FYP FIAT

Comparison of Male Reproductive Performance with Naturally Mated and Superovulated Female Mice

By

CHENG YEOW KOON

F14A0425

A report submitted in fulfillment of the requirements for the degree of Bachelor of Applied Science (Animal Husbandary) with Honours

Faculty of Agro Based Industry

UNIVERSITI MALAYSIA KELANTAN

2018

DECLARATION

I hereby declare that the work embodied in this report is the result of the original research and has not been submitted for a higher degree to any universities or institutions.

____

Student Name: CHENG YEOW KOON

Date:

I certify that the report of this final year project entitled “Comparison of Male Reproductive Performance with Naturally Mated and Superovulated Female Mice” by CHENG YEOW KOON, matric number F14A0425 has been examined and all the correction recommended by examiners have been done for the degree of Bachelor of Applied Science (Animal Husbandary) with Honours, Faculty of Agro-Based Industry, Universiti Malaysia Kelantan.

Approved by:

____

Supervisor Name: DR. RAJA ILI AIRINA BINTI RAJA KHALIF

Date:

FYP FIAT
UNIVERSITI
MALAYSIA
KELANTAN

FYP FIAT

ACKNOWLEDGEMENT

First of all, I would like to show my appreciation to the University Malaysia Kelantan, Jeli for giving my friend and me the chance to finish my experiment in Thailand. The university supplied some of the chemicals, materials and also provided insurance for going to Thailand. Next, I would like to show my gratitude to my supervisor, Dr. Raja Ili Airina binti Raja Khalif for her guidance and support in my final year project implementation. Her guidance gave me the idea of this topic and her support on giving practices in lab mice, helping us to communicate with Princess of Naradhiwas University (PNU) in Naradthiwat, Thailand and chemicals with materials of the experiment support. I would like to thank her and her husband in advance for taking care of me and my friend in Thailand.

Besides, I would like to show my gratitude for the guidance of my co-supervisor in PNU Naradthiwat, who is Dr. Jakkhaphan Chasombat. He gave us his full support by providing a fully completed lab for the mice embryo flushing also for cell culture, all the require equipment and chemicals were also made available. Kak Sura Surasu and Kak Nuseela Ka-jae who were the assistant of the lab indeed helped us in advance, supporting us on finding the material and chemicals in the lab. In addition, I very much appreciate PNU University for everything as they are generously provided like free accommodation and such a good environment for my experiment, thanks to the dean and the lecturers in PNU and also to friendly students in PNU. They have been good tour guides and were kind to help us. Thus, I would like to thank again the people who gave their help and support to me. Without them my project will not be completed successfully.

UNIVERSITI
MALAYSIA
KELANTAN

Comparison of Male Reproductive Performance with Naturally Mated and Superovulated Female Mice

ABSTRACT

Superovulation is integrated in Assisted Reproductive Technology prior cryopreservation. It is a procedure that exogenous gonadotrophins such as pregnant mares' serum gonadotrophin or follicle stimulating hormone followed by human chorionic gonadotrophin administrated to the female mice for producing large numbers of embryos. Superovulation reduces the number of animal used while increase the number of oocyte or embryo production. In this study, the reproductive performance of male mice was conducted using superovulated (SO) mice and naturally mated (NM) mice, also the optimal male semen condition was determined by observing the number of sample presented. The study investigated the factor that affecting the mice performance. Furthermore, the vaginal copulatory plug was an important element for a successful mating. Limitation of the study was investigating on ICR breed mice and limited time for mating 20 female mice in 2 weeks. As result, the SO mice successful to produce more sample (n=106) comparing NM mice (n=75) in number. The semen condition which dilute produce only oocyte (0% embryo produced) while high amount of embryo was discovered in the hard solid condition for SO mice (73% embryo produced) and NM mice (100% embryo produced).

Keywords: Superovulation, Oocyte, Embryo, Vaginal Copulatory Plug, Reproductive Performance.

FYP FIAT

UNIVERSITI MALAYSIA KELANTAN

Perbandingan Prestasi Pembiakan Tikus Jantan dengan Tikus Betina yang Mengawan secara Semulajadi atau Tikus Betina yang Induksi Superovulasi

ABSTRAK

Superovulasi terkamir di aspek krioawetan dalam Pembiakan Teknologi asas Pembantuan. Ia adalah satu prosedur yang menggunakan gonadotropin eksogenus seperti pregnant mares' serum gonadotrophin atau follicle stimulating hormone, diikuti oleh penyuntikan human chorionic gonadotrophin kepada tikus betina untuk menghasil embrio yang banyak. Superovulasi dapat mengkurangkan bilangan haiwan yang digunakan serta meningkat penghasilan oosit atau embrio. Tikus bentina yang induksi superovulasi dan tikus yang mengawan secara semulajadi telah digunakan dalam kajian ini untuk membandingkan prestasi tikus jantan. Keadaan mani yang optimum juga ditentui oleh nombor sampel yang didapati dari tikus betina. Selain itu, punca-punca yang menjejaskan performasi tikus jantan telah disiasat. Salah satu unsur penting untuk memasti pengawaan itu berjaya adalah dengan memerhatikan tiga keadaan air mani iaitu keadaan cair, separuh cair dan keras. Kajian ini hanya mengaji pretasi dan sampel dihasil dari baka tikus ICR dan kekurangan masa untuk mengawan 20 ekor tikus betina dalam 2 minggu menjadi limitasi kajian ini. Keputusan dalam kajian ini menunjukan tikus yang mengalami superovulasi menghasilkan lebih banyak sampel (n=106) berbandingan tikus yang membiak secara semulajadi (n=75) dalam bilangan. Air mani yang cair hanya bagi oosit (0% embrio dihasilkan) tapi banyak embrio didapati dalam tikus induksi superovulasi (73% embrio dihasilkan) dan tikus mengawan secara semulajadi (100% embrio dihasilkan) yang mempunyai keadaan air mani yang keras.

Kata Kunci: Superovulasi, Oosit, Embrio, Vaginal Copulatory Plug, Prestasi Pembiakan.

FYP FIAT

UNIVERSITI
MALAYSIA
KELANTAN

FYP FIAT

TABLE OF CONTENTS

	PAGE
DECLARATION	II
ACKNOWLEDGEMENT	III
ABSTRACT	IV
ABSTRAK	V
TABLE OF CONTENTS	VI
LIST OF TABLES	IX
LIST OF FIGURES	X
LIST OF ABBREVIATION AND SYMBOLS	XII
CHAPTER 1 INTRODUCTION	
1.1 Research Background	1
1.2 Problem Statement	2
1.3 Objective	2
1.4 Scope Study	3
1.5 Significant of Study	3
1.6 Limitation of Study	3
CHAPTER 2 LITERATURE REVIEW	
2.1 Reproduction of Male Mice	5
2.2 Superovulation	7
2.2.1 Follicle stimulating hormone (FSH) and human chorionic gonadotropin (HCG)	8
2.3 Embryo Flushing	9

UNIVERSITI MALAYSIA KELANTAN

2.3.1 Two-cell stages embryo and oocyte 10
2.4 Vaginal Copulatory Plug and Semen Condition 11

CHAPTER 3 METHODOLOGY
3.1 List of Materials and Apparatus 12
3.1.1 Materials 12
3.1.2 Equipment and Apparatus 13
3.1.3 Mouth Pipette for Embryo Transfer 13
3.1.4 Modified Phosphate-buffered saline solution with Calcium Chloride Dihydrate and Magnesium Chloride Hexahydrate (mDPBS with Ca^{2+} and Mg^{2+}) 14
3.1.5 PBI medium 15
3.2 Methods 16
3.2.1 Animals 16
3.2.2 Obtaining Mice Embryo by Superovulation and Naturally Mating Method 16
3.2.3 Naturally Mating and In Vitro Maturation of Mice Embryo by Plug Checking 17
3.2.4 Collection, Culture and Incubation of Embryos 18

CHAPTER 4 RESULT 20

CHAPTER 5 DISCUSSION 29

CHAPTER 6 CONCLUSION
6.1 Conclusion 36
6.2 Recommendation 36

FYP FIAT
UNIVERSITI MALAYSIA KELANTAN

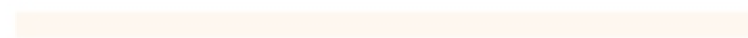
REFERENCES 38

Appendix A 42

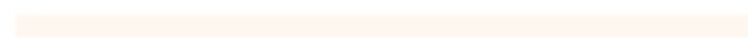
Appendix B 45

FYP FIAT

UNIVERSITI



MALAYSIA



KELANTAN

LIST OF TABLES

No.		PAGE
4.1	Male reproductive performance between NM female mice	20
4.2	Male reproductive performance between SO female mice	22
4.3	The amount of oocyte, embryo and degenerated embryo/ oocyte affect by semen condition in NM female mice in percentages	26
4.4	The amount of oocyte, embryo and degenerated embryo/ oocyte affect by semen condition in SO female mice in percentages	27
A1	The amount of oocyte affects by semen condition in both naturally mated female mice and superovulated female mice in percentages	42
A2	The amount of embryo affects by semen condition in both naturally mated female mice and superovulated female mice in percentages	42
A3	The amount of sample produced in percentages (%) and semen condition from NM female mice after mating	43
A4	The amount of sample produced in percentages (%) and semen condition from SO female mice after mating	44

FYP FIAT

UNIVERSITI

MALAYSIA

KELANTAN

LIST OF FIGURES

No.		PAGE
3.1.3	DIY mouse pipette	14
4.1	Pie chart of male reproductive performance between NM female mice in percentages	21
4.2	Pie chart of male reproductive performance between SO female mice in percentages	22
4.3	Graph of male reproductive performance between NM female mice in percentages	23
4.4	Graph of male reproductive performance between SO female mice in percentages	24
4.5	Oocyte	24
4.6	Polar body	25
4.7	Embryo	25
4.8	Degenerated embryo/oocyte	25
4.9	Graph of amount of embryo affect by semen condition in both NM female mice and SO female mice in percentages	28
4.10	The 2-cell stages embryo failed to further develop into blastocyst	28
5.1	Vaginal plug	31
5.2	Oviduct of the female mice which was normal in shape	32
5.3	Oviduct which was swollen	33
B1	Solution Preparation	45

FYP FIAT
UNIVERSITI MALAYSIA KELANTAN

B2	The processed feed for the mice	45
B3	The cages for the mice with the water bottle and bedding	46
B4	The petri dish use for cell culture	46

UNIVERSITI
MALAYSIA
KELANTAN
FYP FIAT

LIST OF ABBREVIATIONS AND SYMBOLS

NO.	
ART	Assisted Reproductive Technology
PMSG	Pregnant mares' serum gonadotrophin
FSH	Follicle stimulating hormone
hCG	Human chorionic gonadotrophin
&	And
SO	Superovulated
NM	Naturally mated
LH	Luteinizing hormone
LHCGR	LH/ hCG receptor
IU	International units
L	liter
g/L	Gram per liter
g	Gram
$CaCl_2 \cdot 2H_2O$	Calcium chloride dihydrate
$MgCl_2 \cdot 6H_2O$	Magnesium chloride hexahydrate
BSA	Bovine serum albumin
mDPBS	Modified phosphate buffered saline
%	Percentage
μL	Microliter

UNIVERSITI MALAYSIA KELANTAN
FYP FIAT

mL	Milliliter
cc/ mL	Cubic centimeter per milliliter
G	Gauge
mm	Millimeter
cm	Centimeter
Σx	Sum of all the sample observations
n	Number of sample observations/ number of elements in the sample
σ	Standard deviation
$\bar{x}$	Mean
x_i	i th element from the sample
CO_2	Carbon dioxide
PVC	Polyvinyl chloride
DIY	Do it yourself
°C	Degree Celcius
Ca^{2+}	Calcium ions
Mg^{2+}	Magnesium ions
×	Magnification

FYP FIAT
UNIVERSITI MALAYSIA KELANTAN

FYP FIAT

CHAPTER 1

INTRODUCTION

1.1 Research Background

Superovulation is a norm in Assisted Reproductive Technology (ART) prior cryopreservation. Due to it enables to increase in oocyte production, it was used to treat infertility in human (Wei et. al., 2014). It was familiarized in clinical practice on human assisted reproduction also in animal field to preserve endanger species. As in superovulation, it was a procedure that exogenous gonadotrophins such as pregnant mares' serum gonadotrophin (PMSG) or follicle stimulating hormone (FSH) followed by human chorionic gonadotrophin (hCG) administrated to the female mice for producing large numbers of embryos (Redina et. al., 1994; Popova et. al., 2002). This method can surely decrease the number of animal used and yielding high amount of oocyte or embryo for further research, reducing the cost to having extra experimental animal.

The usage of the mice as experimental body was common in life science studies, including used in toxicology, oncology, infection and pharmacology research. Most important it was used in the embryology for the vitrification and the data obtained were contributed to the early accomplishment in the human embryos culture and preserve in vitro (Quinn & Horstman, 1998; Shin et. al., 2017). This also support by Niakan et. al. (2012) as human and mouse embryos give the impression in alike morphology during pre-implantation development, the only differences was the timing

in development, hence mouse was normally used as experimental model before apply on humanity.

1.2 Problem Statement

Although superovulation greatly increased the amount of oocyte or embryo, but it was found to give disadvantages for instance reduced the chance of oocyte maturation and loss of embryo when pregnancy (Redina et. al., 1994; Van derAuwera & D'Hooghe, 2001; Wei et. al., 2014). It shows that superovulation might affect the fertility of the mice. Other than that, the male and female quality might be the obstacle for successful fertilization, as if the male or female facing infertility problem, the embryo would not form or successful develop into blastocyst stages (Jamsai & O'Bryan, 2011). It was important as anything affected the male mice or the female, the result development of the embryo might fail. Hence, in this study, it was focused on the reproductive performance of the male mice by mating to superovulated female mice and mating with another group which was without inducing any hormone. The semen condition was also being investigated on the aspect of fertility rate in female mice.

1.3 Objective

1. To evaluate the reproductive performance of each male mice between naturally mated (NM) and superovulated (SO) female mice base on fertilization rate.
2. To determine the optimal male semen condition with number of oocytes and embryos in female mice.

FYP FIAT

1.4 Scope of Study

This study was focused on the effect of superovulation on the mice by comparing NM female mice with SO female mice. The study was investigating the factor that affecting the mice performance. Also, the vaginal copulatory plug was an important element for a successful mating. Additionally, the semen condition was taken in concern as it might affect the fertilization. Three semen condition which dilute, semi-solid and hard solid condition were investigated for the best fertility rate.

1.5 Significant of Study

The study investigated the effect of hormone induction in female mice at the aspect of fertility rate. Furthermore, occurrence of vaginal copulatory plug which the higher concentration indicated a better production of embryo in number. The optimal conditions were study as it highly affecting the mice fertility rate.

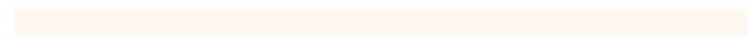
1.6 Limitation of Study

The limitation of this study was used a combination of Follicle Stimulating Hormone (FSH) and Human Chorionic Gonadotrophin (hCG) on the female mice for the superovulation induced. It was focused on the performance of the breed of ICR mice strain. Also, the temperature was not maintained as the lab did not have air condition. Due to the collaboration between Princess of Naradhiwas University (PNU)

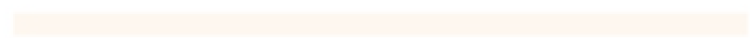
FYP FIAT
UNIVERSITI
MALAYSIA
KELANTAN

and University Malaysia Kelantan, the time of the experiment was limited to be finished in 2-week time.

UNIVERSITI



MALAYSIA



KELANTAN

FYP FIAT

CHAPTER 2

LITERATURE REVIEW

2.1 Reproduction of Mice

The reproductive organs that mainly contribute to the producing of sperm are testes which are important for the spermatogenesis in the epithelial lining of the seminiferous tubes. Vas deferens are the carrier of sperm that will transfer them to the penis. Penis in mice, also named as baculum are functioning like others animal that deliver urine and semen through the urethra, remove out from the inner body. In mice, male has 5 pair of accessory sex glands (Hodges, 2006). Mice has two vesicular glands that produce energy source, which is fructose to the sperm. The secreted fluid from vesicular glands and the coagulating gland makes the copulatory plug to be formed. Cowper's glands are functioning clear the urethra of urine by secretion of fluid and can act as lubricant. Preputial glands will secrete smegma that has antibacterial plus antiviral properties, also moisturizes the glans. Most of the fluids are produced in prostate gland.

Male mice will reach sexual maturity at 7-8 weeks of age while female take around 6 weeks. Gestation time for mice ranging from 19 to 21 days and the average of 10-12 mice will be birth (Katherine E. Quesenberry & Kenneth R. Boschert, n.d.). Mice have a short reproduction cycle and it is use for many type of research like toxicology, oncology, infection and pharmacology. Now, ICR breed is widely

FYP FIAT

commercialize and easily to get. ICR breed have a good performance in breeding purpose (Shin et al., 2017). Moreover, research found that ICR mice got better paternal and maternal characteristic that will increase the successfulness in breeding program (Liang et al., 2014). In this study, the ICR male and female were used for the investigation in the reproductive performance.

In the aspect of reproduction, male infertility causes a serious problem in men also in animals. As stated in Li et al. (2010) and Kumari & Singh (2013), chemical and drugs that are normally use for treatment can cause infertile in male. This proven that technology is one of the major causes to the male reproductive performance. Hence, many study are focusing on the male fertility in mice to find out other reason that affecting the reproduction of the male. Besides, some researcher found that the male fertility might be cause by the reproductive defection (Horan et. al., 2017). While female mice got similar problem facing by male mice. Two ovaries and the female genital tract that includes the oviducts, cervix, vagina and uterus are consist as female reproductive system while male reproductive system consist two testes and one penis, vas deferens and some accessory glands (Yahia et. al., 2013).

Reproductive performance of the mice also influence by changing of temperature, induces stress, modifying the housing space, level of lighting given and handling of the mice (Hansen et al., 2001; Shi et al., 2016). All the cause above are because the mice recognize a threat to it homeostasis and the animal cannot adapt to the environment then reduce the reproduction effectiveness.

FYP FIAT
UNIVERSITI
MALAYSIA
KELANTAN

2.2 Superovulation

Superovulation is a process that exogenous hormones or gonadotrophins administrated to stimulate the ovulation inside female individual like mammals, rodents that purposely to produce greater yields of oocyte and embryos. Superovulation is normally used in genetic studies and it was beneficial as small amount of animal can greatly rose in oocyte yield. It is an important method to doing research on embryo as it can increase the embryo per individual (Luo et. al., 2011). In the different strain of mice strains, they have varied responses to the superovulation protocols. As cited in Armstrong & Opavsky (1988), immature animal can have an earlier ovulation by injection of hormones and the embryos can be successfully grown up. Also, infertility can be treated by inducing superovulation (Guzik et. al., 1999). Usually, follicle-stimulating hormone (FSH) or pregnant mare's serum gonadotropin (PMSG) are used as the hormone follow by another injection of human chorionic gonadotropin (hCG) for superovulation in mice (Redina et. al., 1994; Luo et. al., 2011).

Although superovulation can increase production of embryo, it also causing adverse effect to the animal itself also the quality of the embryo. A failed or late implantation of superovulated mice, embryo lost after superovulation was cited in Van derAuwera & D'Hooghe (2001) that stated superovulation reduce the fertility rate of the mice. Superovulation was found to give greater loss of embryos or fail to develop into another stages as cited in Redina et. al. (1994). Although the mechanisms on how the gonadotropin increased affecting the abnormality of ova to be occur, but some suggestions had made as like degenerative ova occur resulted from fertilization failure. In the research of Redina et. al. (1994), they found that this degeneration occur were

FYP FIAT

possibly due to imbalance of the oestradiol and progesterone concentrations. Further study is still on going on the effect of superovulation to the egg cell and the female itself.

Furthermore, the ages of the mice for superovulation is important as in the study of Kim (1995), the author found out that an individual was having regression in getting a successfully fertilized egg when the age increases. It is because when the individual is getting older, it has a slower respond on detecting the stimulation of the hormone, also the reproductive system declining after the suitable age of mating. Hence a suggestion of suitable mice ages for experiment is ranging from 6-8 weeks old.

2.2.1 Follicle stimulating hormone (FSH) and human chorionic gonadotropin (hCG)

Follicle stimulating hormone (FSH) is use to stimulates the preantral follicles in mice to further growth into preovulatory stages, later inducing the FSH and Luteinizing hormone (LH) receptors on the granulosa cells (Wang & Greenwald, 1993). It is required for folliculogenesis, encompassing initiation, proliferation, differentiation and terminate the ovulation. In their research, they found that FSH will synergizes with LH to trigger oestrogen production that was an essential hormone for normal follicular development.

Human chorionic gonadotropin (hCG) is a vital component that controls the function and maturation of the sex. Mainly, hCG is primarily active in pregnancy and development of the fetal (Dinopoulou et. al., 2016). hCG has some biological similarities with LH, correspondingly in the structure. Hence, both of them bind to the

FYP FIAT

same receptor which called the LH/ hCG receptor (LHCGR). hCG was act as a replacement for the LH surge.

2.3 Embryo Flushing

Embryo flushing is a major component in embryo transfer and associated with superovulation. The embryo from the female mice is flush through using suitable equipment and the embryo can be transfer to the others animal as it improve the conception rate during heat stress (Baruselli et al., 2011). The flushing medium use have two type, which are phosphate or bicarbonate buffer solution ("Training manual for embryo transfer in water-buffaloes", n.d.). 6.9-7.7 pH value is well-suited to the normal mice embryo development and the phosphate buffered saline (PBS) should be warm to the 37°C before the flushing. The serum added in PBS are the protein source for embryo growth and membrane stabilization. PBS can maintain the pH value when exposed to air. As stated in ("Dulbecco's Phosphate Buffered Saline," n.d.), Ca2+ and Mg2+ addition is purposely to facilitate cell binding, which the embryo or oocyte will be stick together for an easier flushing process while preventing them to stick on the petri dish.

When doing flushing in mice, the procedure must be careful as the oviduct and infundibulum are very small in size. The flushing should be done at the direction at the cut end (slit) from the infundibulum, if wrongly flush the oocyte or embryo might stuck at the infundibulum. Hence it is important to flush follow the right direction, which is flush to the exit at oviduct side.

FYP FIAT
UNIVERSITI
MALAYSIA
KELANTAN

2.3.1 Two-cell stages embryo and oocyte

Embryos are the cleavage stages of the blastomere of the egg cells that ranging from 2-cell stage then further develop into compacted morula that self-possessed 8-16 cells. The stage was determined by counting on the number of blastomeres. In good quality embryos, it must exhibit the proper kinetics and had division synchrony which cell division occurred every 18-20 hours (Prados et. al., 2012). In Prados et. al. (2012), they cited that too slow or too fast of cell division suggested that the embryo may have metabolic or chromosomal defects. 2-cell stage embryo was investigate by Illmensee & Levanduski (2010) and the consequence healthy offspring was developed from 2-cell embryo and have a relatively identical size to control live-born mice, it demonstrated that normal adult development was not affect from early embryo transfer. 2-cell embryo was chosen for the experiment due to it require 2 days after the mating and it can saved up the time. 2-cell stage embryo is easy to be notice due to a clear duo blastomere can be observe through microscope.

While for oocyte, it is the largest mammalian cell that are the stages before the fertilization to embryo. It consists of oolemma which is the plasma membrane of the oocyte with a clear ooplasm (Agca, 2000). The zona pellucida is surrounding the oolemma, hence the oocyte is appeared as a layer of circle warping another small circle inside it. Appearance of oocyte indicate the fertilization is not successful.

FYP FIAT

2.4 Vaginal Copulatory Plug and Semen Condition

Vaginal copulatory plug are form resulting coagulation of the male ejaculate (Wei et. al., 2014). The seminal vesicles secrete proteins that will coagulate in the process by enzymes and coagulating gland. Plug are found in few mammalian and also natricine snakes (Martan & Shepherd, 1976). Moreover, plugs are available in rodents and it is a very hard and strongly stick to the vaginal epithelium. The finding in Martan & Shepherd (1976) stated that the selection thru sperm competition involving the copulatory plug, likewise plug is capable to prevent the remating in mice. As in mammalian reproduction, the function of copulatory plug is embrace of preventing the spermatozoa outflow, aid in the transportation of the sperm from vagina to uterus and act as a sperm reservoir.

The semen condition is related to the vaginal copulatory plug as the abnormal seminal vesicle can cause smaller plug to be deposit. As the content in the seminal vesicles reduces, the plugs weight will decrease and then the number of spermatozoa will be gradually decreased (Carballada & Esponda, 1993). The normal plug should be hard solid condition, while the abnormal plug may be semi-solid or even the plug not forming due to the sperm are too dilute. The differences in the plug in different male species as it depends on the ejaculate which is the amount of seminal vesicle secretion and on the mating behavior.

FYP FIAT
UNIVERSITI
MALAYSIA
KELANTAN

CHAPTER 3

METHODOLOGY

3.1 List of Materials and Apparatus

3.1.1 Materials

The ICR strain mice which are 20 female mice and 5 male mice were used in the experiment. The bedding for the mice was Processed Corn Cob and Dried Water Hyacinth Stem. The chemical used are consisted 50 International Units (IU) of Follicle Stimulating Hormone (FSH) and 50 IU of Human Chorionic Gonadotropin (hCG) for hormone injection.10 tablets of Phosphate Buffered Saline Tablets, 1 liter (L) of Distilled Water, 0.036 gram/liter (g/L) Sodium Pyruvate, 1 g/L Glucose, 0.1374 gram (g) Calcium Chloride Dihydrate ($CaCl_2 \cdot 2H_2O$), 0.1 g Magnesium Chloride Hexahydrate ($MgCl_2 \cdot 6H_2O$), 0.06 g/L Penicillin G. (Potassium Salt), 0.05 g/L Streptomycin Sulfate, 1.5 g Bovine Serum Albumin (BSA), 0.005 g Phenol Red were the chemical used to make modified Phosphate Buffered Solution (mDPBS) and PBI Medium was used for flushing procedure, KSOM medium for culture embryos that obtained from ICR female mice and Mineral Oil Light White Oil was used for preventing the evaporation of the medium. 70% ethanol is used for cleaning the working platform and dissecting kits also for cleaning the abdomen area of the mice before dissection.

FYP FIAT
UNIVERSITI MALAYSIA KELANTAN

3.1.2 Equipment and Apparatus

For solution preparation, 0.5-10 µL Clear Tips, 1-200 µl Yellow Reference Tips, 100-1000 µL Blue Tips, Magnetic Stirrer, 15 mL Conical Centrifuge Tube, Vortex Mixer, Plastic Syringe Filter, 10 mL Syringe, 1 L Volumetric Flask, Electronic Balance, Motorized Pipette Filler, 10 mL Disposable Serological Pipettes and Micropipette were used. Dissecting kits were used for the mice dissection. Small cages with water bottles and pellet were prepared for the housing of the mice. Bunsen Burner was used for modify the glass pasture pipette. 1-inch Tip Length 30G Blunt Stainless Steel Dispensing Syringe Needle and 1 cc/mL NIPRO Hypodermic syringe was used for flushing the oviduct. Disposable Sterile Petri Dish (90×15 mm) was used as a platform for embryo flushing. Disposable Sterile Petri Dish (60×15 mm) was used for embryo transfer before culture while Disposable Sterile Petri Dish (35×12 mm) was used for embryo culture. Glass Pasture Pipette 230 mm (Long Form), Plastic Syringe Filter, Parafilm and Small Clear Polyvinyl Chloride (PVC) Tubing were used to make mice pipette for embryo transfer and collection. Carbon Dioxide (CO2) Incubator was used for the embryo culture. Stereomicroscope and Light Microscope were used in the process of flushing, collecting and transferring embryo.

3.1.3 Mouth Pipette for Embryo Transfer

The small clear PVC tubing is cut into two 10 cm long tube. Firstly, one end of the first 10 cm PVC tube is connected to a 100-1000 µL Blue Tips by sealing tight with parafilm as the place for mouth for controlling the suction of the embryos. After that, the second PVC tube was connected to another 100-1000 µL Blue Tips as a connector to the modified glass pipette. Both of the tubes were attached to a same plastic syringe

FYP FIAT
UNIVERSITI MALAYSIA KELANTAN

filter as a connector and parafilm was used to ensure it do not loose when transferring embryo.

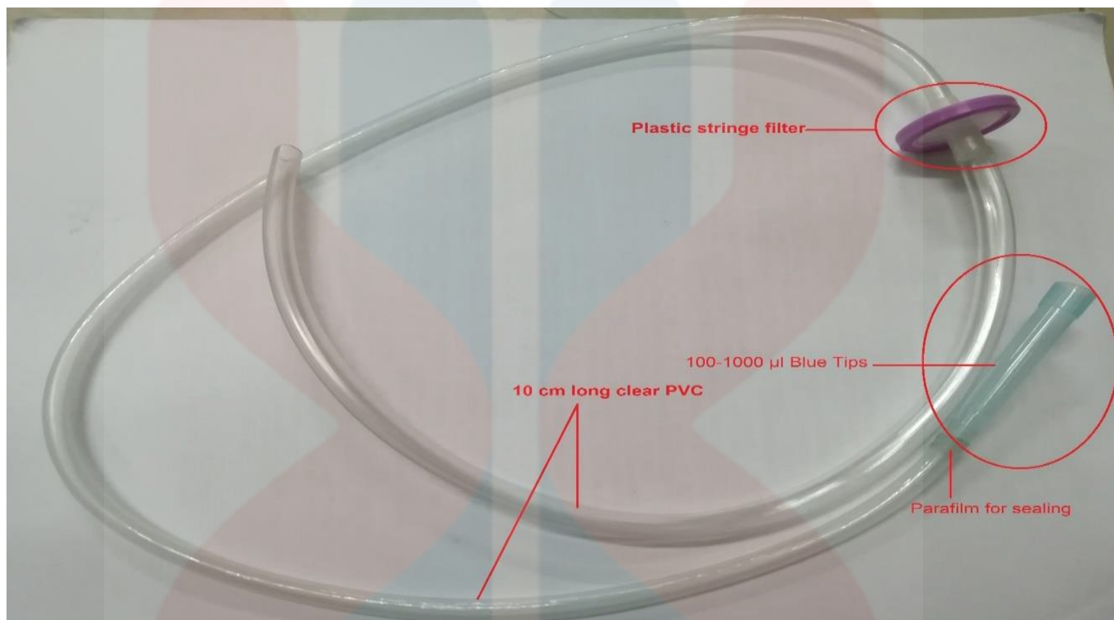


Figure 3.1.3: The DIY mouth pipette.

3.1.4 Modified Phosphate-buffered Saline Solution with Calcium Chloride Dihydrate and Magnesium Chloride Hexahydrate (mDPBS with Ca^{2+} and Mg^{2+})

First, 10 tablet of PBS tablets were dissolved in 700 mL of distilled water to make into PBS solution in a 1 L volumetric flask. Then, 0.036 g/L of sodium pyruvate and 1 g/L of glucose were measured using electronic balance and mixed with the PBS solution by using magnetic stirrer for 30 minutes of mixing and the solution was named as solution A. Solution B was prepared by using 0.1374 g of Calcium Chloride Dihydrate ($CaCl_2 \cdot 2H_2O$) mixed with 2 mL of distilled water in a 15 mL conical centrifuge tube and stirred well using vortex mixer for 15 minutes. The solution A and solution B were mixed up using Motorized Pipette Filler with 10 mL Disposable Serological Pipettes. After that, solution C was prepared using 0.1 g Magnesium

FYP FIAT
UNIVERSITI
MALAYSIA
KELANTAN

Chloride Hexahydrate ($MgCl_2 \cdot 6H_2O$) mixed with 2 mL of distilled water and mixed well by the vortex mixer in centrifuge tubes for 15 minutes. PS stock was made up by using 0.06 g/L penicillin G. and 0.05 g/L streptomycin sulfate. Lastly, solution AB was mixed with solution C and PS stock in another 1 litter of volumetric flask and mixed well with magnetic stirrer. All solution was filter by using 10 mL syringe with syringe filter attached on it after the mixing. 500 mL of mDPBS was then store in the refrigerator and another 500 mL will be used to make PBI medium.

Resources from: Chasombat J. (2014)

3.1.5 PBI medium

500 mL of mDPBS solution was prepared. The solution was mixed with 1.5 g of bovine serum albumin and 0.005 g of phenol red by using magnetic stirrer in a 1 litter volumetric flask.

Resources from: ("PBI medium," 2006).

FYP FIAT
UNIVERSITI
MALAYSIA
KELANTAN

3.2 Methods

3.2.1 Animals

Around 10 female mice which ages reached 6-8 weeks old were used for superovulation and another 10 female mice were used for natural mating with the same ages. 5 male mice were prepared for the mating which is 8 weeks older to ensure a successful mating. The breed that use was ICR breed, and each of the mice had an average 20-28 g of weight. Small cages were used to keep all the mice in it with a water bottle. The mice were distributed into 3 mice per cage for female while male was distributed to one male per cage before the mating. They were housed under a 12 hours of dark-light cycle at room temperature (31 ± 1°C) with ad libitum food and water (Brooks et. al., 2014). Processed corn cob and dried water hyacinth stem were used as the bedding of the mice. The bedding was changed 3 days once to ensure a clean environment for the mice, and the cage was closed tightly to prevent escape of the mice. Feed was not given too much to prevent overfed that may cause obesity. Water bottle was cleaned daily for hygiene.

3.2.2 Obtaining Mice Embryo by Superovulation and Naturally Mating Method

The method is refer to Luo et. al. (2011) with some modification. Intraperitoneal injection of Follicle Stimulating Hormone (FSH) were given to each of the female mice to the abdominal cavity. The given dosage of FSH was 5 International units (IU), total 50 IU was used for 10 mice. After 2 days of post FSH injection (around 48 hours), 5 IU of Human Chorionic Gonadotrophin (hCG) injection was given to each of them. While

FYP FIAT UNIVERSITI MALAYSIA KELANTAN

the naturally mated mice were straight away mate with the male mice without any hormone injection. When holding the mice, the back side of the neck was held tightly to prevent movement of the mice that might cause injury when injection. The mice head was faced to the ground while the leg was facing upper side for ensure the organ can move downward for a better injection. Alcohol wipe was used to wipe the abdominal side before and after the injection for disinfection purpose.

3.2.3 Naturally Mating and In Vivo Maturation of Mice Embryo by Plug Checking

The protocol were modified base to Ishida et. al. (1997) and Luo et. al. (2011). For the female mice under hormone induced group after the hCG injection, the female mice were straight away separating to 5 ICR breed male mice for mating, ratio of female to male of 2:1. The male mice were named as male A, male B, male C, male D and male E. The male mice were remained in the same cages as to reduce the stress to the male mice for increase the chance of mating. Before mating, 2 female mice were transfer and co-cage with the male mice respectively. The two female mice were caged overnight with the male mice and were examined for vaginal plug (copulatory plug in vagina) in the next morning. The semen condition from the vagina of the mice were observed and recorded for whether it was hard solid, semi-solid or dilute condition. The semen condition was recorded base on the observation on the vaginal plug which the dilute condition was about 90% of the sperm is liquid form, semi-solid which the sperm are 50% solid (coagulated sperm) and 50% liquid condition while hard solid was more than 85% solid in content. The naturally mating group of female mice also followed above procedures for the mating.

FYP FIAT
UNIVERSITI MALAYSIA KELANTAN

3.2.4 Collection, Culture and Incubation of Embryos

Firstly, the mice were manually killed through spinal cord dislocation using a steel bar for prevent pain and for animal welfare. Gloves and mask was worn to prevent contamination and for hygiene purpose. After ensuring the death of the mice, alcohol wipe was used to clean the outer layer skin at the abdominal part. A clear cut was made and the skin was torn to both side for a clear view to find the oviduct. The fats, intestines and others unwanted part were taken out. Then the cervical dislocation was processed under stereomicroscope and using dissecting sets. Two cell stages embryo were flushed using a pre-warmed mDPBS contained in 1 cc/mL NIPRO Hypodermic syringe with 1 inch tip length 30G blunt stainless steel dispensing syringe needle from the ampullary end of the oviducts (infundibulum) by sacrificing the female mice after 48 hours (Legge & Sellens, 1994). The embryo or oocyte was then transferred to a PBI medium with 0.4 % of Bovine Serum Albumin (BSA) using the DIY mouth pipette.

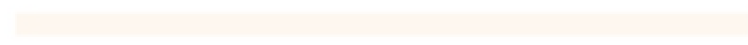
The embryos were collected in a petri dish, all of the embryos were observed under the light microscope and distributed into three groups either oocyte or polar body (considered as oocyte), embryos (starting from two-cell stages and before blastocyst) and the third group was degenerated oocyte or embryo. The amount of the sample was recorded for both superovulation group and naturally mated group. The embryos were straight cultured in KSOM medium with mineral oil. Mineral oil was purposely to reduce the evaporation rate of the culture medium in the incubator.

The medium was prepared earlier as the KSOM was store in freezer. KSOM medium was warmed before used as cool condition affect the survivability of the embryo. The KSOM medium was transferred by using micropipette which 4 drops

FYP FIAT

(each 30 μL) of KSOM media were evenly distributed in the culture dish. Then the medium with embryo was cultured in incubator at 37°C, with atmosphere of 5% carbon dioxide (CO2) in air. After 48 hours of culture, the embryos were taken out and observed for the development status under microscope.

UNIVERSITI

MALAYSIA

KELANTAN

FYP FIAT

CHAPTER 4

RESULT

In this study, total sample of 181, which included the oocyte, embryo and degenerated embryo /oocyte for both superovulation and naturally mated group were collected. For each male, the performance of them were recorded in both mating with superovulated (SO) female mice or with naturally mated (NM) mice. Male reproductive performance between NM female mice was shown in Table 4.1. Male A (n=14) and male E (n=13) gave higher embryos compared to male B (n=5) and male D (n=2). It is also shown male C was unable to fertilize any oocyte which gave no embryo (n=0).

Table 4.1: Male reproductive performance between NM female mice (n=10).

*Total sample in NM mice=75

Male	Oocyte	Embryo	Degenerated embryo/oocyte
A	0	14	0
B	2	5	3
C	3	0	0
D	6	2	0
E	19	13	8
Total	30	34	11

As shown in Figure 4.1, the total sample collected from naturally mated (NM) female mice were 75 sample (n=10), approximately 40% oocyte, 45.33% embryo and

FYP FIAT
UNIVERSITI MALAYSIA KELANTAN

15.67 % degenerated embryo/oocyte. From the figure, it shown almost the same percentages in oocyte (40%) and embryo (45.33%) for the NM female mice.

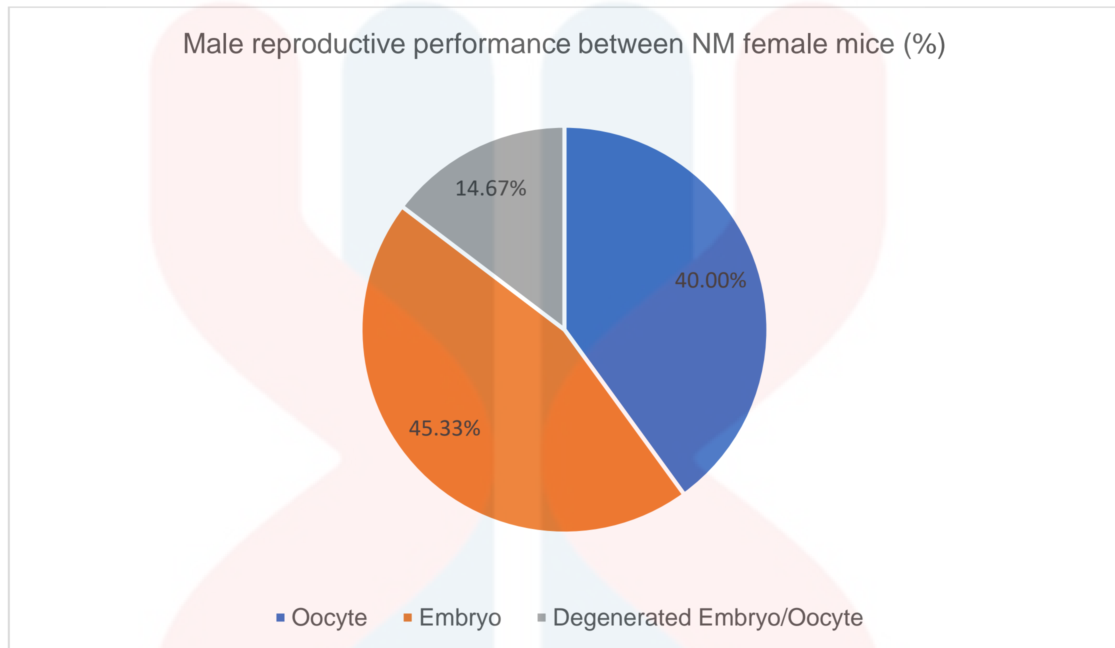


Figure 4.1: Pie Chart of male reproductive performance between NM female mice in percentages.

The superovulated (SO) mice group, result shown that in total number of sample was 106. In table 4.2, Male D produced the most embryo (n=14), followed by Male C (n=10) which was the second. While the Male A and Male B gave no embryo at all (n=0).

In oocyte number, Male B produced the highest in number of oocyte after mating (n=26), Male E was another (n=21) that had higher number of oocyte. Male C (n=6) and Male D (n=4) had low number of oocyte after mating with SO mice.

FYP FIAT
UNIVERSITI
MALAYSIA
KELANTAN

Table 4.2: Male reproductive performance between superovulated (SO) female mice (n=10).

*Total sample in SO mice=106

Male	Oocyte	Embryo	Degenerated embryo/oocyte
A	14	0	0
B	26	0	5
C	6	10	0
D	4	14	0
E	21	6	0
Total	71	30	5

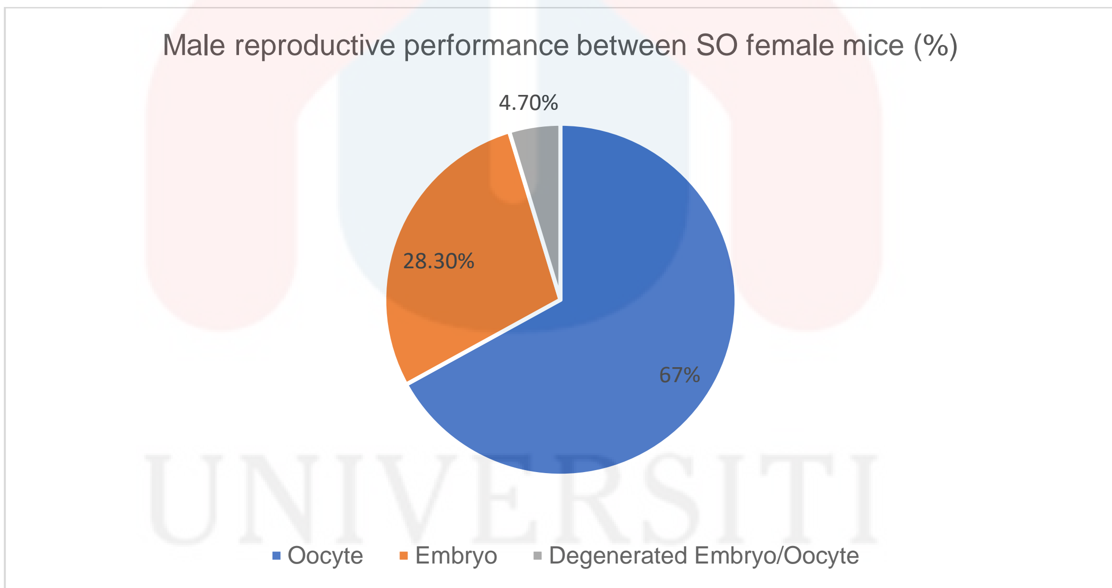


Figure 4.2: Pie Chart of male reproductive performance between SO female mice in percentages.

Percentages of oocyte (67%), embryo (28.3%) and degenerated oocyte (4.7%) were shown in Figure 4.2. The total sample of SO mice (n=106) was higher than the NM mice (n=75) base on the same male mice used.

FYP FIAT

UNIVERSITI MALAYSIA KELANTAN

The performance for the male mice mating with NM female mice were shown below in Figure 4.3. As observation from here, Male A gave the most embryo after

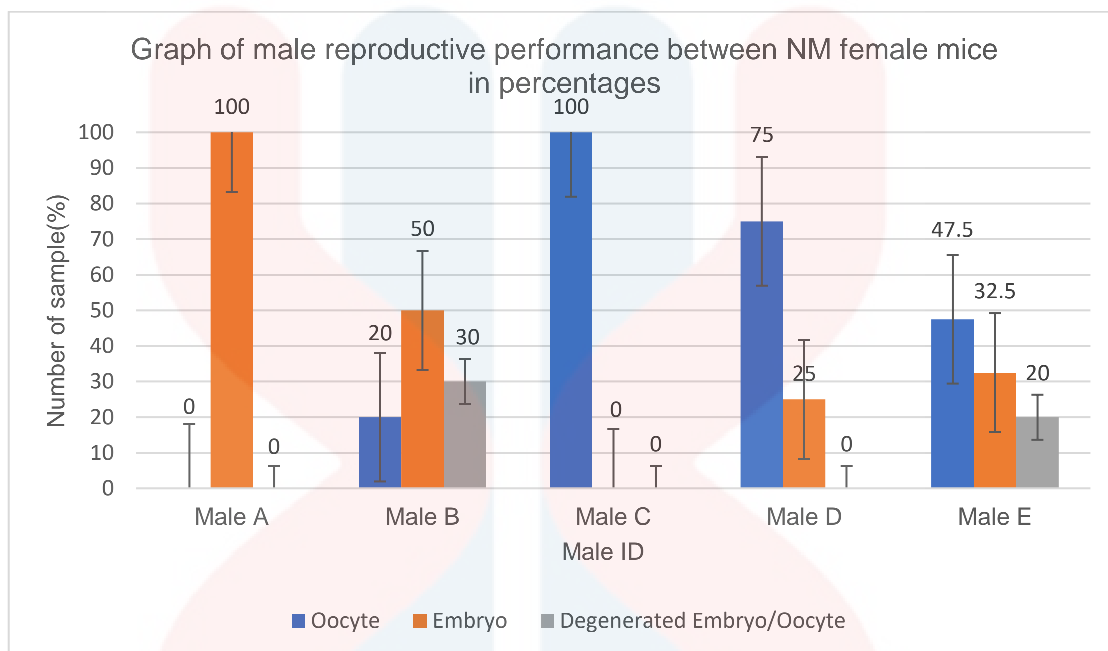


Figure 4.3: Graph of male reproductive performance between NM female mice in percentages.

mating with female mice without hormone injection, followed by the second-best performance which was Male B. Meanwhile, the Male C had the poorest performance as it gave 0 embryo after the mating. Male D and Male E had similarity in the pattern as oocyte was greater than embryo and the degenerated was lowest among that.

FYP FIAT

UNIVERSITI MALAYSIA KELANTAN

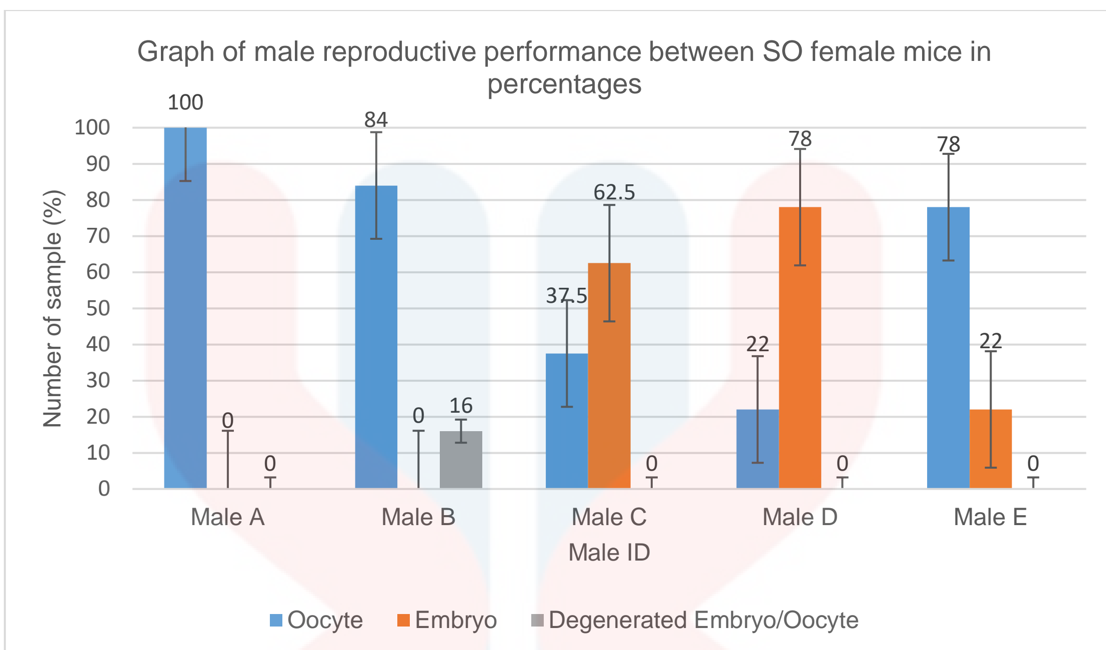


Figure 4.4: Graph of male reproductive performance between SO female mice in percentages.

For the SO female mice group, Male D had a better performance due to it produced highest amount of embryo, followed by Male C which was the second and male E was the third one as shown in Figure 4.4. Male A and Male B produce only oocyte and without any embryo was found. The sample was grouping base on following observation in the Figure 4.5- Figure 4.8 which included oocyte and polar body stated as oocyte, embryos and degenerated oocytes/embryos.

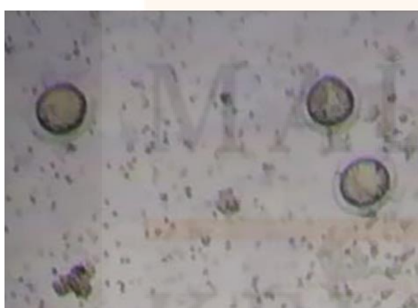


Figure 4.5: Oocyte- which only a circle cell without blastomere in it.

FYP FIAT UNIVERSITI MALAYSIA KELANTAN

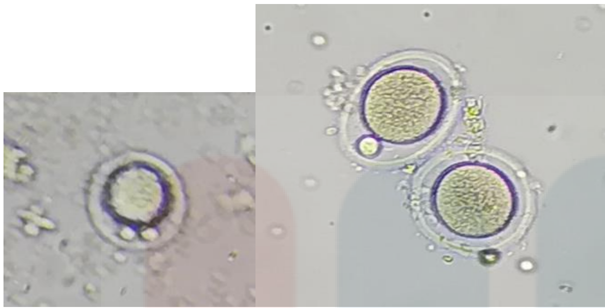


Figure 4.6: Polar body- which can obviously observe, left= round in shape and two white spots (polar body) at the bottom; right= only one polar body on the top of the cell.

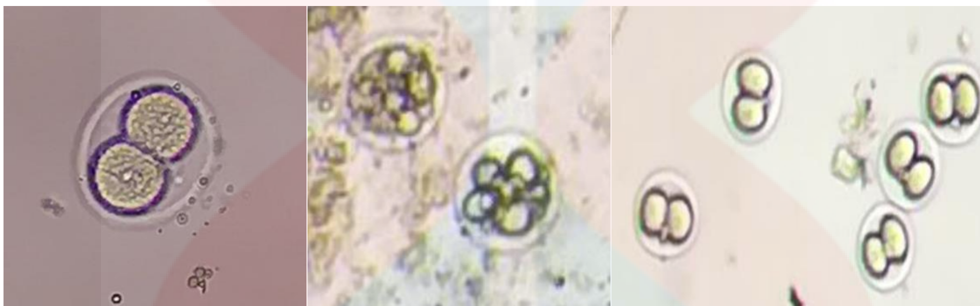


Figure 4.7: Embryo- left= 2-cell stages embryo which was the cleavage stage (2 blastomere); middle= 8-cell stages embryo; right= 2-cell stages embryo.

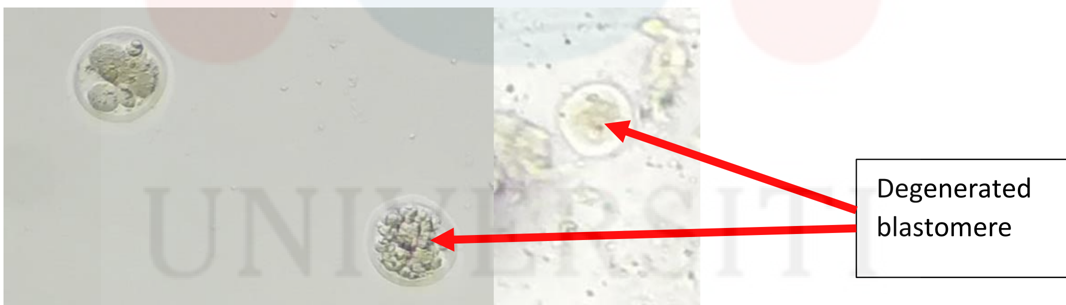


Figure 4.8: Degenerated Embryo/Oocyte)- left= degenerated embryo which the cell inside was darkening (starting to degenerate); right= degenerated oocyte which the inner circle (cytoplasm) was fully degenerated and only the zona pellucida was remained.

*all of the figure was observed using 10x magnification under microscope.

To further investigate the vaginal copulatory plug (semen coagulation) condition affecting the fertility, the semen condition was recorded. Overall, the three conditions of the semen were dilute, semi-solid and hard solid. The condition was decide based

FYP FIAT
UNIVERSITI
MALAYSIA
KELANTAN

on the observation on the sperm condition in the vagina plug. Dilute condition meaning that the vaginal plug was not really successful formed but some liquid form of semen in pale white color can be observed at the vagina of the female mice, almost 90% of the semen was in liquid form. Semi-solid condition was decided as the semen is 50% in solid form while another 50% was in liquid form, when the spatula used to check for the plug, the semen was found to have some liquid form sperm and some hardening substances stuck at the vagina site. For the hard solid condition, there were almost no liquid semen can be observed in the vagina, a fully formed vaginal plug which was hard (85% of solid semen and above) and can be clearly observed which it was stick on the vagina site of the female mice.

As in Table 4.3, for NM female mice, the dilute semen condition was found to give 0 embryo, while it got highest percentages of oocytes amount all the condition. The semi-solid condition got slightly differ value between oocyte and embryo which were 35% and 47%.

Table 4.3: The amount of oocyte, embryo and degenerated embryo/oocyte affect by semen condition in NM female mice in percentages (n=10).

Semen condition	Oocyte (%)	Embryo (%)	Degenerated embryo/oocyte (%)
Dilute	89	0	11
Semi-Solid	35	47	18
Hard Solid	0	100	0

This indicates that almost half percentages of the sample can be estimated either fertilized or unfertilized base on semi-solid condition. While hard solid condition

FYP FIAT
UNIVERSITI MALAYSIA KELANTAN

got 100% result of getting embryo. For the SO mice, the amount of oocyte, embryo and degenerated embryo/oocytes were recorded in Table 4.4.

Table 4.4: The amount of oocyte, embryo and degenerated embryo/oocyte affect by semen condition in SO female mice in percentages (n=10).

Semen condition	Oocyte (%)	Embryo (%)	Degenerated embryo/oocyte (%)
Dilute	71	0	29
Semi-Solid	89	11	0
Hard Solid	27	73	0

The dilute semen condition was identical to naturally mated female mice which it gave most to the embryo for 71%. Also, the hard solid gave the most embryo which was 73%. While the semi-solid condition was differed as SO mice got higher percentage of oocyte while embryo percentage was low. So, to comparing the semen condition either which one gave better fertility rate, a table of semen condition affecting the embryo number in percentages of both NM female mice group and SO female mice were recorded in Figure 4.9 which the graph shown that dilute condition had 0% in embryo number, meanwhile in hard solid condition both group had higher percentages of embryo which were 41% and 80% respectively. The semi-solid condition was having higher percentages in NM female which was 59% comparing the SO female mice which was 20%.

FYP FIAT
UNIVERSITI
MALAYSIA
KELANTAN

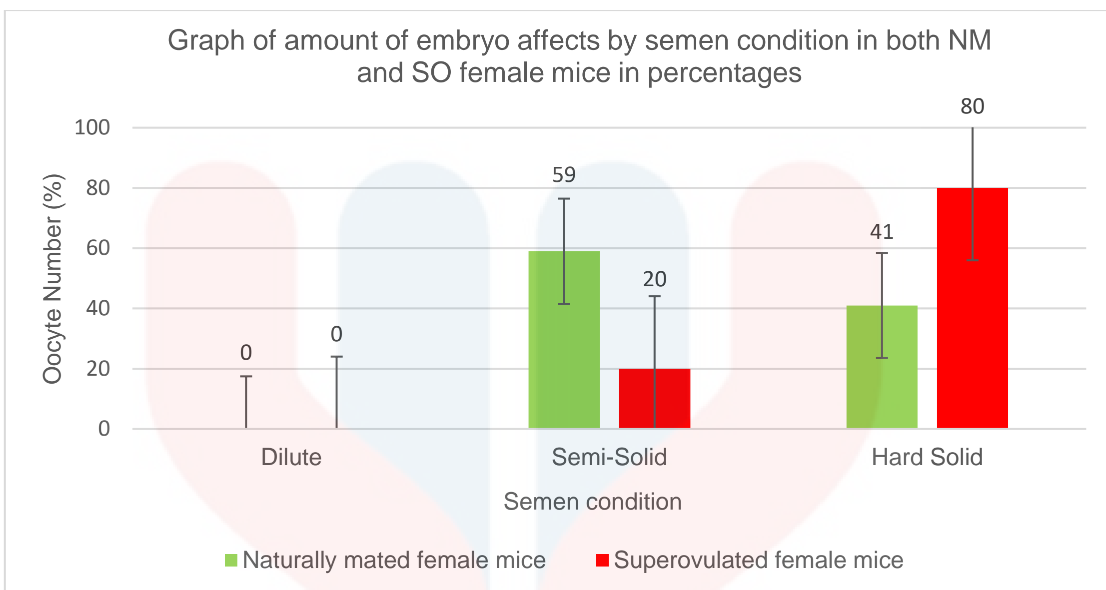


Figure 4.9: Graph of amount of embryo affects by semen condition in both NM female mice and SO female mice in percentages.

2-cell stages embryos were culture for further investigation, all of the sample were not developed into blastocyst. Below was the figure of the embryos (Figure 4.10) that grown in KSOM media and failed to develop after 48 hours of culture.

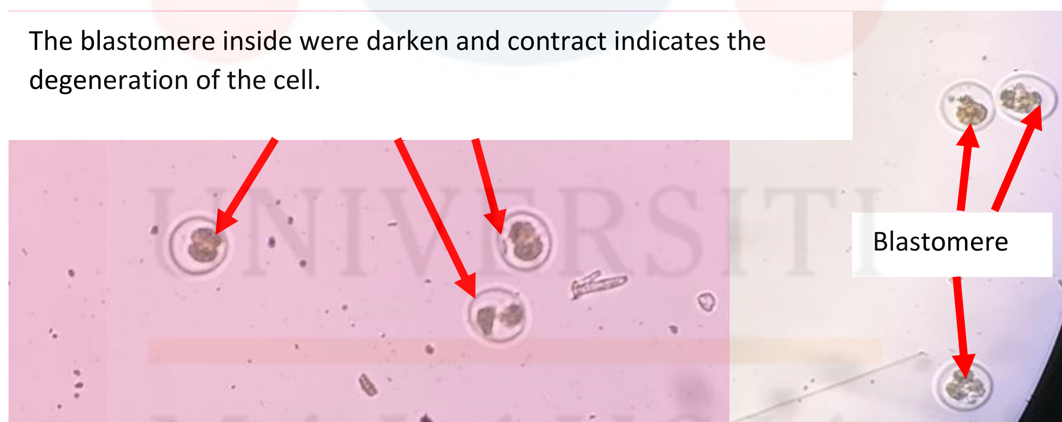


Figure 4.10: The 2-cell stages embryo failed to further develop into blastocyst.

FYP FIAT
UNIVERSITI MALAYSIA KELANTAN

CHAPTER 5

DISCUSSION

A total of 5 ICR males were mated to 10 superovulated (SO) female ICR mice and 10 naturally mated (NM) female mice from the same strain. The first finding of this study, SO female obtained higher number of sample oocyte, embryo and degenerated embryo/oocyte (n=106). This was compared to NM female mice, they only gave total number of 75 sample (n=75). The number of oocyte was found to be additional 41 sample in SO female mice while the number of embryo only a little dissimilarity between the NM mice and the SO mice which was 34 and 30. This proven that superovulation induced the production of larger numbers of oocytes (Legge & Sellens, 1994), but it did not effecting the fertilization rate of the mice base on the result.

In the perspective of the percentages of the oocyte and the embryo, the total percentages in oocyte and embryo in NM mice were relatively close which only 5 % of differences while in SO mice were 39 % in contrasts. Although the total sample number were increased, but the embryo percentages was less dissimilarity due to adverse effect on the oocyte maturation from the hormonal treatment that was cited in Van derAuwera & D'Hooghe (2001) and Wei et. al. (2014).

Next, another finding that had been observed were different performance from Male A to Male E. In the NM female mice mating, Male A had the most number in embryo (n=100%) while Male C had poor performance as only oocyte was found

FYP FIAT

(n=100%). Male B and Male E had a greater percentage of degenerated embryo/oocyte which it would not have any further development. Normally, the occurrence of degenerated oocyte was due to some of them were apoptosis before successful fertilized (Perez et. al., 2000). They found that the degenerated embryo might due to endocrine, genetic, physiological and also environmental effect, and usually occur in early embryo stage. By comparing the male performance in SO mice, the Male A had an adverse result which it gave only 100% embryo. Else, Male B was not giving any embryo (n=0%) and yield 84% of oocyte that shown opposing result too. Male C was divergent in performance as it gave 62.5% of embryo whereas Male D got the most percentages in producing embryo (n=78%). Only Male E maintained the same pattern as the oocyte number was higher than embryo (n=78%) and degenerated embryo/oocyte (n=0%) was the lowest among that.

The result shown all the male gave embryo after mating with either SO female mice or NM female mice. Regarding of male infertility, it could be the reason of sperm defective, blockage in tract obstruction, sexual disorders or inflammation (Jamsai & O'Bryan, 2011). Although some of the mice from the first batch were not producing fertilize egg, the second mating shown some opposite outcome, hence the male mice were not facing infertility problem, and further investigation was made in the semen condition.

Nevertheless, to find out whether the semen condition got affecting the fertility rate of the egg, vaginal copulatory plug of each female mice was recorded base on the coagulation condition of the male mice semen. The hard solid condition was discovered to give the best fertility rate of the oocyte and it was the third finding of this research. As comparing both of the group in embryo number, diluted semen gave no embryo at

FYP FIAT
UNIVERSITI
MALAYSIA
KELANTAN

all (0%), while in semi-solid condition SO got lesser percentages (20%) compare to NM female mice (59%). In hard solid condition, SO female mice gave higher percentages (80%) compared NM female mice (41%). This was because the diluted semen got lesser number of sperm in it, causing the sperm amount was not enough to fertilize the egg. As stated by Martan & Shepherd (1976), the main function of the copulatory plug included it act as a stopper for preventing outflow of the sperm from the vagina, act as a reservoir for the sperm to be gradually release and it was a facilitation for transporting

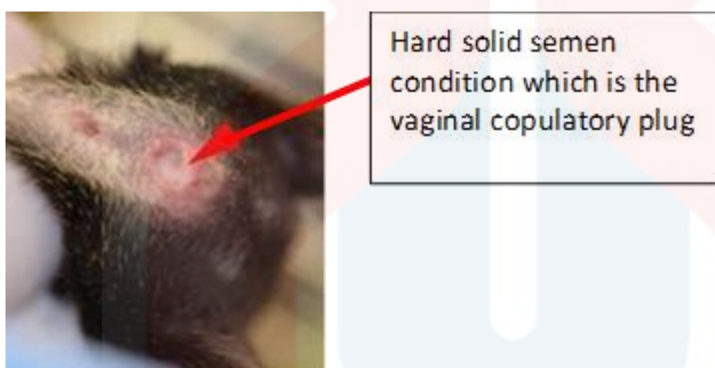


Figure 5.1: Vaginal Plug- the white coagulant was the vaginal copulatory plug of the mice (Qiu et. al., 2016).

the sperm into the uterus. The plug was formed by protein secreted from the male ejaculate and it should be in hard condition as shown in Figure 5.1. If the semen was dilute, causing it fail to form a normal plug, then the sperm might be outflow and causing the fertilization rate to be reduced. In a study, it stated that normally rodent copulatory plug were hard and robust, it consisted a ranging condition which was temporary semi-solid condition or very robust coagulation (Schneider et. al., 2016). Despite the fact, hard solid surely can give a better fertilization rate from the statement above. Then the semi-solid condition got 50% of chances to get successful fertilization due to the plug was consider as abnormal (Dean, 2013).

FYP FIAT
UNIVERSITI
MALAYSIA
KELANTAN

Other than that, this study also showed that fertility rate was affected by varies reason, no matter it happened on male or female mice. Base on the researches, stress, environment effect, light intensity and male or female infertility were the cause of the reduced in breeding performance. As stress may occur during handling either in male or female mice, due to mostly the laboratory mice were minimally handled and when doing any hormone injection or transferring them in the duration, they felt uncomfortable and scare (Fridgeirsdottir et. al., 2017). Furthermore, hormone injection may cause stress to appear due to technical problem and lack of experiences in the research, hence causing some of the mice have injury in the oviduct (swollen oviduct).

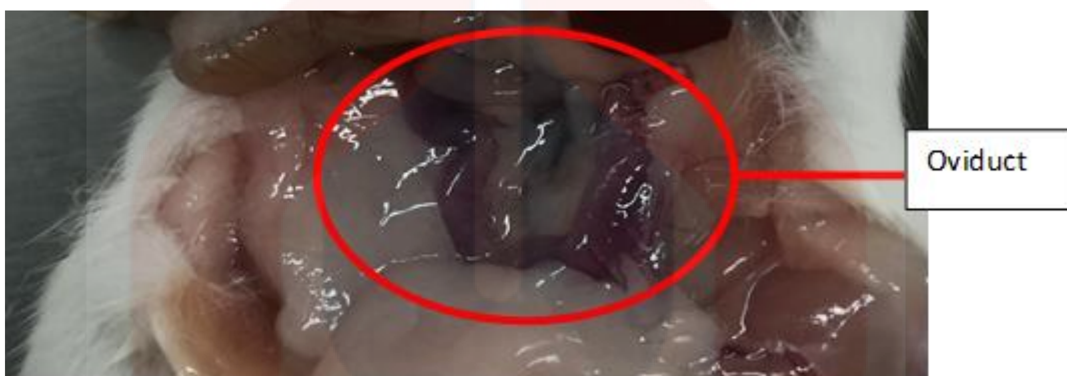


Figure 5.2: Oviduct of the female mice which was normal in shape.

As from the observation in the Figure 5.2 and Figure 5.3, obviously that the oviduct in the Figure 5.3 was swollen as it was reddish in color. This occur during the poor technical injection on the intraperitoneal side on the mice during the process. Hence, this was causing much stress to the mice due to inner injury making some of them did not want to be mated or the sperm fail to reach to fertilization site.

FYP FIAT
UNIVERSITI MALAYSIA KELANTAN

Figure 5.3: Oviduct which was swollen.

Some factor may affect the superovulation and further causing individual itself to decrease in fertility rate. In the aspect of heat stress, researchers found that females that exposed to heat stress after fertilization caused quality and quantity of embryo to decline in mice (Takahashi, 2012). Additionally, the author also found that hyperthermia can inhibiting the spermatogenesis in mice, as it had several effects that was adverse on reproductive tissue in mice. As stated in Hamid et. al. (2012), heat stress caused not only decrease in semen quality, but also decrease embryo quality. It has an adverse effect on the spermatogenesis and oocyte development and maturation. The statement was supported by observation on different animal including cattle, sow and mice that pregnancy of the animal is lower at summer comparing autumn. Furthermore, as cited by Takahashi (2012), the oocytes that exposed to high temperature inhibits the rate of metaphase II stages in mice, hence the subsequent embryos might develop slowly or abnormally. In the experiment, all the mice were kept in a room temperature of average 31 °C.

As comparing to the standard temperature for housing the mice in laboratory (20–22°C), it was considered hot for the mice as it was 11°C higher that the standard temperature (Speakman & Keijer, 2013). This can be considered as a stressor for the

FYP FIAT

mice to reach a homeostasis to the thermal neutrality temperatures (26-34°C), eventually the mice will have wide ranging effects (Gaskill et. al., 2009). The mice were not comfortable with the hot condition hence stress occurred and affecting the breeding performance. As stated in Hansen et. al. (2001), environment that is too warm for the animal is found to induce stress which are call heat-stress causing hyperthermia, the female animal will decrease in fertility while in male animal the sperm quantity and quality reduce.

Besides, lighting was important for the successful mating of the mice. In a study from Weihe et. al. (1969), they conclude that a dim light gave better reproduction of mice compared bright light. As a norm, mice were grouping as nocturnal animals as it will be active in night, including mating. This was because the light intensity adjusting the activity based on the biological clock which generates endogenous times in physical and behavior of the mice (Kramer & Birney, 2001; Peirson et. al., 2017). Mice were active in night as they hunt for food and performing normal biological processes, as increasing light intensity, it might affect the regulation of hormones also the modulation of sleep, causing the mess up of time clock in the mice that might affect the reproductive performance. This was supported by Castelhano-Carlos & Baumans (2009) which the red light was discovered that it made a change in the time of ovulation in mice. This was proven that minimizing the light intensity surely can get a better reproductive result in mice.

For the last finding, the embryo that culture in KSOM medium was failed to develop due to some reasons. As in the procedure of transferring the embryo, then cultured in vitro, it was fewer than 50% of chances for the successful development of the early embryo stage (Betts & Madan, 2008). Also, Betts & Madan (2008) stated that

FYP FIAT
UNIVERSITI MALAYSIA KELANTAN

apoptosis, which was the procedure to purge the unnecessary or damaged cells in the early embryo, will lead embryo to be fail develop if the apoptosis was vigorous. Hence, the early embryo were failed to culture to the later stages. Also, the time of the transferring of embryo was too long as the microscope that used was the inverted microscope that show different direction from the observation. This truly increased the difficulty for the collection of sample, plus the working platform was limited as the modified glass pipette was hard to reach the sample.

FYP FIAT

UNIVERSITI MALAYSIA KELANTAN

CHAPTER 6

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

As in this experiment, it concluded that the superovulation was producing more sample number comparing naturally mated female mice. The male and female mice were not facing infertility problem as all of the mating comprises fertilize egg (embryo). Each male mice performance was not significantly shown that which mice got the most embryo yield. From the investigation on the vaginal copulatory plug, it concluded that the hard solid condition was the best indicator for the effective fertilization in the female mice, while the dilute semen condition had higher failure in fertilizing the egg cell. Some feature also been investigated as it affecting the reproduction performance of the mice.

6.2 Recommendation

In contrast, to ensure a successful fertilization in mice, environment factor, temperature, light intensity, handling of the animals should be taken into concern as this will change their life style and most importantly breeding performance. Food and water supply should be sufficient and should not overfed as the mice would not stop eating that might causing obesity, which further affect the breeding performance.

FYP FIAT
UNIVERSITI
MALAYSIA
KELANTAN

Indeed, a suitable temperature and a good housing with better ventilation provide can reduce the chance of causing heat stress to the mice and reduce the chances of mice to get diseases. The housing space should be sufficient for the mice as too peek of mice in a small space inducing a lot of stress and competition that might making them fight each other and affect the reproductive performance.

12 hours of light with dark cycle can be maintained to raise the mice to regulate their normal activity as night time are important for the mice mating. Minimal handling is important to reduce stress applying on mice. Moreover, the mice should be rest for a week to let them to feel comfortable to the new environment as there are a lot of stress occur during the transportation from one place to another place. Flushing on later embryo stages such as blastocyst will surely increase the number of successful development when culture, as it reduces a lot of risk like apoptosis that commonly happen in early embryo stage. Number of mice can be increase or adding more replication can be done to get a more accurate data.

FYP FIAT

UNIVERSITI
MALAYSIA
KELANTAN

REFERENCES

Agca, Y. (2000). Cryopreservation of oocyte and ovarian tissue. *Ilar Journal*, *41*(4), 207–220. https://doi.org/10.1093/ilar.41.4.207

Armstrong, D. T. & Opavsky, M. A. (1988). Superovulation of immature rats by continuous infusion of follicle-stimulating hormone. *Biology of Reproduction*, *39*(3), 511–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3143425

Baruselli, P. S., Ferreira, R. M., Sales, J. N. S., Gimenes, L. U., Sá Filho, M. F., Martins, C. M., …Bó, G. A. (2011). Timed embryo transfer programs for management of donor and recipient cattle. *Theriogenology*, *76*(9), 1583–1593. https://doi.org/10.1016/j.theriogenology.2011.06.006

Betts, D. H. & Madan, P. (2008). Permanent embryo arrest: Molecular and cellular concepts. *Molecular Human Reproduction*, *14*(8), 445–453. https://doi.org/10.1093/molehr/gan035

Brooks, E., Patel, D. & Mercè Canal, M. (2014). Programming of mice circadian photic responses by postnatal light environment. *PLoS ONE*, *9*(5). https://doi.org/10.1371/journal.pone.0097160

Carballada, R. & Esponda, P. (1993). Structure of the vaginal plugs generated by normal rats and by rats with partially removed seminal vesicles. *Journal of Experimental Zoology*, *265*(1), 61–68. https://doi.org/10.1002/jez.1402650109

Castelhano-Carlos, M. J. & Baumans, V. (2009). The impact of light, noise, cage cleaning and in-house transport on welfare and stress of laboratory rats. *Laboratory Animals*, *43*(4), 311–327. https://doi.org/10.1258/la.2009.0080098

Chasombat, J. (2014). *In Vitro Embryo Production from Vitrified/ Thawed Ooctyes in Thai Native Cattle*.

Dean, M. D. (2013). Genetic Disruption of the Copulatory Plug in Mice Leads to Severely Reduced Fertility. *PLoS Genetics*, *9*(1), 1–7. https://doi.org/10.1371/journal.pgen.1003185

Dinopoulou, V., Drakakis, P., Kefala, S., Kiapekou, E., Bletsa, R., Anagnostou, E., …Loutradis, D. (2016). Effect of recombinant-LH and hCG in the absence of FSH on in vitro maturation (IVM) fertilization and early embryonic development of mouse germinal vesicle (GV)-stage oocytes. *Reproductive Biology*, *16*(2), 138–146. https://doi.org/10.1016/j.repbio.2016.01.004

Dulbecco's Phosphate Buffered Saline. (n.d.). Retrieved January10, 2018, from https://www.sigmaaldrich.com/catalog/product/sigma/d8537?lang=en®ion=MY

Fridgeirsdottir, G. A., Hillered, L. & Clausen, F. (2017). Escalated handling of young C57BL / 6 mice results in altered Morris water maze performance, *9734*(December). https://doi.org/10.3109/03009734.2013.847511

Gaskill, B. N., Rohr, S. A., Pajor, E. A., Lucas, J. R. & Garner, J. P. (2009). Some like it hot : Mouse temperature preferences in laboratory housing, *116*, 279–285. https://doi.org/10.1016/j.applanim.2008.10.002

Guzik, D. S., Carson, S. A., Coutifaris, C., Overstreet, J. W., Factor-Litvak, P., Steinkampf, M. P., …Canfield, R. E. (1999). Efficacy of superovulation and intrauterine insemination in the treatment of infertility. *The New England Journal of Medicine*, *340*(3), 177–183.

FYP FIAT

Hamid, H. Y., Abu Bakar Zakaria, M. Z., Yong Meng, G., Haron, A. W. & Mohamed Mustapha, N. (2012). Effects of Elevated Ambient Temperature on Reproductive Outcomes and Offspring Growth Depend on Exposure Time. *The Scientific World Journal*, *2012*, 1–6. https://doi.org/10.1100/2012/359134

Hansen, P. J., Drost, M., Rivera, R. M., Paula-Lopes, F. F., Al-Katanani, Y. M., Kringinger, C. E. & Chase, C. C. (2001). Adverse impact of heat stress on embryo production. *Theriogenology*, *55*(0), 91–103.

Hodges, J. "Bella." (2006). Breeding Guide: Male Reproductive System. Retrieved December13, 2017, from http://ratguide.com/breeding/anatomy/male_reproductive_system.php

Horan, T. S., Marre, A., Hassold, T., Lawson, C. & Hunt, P. A. (2017). Germline and reproductive tract effects intensify in male mice with successive generations of estrogenic exposure. *PLoS Genetics*, *13*(7), 1–23. https://doi.org/10.1371/journal.pgen.1006885

Illmensee, K. & Levanduski, M. (2010). Embryo splitting. *Middle East Fertility Society Journal*, *15*(2), 57–63. https://doi.org/10.1016/j.mefs.2010.05.001

Ishida, G. M., Saito, H., Ohta, N., Takahashi, T., Ito, M. M., Saito, T., …Hiroi, M. (1997). The optimal equilibration time for mouse embryos frozen by vitrification with trehalose. *Human Reproduction (Oxford, England)*, *12*(6), 1259–62. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9222013

Jamsai, D. & O'Bryan, M. K. (2011). Mouse models in male fertility research. *Asian Journal of Andrology*, *13*(1), 139–151. https://doi.org/10.1038/aja.2010.101

Katherine E. Quesenberry & Kenneth R. Boschert. (n.d.). Breeding and Reproduction of Mice. Retrieved December12, 2017, from http://www.msdvetmanual.com/all-other-pets/mice/breeding-and-reproduction-of-mice

Kim, S. (1995). Female Aging and Superovulation Induction for IVF, *21*, 75–82.

Kramer, K. M. & Birney, E. C. (2001). Effect of Light Intensity on Activity Patterns of Patagonian Leaf Eared Mice, Phyllotis Xanthopygus. *Journal of Mammalogy*, *82*(2), 535–544. https://doi.org/10.1644/1545-1542(2001)082<0535:EOLIOA>2.0.CO;2

Kumari, M. & Singh, P. (2013). Study on The Reproductive Organs and Fertility of The Male Mice following Administration of Metronidazole. *International Journal of Fertility & Sterility*, *7*(3), 225–238.

Legge, M. & Sellens, M. H. (1994). Optimization of superovulation in the reproductively mature mouse. *Journal of Assisted Reproduction and Genetics*, *11*(6), 312–318. https://doi.org/10.1007/BF02215719

Li, H., Li, H.-B., Zhang, M., Yan, F., Zhang, Z.-X. & Li, Z.-L. (2010). Effect of apigenin on the reproductive system in male mice. *Health*, *2*(5), 435–440. https://doi.org/10.4236/health.2010.25065

Liang, M., Zhong, J., Liu, H. X., Lopatina, O., Nakada, R., Yamauchi, A. M. & Higashida, H. (2014). Pairmate-dependent pup retrieval as parental behavior in male mice. *Frontiers in Neuroscience*, *8*(8 JUL), 1–10. https://doi.org/10.3389/fnins.2014.00186

FYP FIAT
UNIVERSITI MALAYSIA KELANTAN

Luo, C., Zuñiga, J., Edison, E., Palla, S., Dong, W. & Parker-Thornburg, J. (2011). Superovulation strategies for 6 commonly used mouse strains. *Journal of the American Association for Laboratory Animal Science : JAALAS*, *50*(4), 471–478. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3148645&tool=pmcentrez&rendertype=abstract

Martan, J. & Shepherd, B. A. (1976). The role of the copulatory plug in reproduction of the guinea pig. *Journal of Experimental Zoology*, *196*(1), 79–83. https://doi.org/10.1002/jez.1401960108

Niakan, K. K., Han, J., Pedersen, R. A., Simon, C. & Pera, R. A. R. (2012). Human pre-implantation embryo development. *Development*, *139*(5), 829–841. https://doi.org/10.1242/dev.060426

PB1 medium. (2006). *Cold Spring Harbor Protocols*, *2006*(8), pdb.rec10363-rec10363. https://doi.org/10.1101/pdb.rec10363

Peirson, S. N., Brown, L. A., Pothecary, C. A., Benson, L. A. & Fisk, A. S. (2017). Light and the laboratory mouse. *Journal of Neuroscience Methods*. https://doi.org/10.1016/j.jneumeth.2017.04.007

Perez, G. I., Trbovich, A. M., Gosden, R. G. & Tilly, J. L. (2000). Reproductive biology: Mitochondria and the death of oocytes. *Nature*, *403*(6769), 500–501. https://doi.org/10.1038/35000651

Popova, E., Krivokharchenko, A., Ganten, D. & Bader, M. (2002). Comparison between PMSG- and FSH-induced superovulation for the generation of transgenic rats. *Molecular Reproduction and Development*, *63*(2), 177–182. https://doi.org/10.1002/mrd.10173

Prados, F. J., Debrock, S., Lemmen, J. G. & Agerholm, I. (2012). The cleavage stage embryo. *Human Reproduction*, *27*(suppl 1), i50–i71. https://doi.org/10.1093/humrep/des224

Qiu, L., Lai, W., Stumpo, D. & Blackshear, P. (2016). Mouse Embryonic Fibroblast Cell Culture and Stimulation. *BIO-PROTOCOL*, *6*(13). https://doi.org/10.21769/BioProtoc.1859

Quinn, P. & Horstman, F. C. (1998). Is the mouse a good model for the human with respect to the development of the preimplantation embryo in vitro? *Human Reproduction (Oxford, England)*, *13 Suppl 4*(December), 173–183.

Redina, O. E., Amstislavsky, S. Y. & Maksimovsky, L. F. (1994). Induction of superovulation in DD mice at different stages of the cycle. *Journal of Reproduction and Fertility*, (102), 263–267.

Schneider, M. R., Mangels, R. & Dean, M. D. (2016). The molecular basis and reproductive function(s) of copulatory plugs. *Molecular Reproduction and Development*, *83*(9), 755–767. https://doi.org/10.1002/mrd.22689

Shi, X., Wang, T., Qiu, Z. L., Li, K., Li, L., Chan, C. P. S., …Quan, S. (2016). Effects of mechanical stresses on sperm function and fertilization rate in mice. *Systems Biology in Reproductive Medicine*, *62*(2), 152–159. https://doi.org/10.3109/19396368.2015.1115915

Shin, H., Cho, Y. M., Shin, H. J., Kim, H. D. & Choi, K. M. (2017). Comparison of commonly used ICR stocks and the characterization of Korl : ICR, *6055*(1), 8–14.

FYP FIAT

Speakman, J. R., &Keijer, J. (2013). Not so hot: Optimal housing temperatures for mice to mimic the thermal environment of humans. *Molecular Metabolism*, *2*(1), 5–9. https://doi.org/10.1016/j.molmet.2012.10.002

Takahashi, M. (2012). Heat stress on reproductive function and fertility in mammals. *Reproductive Medicine and Biology*, *11*(1), 37–47. https://doi.org/10.1007/s12522-011-0105-6

Training manual for embryo transfer in water-buffaloes. (n.d.). Retrieved December13, 2017, from http://www.fao.org/docrep/004/T0120E/T0120E06.htm

Van derAuwera, I. & D'Hooghe, T. (2001). Superovulation of female mice delays embryonic and fetal development. *Human Reproduction (Oxford, England)*, *16*(6), 1237–1243.

Wang, X. N. & Greenwald, G. S. (1993). Synergistic effects of steroids with FSH on folliculogenesis, steroidogenesis and FSH- and hCG-receptors in hypophysectomized mice. *Journal of Reproduction and Fertility*, *99*(2), 403–413. Retrieved from http://www.reproduction-online.org/content/99/2/403.full.pdf

Wei, Z. T., Lu, X. L., Zhang, G., Yu, J., Li, H., Jia, G. H., …Zhang, J. M. (2014). The long-term effects of superovulation on fertility and sexual behavior of male offspring in mice. *Journal of Assisted Reproduction and Genetics*, *31*(5), 555–560. https://doi.org/10.1007/s10815-014-0191-6

Weihe, W. H., Schidlow, J. & Strittmatter, J. (1969). The effect of light intensity on the breeding and development of rats and golden hamsters. *International Journal of Biometeorology*, *13*(1), 69–79. https://doi.org/10.1007/BF02329580

Yahia Hamid, H. & Zuki Abu Bakar Zakaria, M. (2013). Reproductive characteristics of the female laboratory rat. *African Journal of Biotechnology*, *12*(19), 2510–2514. https://doi.org/10.5897/AJB12.2046

FYP FIAT

APPENDIX A

Table A1: The amount of oocyte affects by semen condition in both naturally mated female mice and superovulated female mice in percentages (n=10).

	Naturally mated female mice	Superovulated female mice
Semen condition	Oocyte (%)	Oocyte (%)
Dilute	52	17
Semi-Solid	48	70
Hard Solid	0	12

Table A2: The amount of embryo affects by semen condition in both naturally mated female mice and superovulated female mice in percentages (n=10).

	Naturally mated female mice	Superovulated female mice
Semen condition	Embryo (%)	Embryo (%)
Dilute	0	0
Semi-Solid	59	20
Hard Solid	41	80

FYP FIAT
UNIVERSITI
MALAYSIA
KELANTAN

Table A3: The amount of sample produced in percentages (%) and semen condition from NM female mice after mating.

Male ID	Female ID	Semen Condition	Oocyte	Embryo	Degenerated Embryo/ Oocyte
A	#A1	Hard Solid	0	11	0
	#A2	Hard Solid	0	3	0
B	#A3	Dilute	2	0	3
	#A4	Semi-Solid	0	5	0
C	#A5	Dilute	3	0	0
	#A6	-	Failed plug, mate with male E		
D	#A7	Semi-Solid	6	0	0
	#A8	Semi-Solid	0	2	0
E	#A9	Semi-Solid	8	0	0
	#A10	Dilute	0	10	0
	#A6	Semi-Solid	1	13	8

FYP FIAT

UNIVERSITI MALAYSIA KELANTAN

Table A4: The amount of sample produced in percentages (%) and semen condition from SO female mice after mating.

Male ID	Female ID	Semen Condition	Oocyte	Embryo	Degenerated Embryo/ Oocyte
A	#B1	Semi-Solid	14	0	0
	#B2	-	Failed plug, mate with male E		
B	#B3	Semi-Solid	15	0	0
	#B4	Dilute	11	0	5
C	#B5	Hard Solid	6	10	0
	#B6	Hard Solid	0	0	0
D	#B7	Dilute	1	0	0
	#B8	Hard Solid	3	14	0
E	#B9	Semi-Solid	7	3	0
	#B10	Semi-Solid	6	0	0
	#B2	Semi-Solid	8	3	0

FYP FIAT
UNIVERSITI MALAYSIA KELANTAN

FYP FIAT

Appendix B

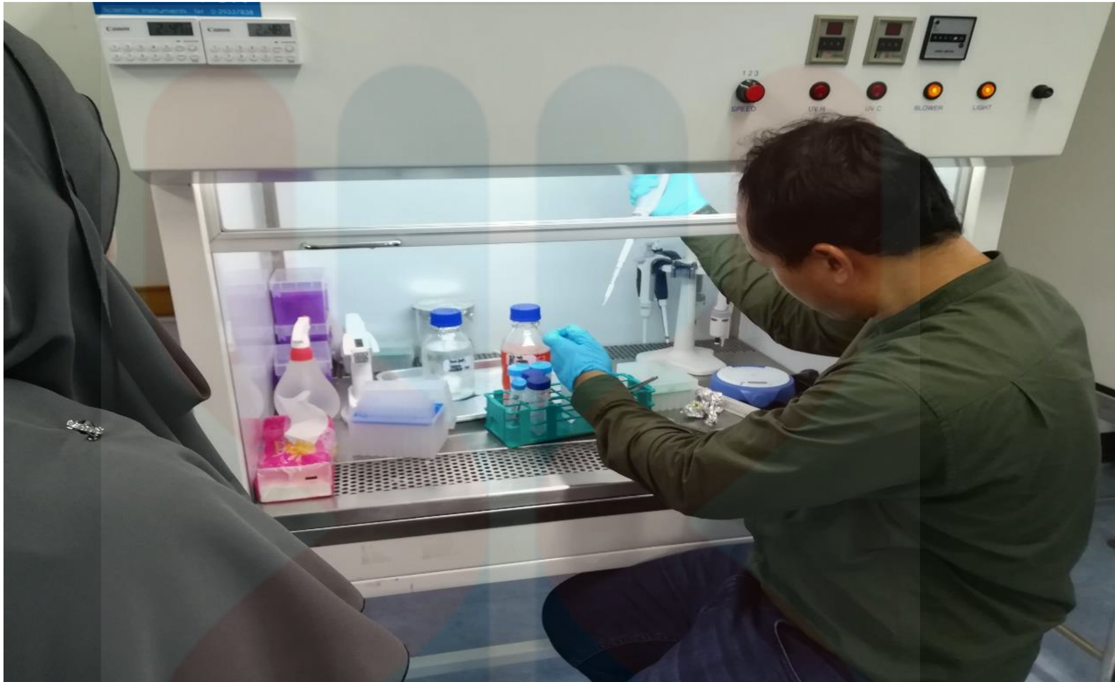


Figure B1: Solution Preparation (Dr. Jakkhaphan Chasombat from PNU, Thailand giving some guidance before starting our experiment).

Figure B2: The processed feed for the mice.

UNIVERSITI MALAYSIA KELANTAN

FYP FIAT

Figure B3: The cages for the mice with the water bottle and bedding.

Figure B4: The petri dish use for cell culture (the 4 drops are the KSOM medium use for embryo culture and covering with mineral oil).

UNIVERSITI
MALAYSIA
KELANTAN