peres, D.F. Moses and H. Baldassare. 1994. Fluorescent Staining as a Method of Assessing Membrane Damage and Post Thaw Survival of Ram Spermatozoa. Theriogenology. 46: 483-489.

VanderVoort A. Chaterine. 2004. High Quality Sperm For Nonhuman Primate: Production and Assesment. Reproduction Biology Endrocrinology Journam: 33: 1-4.

Younglai, E.V., D. Holt, P. Brown, A. Jurisicova,and R.F Casper. 2001. Sperm *Swim up method* and DNA Fragmentation. Human Reproduction 16 : 1950 – 1953.

Yovich, J.L. 1995. Sperm preparation for assisted conception. In: Grudzinskas JG, Yovich JL. Gametes The Spermatozoon. New York: Cambridge University Press. Cambridge.

IR-PERPUSTAKAAN UNIVERSITAS AIRLANGGA

APPENDICES

Appendix 1. Composition of Ram's Semen

		Mean
Weight dry		14820
Chloride		87
Sodium		
	in whole semen	192
	in plasma seminal	178
	in spermatozoa	111
Potassium		
	in whole semen	92
	in plasma seminal	89
	in spermatozoa	132
Magnesium		
	in whole semen	8.8
	in plasma seminal	5.8
	in spermatozoa	13.3
Calcium		9
Phosphor inorganic		12
Total nitrogen		875
Non protein nitrogen		57
Urea		44
Uric acid		11
Ammoniac		2
Fructose		247
Lactate acid		36
CO_2		16
Citrate acid		137
Ascorbic acid	H	5

* in mg/100 ml

Source: Hafez, 2000

Appendix 2. Composition of Brackett and Oliphant (BO) mediun

Medium A		
	NaCl	4309,02 mg
	KCl	197,40 mg
	$CaCl_2.2H_2O$	217,10 mg
	$NaH_2PO_4.2H_2O$	84,00 mg
	$MgCl_2.6H_2O$	69,70 mg
	Phenol red 0,5 %	0,1 ml
	Distilated water	500 ml
Medium B		
	NaHCO3	2587,3 mg
	Phenol red	0,5 %
	Distilated water	200 ml
Medium BO		
	1. Medium A	76 ml
	2. Medium B	24 ml
	3. Glucose	150 mg
	4. Sodium Piruvat	13,7 mg
	5. Gentamycin	

Appendix 3. Hypo Osmotic solution.

1. Weighing 13,52 gram fructose + 13,52 gram Na citrate H_2O
2. The ingredient above mixed until homogeny ad 1000ml aqua

Sumber : Suherni (2008)

Appendix 4. The Percentage of Spermatozoa Plasma Membrane Integrity after Treatment

Swim-up Time	Replicates (%)					
	1	2	3	4	5	6
T1 (15 minutes)	80	81	73	79	80	87
T2 (30 minutes)	35	47	44	54	47	63
P2 (45 minutes)	34	31	35	53	43	49

Appendix 5. Data Analyze of Plasma Membrane Integrity

Summarize

Case Processing Summary[a]

	Cases					
	Included		Excluded		Total	
	N	Percent	N	Percent	N	Percent
Plasma_Membrane_Integrity * treatment	18	100.0%	0	.0%	18	100.0%

a. Limited to first 100 cases.

Case Summaries[a]

			Plasma_Membrane_Integrity
treatment t1	1		63.44
	2		64.16
	3		58.69
	4		62.72
	5		63.44
	6		68.87
	Total	N	6
		Mean	63.5533
		Std. Deviation	3.25598

t2	1		36.27
	2		43.28
	3		41.55
	4		47.29
	5		43.28
	6		52.53
	Total	N	6
		Mean	44.0333
		Std. Deviation	5.48399
t3	1		35.67
	2		33.83
	3		36.27
	4		46.72
	5		40.98
	6		44.43
	Total	N	6
		Mean	39.6500
		Std. Deviation	5.21036
Total	N		18
	Mean		49.0789
	Std. Deviation		11.58687

a. Limited to first 100 cases.

ONEWAY ANOVA

Plasma_Membrane_Integrity

	Sum of Squares	df	Mean Square	F	Significance
Between Groups	1943.227	2	971.613	42.977	.000
Within Groups	339.117	15	22.608		
Total	2282.344	17			

Post Hoc Tests

Homogeneous Subsets

Plasma_Membrane_Integrity

Duncan[a]

Treatment	N	Subset for alpha = 0.05	
		1	2
t3	6	39.6500	
t2	6	44.0333	
t1	6		63.5533
Significance		.131	1.000

Means are displayed ...

Plasma_Membrane_Integrity

Duncan[a]

Treatment	N	Subset for alpha = 0.05	
		1	2
t3	6	39.6500	
t2	6	44.0333	
t1	6		63.5533
Significance		.131	1.000

Means are displayed ...

a. Uses Harmonic Mean Sample Size = 6.000

Means Pl