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PROPERTIES AND EFFECTS OF DIFFERENT EGG YOLKS WITH HONEY ADDED IN EXTENDER ON SHEEP SEMEN

By

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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.

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Student

Name: HENG CHUI LING

Date:

I certify that the report of this final year project entitled “Properties and Effects of Different Egg Yolks with Honey added in Extender on Sheep Semen” by Heng Chui Ling, matric number F14A0082 has been examined and all the correction recommended by examiners have been done for the degree of Bachelor of Applied Science (Husbandry Science) with Honours, Faculty of Agro-Based Industry, Universiti Malaysia Kelantan.

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PROPERTIES AND EFFECTS OF DIFFERENT EGG YOLKS WITH HONEY ADDED IN EXTENDER ON SHEEP SEMEN

ABSTRACT

The aim of this study was to investigate the properties and effects of chicken, quail and duck egg yolks with or without honey and stingless bee honey as supplementation added in extender on sheep semen. The extended semen were chilled for three periods (two hours, one day and two days).The tested sheep semen quality included semen volume, morphology, sperm concentration, general motility, progressive motility and sperm viability. For comparison among different extenders, the semen quality was analyzed by using Computer-assisted sperm analysis (CASA) and the results were plotted. It was observed that after chilling for two hours quail egg yolk with honey showed the best results in terms of sperm concentration (1.218 × 109 /ml), general motility (86%), progressive motility (67%) and viability (93%). Honey was found to be able to supply sugar to the quail egg yolk which is lack of sugar. Duck egg yolk had the highest viscosity among three egg yolks. After chilling for one day, duck egg yolk extender showed the best results in terms of general motility (63%), progressive motility (33%), viability (86%) and sperm concentration (1.421 × 109 / ml), whereas after chilling two days quail egg yolk extended semen showed the best results with respective parameters of 45%, 30%, 67% and 0.9013 × 109 /ml, respectively. Honey had pH about 3.9, which caused the semen to turn acidic and less favorable for sperm survival. In conclusion, these findings are useful to be considered in future experiments involving semen extender. However, further experiment is necessary to improve the present protocol for better sperm preservation.

Keywords: Sheep semen, chilling, different types of egg yolk with honey extender, properties of honey

SIFAT DAN KESAN KUNING TELUR YANG BERLAINAN DENGAN MADU DALAM MEDIA PELANJUT MANI BIRI-BIRI

ABSTRAK

Tujuan penyelidikan ini adalah untuk mengkaji sifat-sifat dan kesan kuning telur ayam, puyuh dan itik dengan atau tanpa madu dan madu kelulut sebagai suplemen dalam media pelanjut mani biri-biri. Mani biri-biri dilanjutkankan dan disejukkan untuk tiga tempoh (dua jam, satu hari dan dua hari). Kualiti air mani yang diuji termasuk isipadu, morfologi, konsentrasi sperma, motilitas umum, motilitas progresif dan kadar survival. Kualiti mani dianalisa dengan menggunakan analisis sperma yang dibantu komputer (CASA) dan keputusan diplotkan ke dalam jadual untuk dikaji dan dibanding. Dari maklumat yang didapati, selepas penyejukan selama dua jam kuning telur puyuh dengan madu menunjukkan hasil terbaik dengan kepekatan sperma 1.218×10^9 / ml, motilitas umum 86%, motilitas progresif 67% dan kadar survival 93%. Madu didapati mampu membekalkan gula kepada kuning telur puyuh yang kekurangan gula. Kuning telur itik mempunyai kelikatan tertinggi di antara tiga kuning telur. Selepas penyejukan untuk satu hari, media pelanjut kuning telur itik adalah yang terbaik dengan motilitas 63%, motilitas progresif 33%, kadar survival 86% dan konsentrasi sperma 1.421×10^9 / ml manakala media pelanjut kuning telur puyuh adalah lebih baik dengan 45%, 30% 67% dan 0.9013×10^9 / ml selepas penyejukan dua hari. Madu mempunyai pH kira-kira 3.9, menyebabkan air mani menjadi asid dan kurang sesual untuk survival sperma. Hasil daripada kajian ini adalah pembuktian saintifik asas kepada sifat semula jadi kuning telur dan madu yang berlainan apabila diterapkan dalam media pelanjut mani dan membantu para penyelidik untuk mengkaji lebih lanjut mengenai pemeliharaan sperma yang lebih baik pada masa depan.

Kata kunci: Mani biri-biri, penyejukan, media pelanjut telur kuning yang berlainan dengan madu, sifat madu

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LIST OF ABBREVIATIONS AND SYMBOLS

IBVK	Institute Biodiversiti Veterinar Kebangsaan
AV	Artificial vagina
ml	Micro litre
mg	Milligram
g	Gram
%	Percentage
℃	Degree Celcius
°F	Degree Fahrenheit
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1 Research Background

Malaysia livestock industry is common with cattle; swine and poultry sector especially chickens farming but ruminant sector is quite lagging and underdeveloped. Due to active participation of the private sector, pig and poultry have optimally achieved domestic self-sufficiency. The ruminant sector which is dominated by cattle for meat production is hard to meet rising local demand and has to import beef from other countries. The ruminant sector is found growing steadily over the years due to the participation of government land development agencies in cattle and sheep rearing integrated with plantation crops, although it is still lagging in meeting local demand (Devendra, 2006).

In Malaysia, the sheep industry is under-developed if compared to other countries and various factors contribute to this situation. This is because the rural areas farmers commonly only have a small number of sheep and they are limited to access high quality ram. Furthermore natural service of sheep breeding is not popular yet. Limitation access to the knowledge of modern farming makes the farmers are difficult to improve their way of farming. They majorly stick to the traditional farming and they are still practicing slowly pace sheep farming. Their sheep breed in slower rate and the farmers are unable to speed up the breeding in efficient and safe way though.

According to Ministry of Agriculture and Agro-Based Industry Malaysia (MOA), the third National Development Policy (DPN 3) is structured to improve food production from local production including sheep meat commodity production and at the same time Department of Veterinary Services Malaysia (JPV) would analyses and evaluates the part that the industry should play. JPV has found certain values in sheep production and should be focused on its future potential. Goat and sheep meat demand in year 2010 had reached 21.30 metric tons but local production had only 2.18 metric tons. The rate of self-sufficiency has only 10.23%, it would have a very promising space and chance for all producers whom have involved in this industry.

In Malaysia, the word “mutton” usually means both goat and lamb meat, however this word actually refers only to lamb meat. Due to this issue, statistics on goat and sheep meat are often lumped together under the heading of mutton (Kaur, 2010).

To overcome the problem of mutton shortage, artificial insemination (AI) is the effective practice on fields (Allison & Hagevoort, 2014). AI is popular practice in numerous countries especially goat, cattle and sheep. Before successful AI, semen extender which is consisted of Trisaminomethane (TRIS), glucose, citrate, and yolk extender can give sufficient protection to the sperms for up to 192 h after collection. The semen is then analyzed by using *in vivo* insemination to live sheep. The conception rate after insemination is about 50% (Paula, 2016).

In ram semen preservation, different extenders with various ingredients such as skim cow milk, sodium citrate glucose yolk, lactose yolk, saccharose EDTA, calcium nitrite yolk, raffinose yolk, Spermasol and TRIS-yolk have been tested and used (Witco Chemical Corp., Oakland, NJ, USA). The egg yolk lecithin in TRIS-yolk extender can be hydrolysed into fatty acids by the egg yolk-coagulating enzyme

(phospholipase) in semen. Therefore the lysolecithin might affect the ram semen quality (Leboeuf et al., 2000). On the other hand, Abdelhakeam et al. (1991) suggested that the glycerol-containing extender can affect the semen quality negatively in major number of rams because it causes reduction in semen parameters. Glycerol would cause degrading effects on sperm membrane of rams when it is used as cyroprotectant. Not only that, long term storage of frozen sheep semen usually result in decreasing motility, viability, and fertility and impaired transport because of ultrastructural, biochemical, and functional damage to the spermatozoa (Qureshi et al., 2013). Since this type of research work and related fields are popular and heating in western countries, there are numerous types of semen extenders are developed. Then the present study was designed to investigate the effect of various egg yolk extenders with honey on the sheep semen quality.

In this research, the egg yolks from various animal species were used to produce semen extender since different egg yolks do have different nutritional values, composition, properties, antibodies, proteins, lecithin and special effects that might affects the semen quality prior application of AI. Honey is utilized as supplement of sugar to find out the advantages and disadvantaged of its addition in semen extenders. This research aims to find out and compare the egg yolks with or without honey to obtain the best result in extender on sheep semen.

1.2 Problem Statement

The successful conception rate of the female sheep through AI is about 50%, depending on the fertility of both the recipient and the male (semen), accuracy of heat detection, semen processing and insemination technique (Muasya et al., 2007). The ram needs to be in optimum health status prior semen collection. However, sperm quality would vary due to other factors such as semen collecting technique, storage technique after collection and other external factors instead of just concerning about the ram's health. This study is conducted to research how egg yolk and honey can act as extender to maximize the sperm quality during storage period for usage afterwards. Besides this study aims to find out the best egg yolk extender of semen to bring best results of sperm count, sperm motility and others concerned readings.

1.3 Objectives

1. To determine the sperm quality after application of different egg yolks with supplementation of honey extenders.
2. To determine the effects of different egg yolks with honey extenders on sperm quality at different storage periods.

1.4 Scope of Study

The three types of egg yolks: chicken, duck and quail egg yolk and two types of honey: honey and stingless bee honey are chosen to be processed as the extender in sheep semen during storage to maximize its quality by sperm count, sperm concentration, morphology, sperm motility and viability. Thus, the semen will then analyzed by semen analyzer that is available in Institut Biodiversiti Veterinar

Kebangsaan, Jerantut (IBVK) to obtain the readings and make comparisons among different extender-added samples.

This study intends to find out the benefits of egg yolk and honey with its appropriate properties to be applied in sheep semen extender in terms of increasing the sperm count, maintaining good sperm morphology, increasing sperm activeness and viability in 3 different periods: within 2 hours, 1 day and 2 days.

The processing technique of the egg yolk that is applied to the extender in sheep semen is studied and modified to improve the effects and benefits. The egg yolk is filtered, emulsified and studied its content of antibodies and nutrients before added to the extender in semen. The egg yolk emulsification is carefully conducted without mixing with membranes, albumin and other unwanted content to increase the purity of egg yolk substances. Then honey is added into the extenders as well.

The semen samples that are added with extender mixed with different egg yolks are then examined and analyzed by Sperm Class Analyzer (SCA) with Computer Assisted Semen Analysis (CASA) to obtain the readings needed for comparisons ("SCA® CASA System", 2017).

1.5 Limitation of Study

The study of egg yolks variations is only involving livestock animals' egg yolks due to limitation of sample sources. There are only few livestock animals available in UMK Jeli and egg-producing animals at here include chickens, ducks and quails. Besides, these animals are easier to handle and convenient to obtain appropriate samples for study.

Sperm Class Analyzer (SCA) is applied during semen analysis on basic parameters such as sperm motility and concentration in an animal semen sample which is only available in UMK PC.

Due to facility limitation, there is only one ram available in Agropark UMK Jeli currently. The source of semen collection would be limited.

1.6 Significance of Study

The research is focusing on the study of different animal species egg yolks and honey o obtain its reading on nutritional values, properties and effects on sheep semen. The egg yolks are processed to play roles as the extender on sheep semen to prolong the sperm longevity, increase the sperm count and as the source of nutrients to help the sperms to survive longer whereas supplementation of honey is studied its advantages and disadvantaged on semen quality, too. Then the variation of egg yolks is studied and compared to obtain the best result on the sheep semen.

CHAPTER 2

LITERATURE REVIEW

2.1 Livestock Industry in Malaysia

In Malaysia, livestock farming is extremely important as the booster in development of economy of this country. Malaysia is a developing agricultural-based country since centuries of years ago. It provides massive employment, supplies the local demand of meat, milk and dairy products. Both Peninsular of Malaysia and East Malaysia do have ample of agricultural lands that are blessed with countless benefits such as natural disaster free, hot climate but high humidity, nutrients rich soil, constant water source and high yield crop breeds. These advantages contribute to the high productivity in the livestock animal industry in Malaysia such as non-ruminants like poultry and swine, ruminants like cattle, goats, sheep and deer.

Animal meat is the essential and sole source of animal protein in the Malaysian population diet (Kaur, 2010). The demand for meat consumption is always increasing over the years proportional to the increasing population in Malaysia. In year 2017, poultry consumption is up to 42.471 kg per capita, beef consumption is 5.88 kg per capita whereas mutton consumption is 0.865 kg per capita only (Agricultural output - Meat consumption - OECD Data, 2017). Besides poultry industry, the ruminant livestock animal industries are still conducted in small scale in Malaysia until 2017 (Mohamed, 2007). Poultry production in Malaysia is optimum and meets very high self-sufficiency. But ruminant-based livestock production is very low in supplying local demands especially beef and mutton supplies. Malaysians have relatively higher demand than the production available in the country. However,

improving development is obvious in recent years, even though it is still unable to meet the local demand. Malaysia has to depend on the importation of beef, mutton and dairy products from abroad especially India, Australia and New Zealand to meet high local demand. The levels of self-sufficiency (SSL) in 2014 for beef, mutton and milk were 24.84%, 13.10% and 12.93% respectively. The lagging of ruminant sector is usually due to several factors like land resources shortage, high feed price, cheaper import substitutes, poor private-sector involvement (Shanmugavelu, 2014), poor disease prevention and control (Mohamed, 2007), and lack of quality breeds, lack of expertise and workforce (MOA, 2017).

2.1.1 Sheep Industry in Malaysia

In general Malaysian population, sheep meat is considered as unpopular, uncommon and its per capita consumption has remained stagnant compared to other animal meat consumption. In Malaysia, the word “mutton” is usually used to refer both goat and lamb meat, although the term technically only refers to lamb meat. Therefore the statistics on goat and sheep meat are often lumped together under the heading of mutton and furthermore causing the sheep industry development is difficult to be studied. However, the low production of sheep meat and high importation from other countries is giving motivating and encouraging development in this industry. Policy makers should be concerned that lagged development might cause higher importation and they should structure the current undergoing livestock policies besides focus on meat segments to fully utilize the market potential domestically and internationally.

In less than two decades, mutton production has been tripled from 666 tons in 1990 to 1958 tons in 2008. The domestic self-sufficiency level was only at 8.8% then increased to 10% respectively. This is the main reason of high dependence of

Malaysian on importation from overseas. In 2003, Malaysia has imported over 10 thousand tons of mutton valued about RM90 million. By 2007, the import was then risen to 16 thousand tons which valued at RM160 million (Kaur, 2010). It would be high possibility that the importation is still rising and would be higher in future since the Malaysian population is rising all the time.

2.1.2 Sheep Meat Consumption in Malaysia

A small portion of Malaysian population loves the strong distinctive flavor of mutton. It is unpopular and uncommon preference to be served in many dishes or cuisines in general population because they have misconception that the meat is high in cholesterol and saturated fats. This wide spread misconception can cause the low consumption of mutton which is only about 1 kg per capita consumption per annum over the last 20 years. However the per capita consumption of beef has increased 79% whereas poultry meat has increased by 83% within 20 years (FAMA, 2017).

Since year 1997, the mutton prices had been doubled at farm level which is about RM7 per kg to RM15 per kg in year 2008 (FAMA, 2017). On the other hand, the wholesale prices increased from about RM12 to RM23 respectively. The retail price was increasing in slower pace which is only from about RM15 to RM26 (Kaur, 2010). It is common to find that sheep meat is more expensive than chicken meat at all time. Sheep meat is more exotic and uncommon in retail stores as well causing it to have higher price than other livestock meat.

Beef and mutton production have been observed doubled in last 20 years. The main contributor to this improving development is the involvement of private sector in cattle and sheep farming. Besides, they contribute a lot in sharing of livestock industry expenses, especially the ruminant sub-sector as targeted by the

Ninth Malaysia Plan (9MP). To achieve the main aims in 9MP, the Malaysian government has allocated about RM5 hundred million as the budget for agriculture development such as livestock sector (Mohamed, 2007). This plan is very encouraging the domestic beef and mutton production and development since the local entrepreneurs are given chance to start business and their financial problem can be settled by applying loans from the government.

2.2 Artificial Insemination in Sheep

Artificial insemination (AI) is a technique that includes both male semen collection process and semen transfer to the reproductive tract of the female. Ewes can be inseminated with either fresh semen or with frozen semen which is prepared commercially or by farm. AI is practiced to eliminate or reduce the cost of maintaining rams. AI helps to increase the rate of genetic improvement and the number of females to be bred at the same day to obtain large amount of products at the same period with the assist of estrus synchronization by using CIDR (Leboeuf et al., 1998). This method needs various factors to be cooperative with to increase conception rate of does, including accurate estimation of doe reproductive cycle, heat detection, time of insemination, semen thawing and proper insemination procedures. These are usually done by experienced technicians instead of newbies since these procedures require high portion of skills and experience to conduct. The sheep might be hurt if the handler does not conduct certain part well due to lack of experience.

2.2.1 Sperm Cell Preservation

Sperms are the tiniest catabolic body cells. They consume the stored energy within semen and cell body when they are undergoing metabolic processes or moving. They start to age and die over the time. Eventually they become immobilized,

unable to move and fertilize the egg cells (Laboeuf, 1998). Besides, during exposure to the extreme conditions, the sperms will irreversibly deteriorate and become useless. The sperms have very limited anabolic or healing capacity causing the processing of semen shall be handled very careful to avoid any risk of exposure to damaging condition. Therefore to overcome the difficulties during semen storage, the semen catabolism should be minimized to obtain the highest similarity of thawed semen quality to the freshly ejaculated semen. The semen shall be able to be packaged and stored with highest viable cells which are capable in fertilization afther thawing (Laboeuf, 2000).

The processing of semen should be concerned at several aspects such as holding time of semen after collection in room temperature, the ingredients of extender, types of antibiotics, cooling rate of extended semen, the duration of thawing and method of thawing. To achieve the demanded semen quality, all factors that contribute to the result variation should be managed properly (Laboeuf, 2000).

2.2.2 Semen collection

Semen collection can be done by using either one of the techniques such as artificial vagina (AV), digital manipulation or electro ejaculation (Semen Collection, 2017). Upon choosing the correct way to collect semen, the person shall consider the species of animal, animal body size and the location of collection site.

These three ways require an artificial vagina which is a double walled device with an opening at one end and collection tube at the other. Warm water is inserted at the inner lining whereas the outer layer is covered with water soluble lubricating jelly. The ram which is ready to collect semen may be allowed to mount a ewe whereas the person can manually directing the ram's penis into the artificial vagina. The penis

cannot be contacted directly. The person can grasp the ram's sheath to direct the penis. An amount of 0.5 ml to 1 ml can be collected during ejaculation. Then remove the artificial vagina and tilt it upwards to allow the semen running into the collection tube entirely. The second method is by training a ram to mount on a dummy instead of a live doe. The semen collection is conducted with the similar steps. To have more aggressive mounting, the person can put some vaginal mucus scrapings of a ewe that is in heat onto the dummy to attract the ram. The last method: electro-ejaculation (Patel, 1967) does not require the ram to mount on an object. The electrode unit with electricity conducting rings is pushed into the ram's rectum to give minor electric stimulation that brings ejaculation. It is effective to get good quantity and quality of samples by using this method but the sperm concentration usually will be lower than others.

2.2.2.1 Artificial Vagina (AV)

Artificial vagina (AV) is a useful device to collect semen from various species of animals. However the subject male has to be conscious, not easily frightened when contacting with people, and more interested in cooperating to ejaculate than attacking people (Patel, 1967).

The AV mechanism emphasizes on the uses of both thermal and mechanical stimulation to cause ejaculation of the male animal. AV is consisted of a tube with outer lining made by rubber to hold warm water with lubricated inner lining before use. Then the outer liner is filled with water which is higher a bit than body temperature then pressurized with air pump (Huat, 1973).

2.2.3 Semen Chilling

Semen chilling is carried out at about 5°C for few days. Post chilled semen is then thawed in water bath of 37°C for 45 seconds (Lemma, 2011) before evaluation on sperm general motility, progressive motility and viability. This cooling method is a low cost method as it does not require usage of cryotank and liquid nitrogen. This cooling method is more suitable for practice of local farmers.

2.2.3.1 Short-Term Storage

Most semen is preserved in a frozen state. But in chilling method, semen is stored with extenders in liquid state. In order to store up to few days, suitable extenders must be used. An ideal extender has to be able to provide sufficient nutrients, contain protectants to protect the sperms against the harmful effects of cooling and freezing; to provide a media to prevent shifts in pH because of formation of lactic acid; to maintain the proper osmotic pressure and electrolyte balance, to provide antibiotics that inhibit bacterial growth, to be able to dilute the sperm into larger volume so that multiple inseminations can be performed; and to provide a good condition to enable the sperms to undergo smooth metabolic activities. The extenders usually consist of egg yolk, milk, TRIS or a combination of the two as the basic ingredients. The fresh egg yolk can provide primary protection like lipoprotein and lechithin for the sperm cells against cold shock. The milk extender contains milk protein and casein which are efficient in protection against cold shock (Huat, 1973).

2.2.4 Thawing of Semen and Deposition of Semen in ewes

The semen is thawed to about body temperature before application of artificial insemination. Frozen or chilled semen need to be be thawed according to the professional recommendations. The chilled semen is thawed in water bath machine with temperature of about 35°C. After thawing, the semen must be kept warm and not be exposed to sunlight or water during the thawing and inseminating process to prevent damaging or killing sperm cells. Then pull the plunger back 4 to 6 inches on the insemination gun and place the straw into the gun with the cotton plug toward the plunger (Louis, 2016). After the straw has been installed in the gun, the sealed end of the straw must be cut off with scissors. Then the cover sheath should be placed over the insemination gun and secured with an O ring. After preparation of insemination gun the ewe's hind legs are lifted carefully. The speculum is lubricated well then cleaning the ewe's vulva with a clean paper towel. The speculum is inserted upwardly angle to prevent the ewe's irritation at its vagina part. The cervix is found by using the light in speculum and it should be reddish purple colour and if it is on heat, there will be presence of white mucus (Louis, 2016).

The speculum should be located at the right centre over the opening of the cervix. Then push the insemination gun into the speculum and carefully thread it into the opening of the cervix. Apply a circular motion and slightly pressure the insemination gun to pass through the rings of the cervix but not too deep into the cervix. Then deposit the semen slowly by pushing the plunger forward. After done, the insemination gun is pulled out slowly and removes the speculum carefully (Louis, 2016).

2.3 Semen Extender

Semen extender is a liquid medium which is mixed with semen to preserve its fertilizing ability. It acts as a diluent to protect the sperm cells from their own toxic by-products, and it protects the sperm cells from cold shock and osmotic shock during the chilling and storage (the sperm is chilled to decrease metabolism activities so that allow it to live longer). The extender helps the semen to be transferred to the female, rather than putting the male and female together to mate to produce offsprings (Preparing for Breeding, 2017). Certain extender with special ability can obtain cryogenic preservation which can help to freeze the sperms (frozen semen) then easier for transportation and later usage.

The application of extender to semen intends to protect the sperms against risk of damage by toxic seminal plasma, as well as providing nutrients and cooling buffers if the semen needs to be cooled for long storage. For freezing extenders, it is usual to add in one or more penetrating cryoprotectants such as glycerol, DMSO and dimethylformamide to protect the sperms from freezing defects. Egg yolk, which has cryoprotective properties, is also a common component, too (Vera et al., 2009).

2.3.1 Egg Yolks

Eggs contain high quality protein with little energy content. One whole egg has about 5 grams of protein in only approximately 68 calories. Eggs contain choline, which is essential and cannot be sufficiently produced by body. Deficiency of choline also brings deficiency in another essential nutrient, folic acid. Egg yolks contain cholesterol, the fat and saturated fat of the egg, several fat-soluble vitamins, essential fatty acids and other nutrients. An egg yolk has around 50 calories, 4 to 5 grams of total fat and about 1.5 grams of saturated fat, 210 mg of cholesterol, 8 mg of sodium, and almost 3 grams of protein.

The table in Appendix A.1 by the USDA shows the nutritions of the egg yolk, along with all of the percentage of total nutrition found in the yolk. (The Nutritional Value of Egg Whites Versus Egg Yolks: What Do You Use? – A Healthier Michigan, 2017).

2.3.2 Effects of Egg Yolk in Semen Extender

The most commonly used extenders for the use and commercialization of animal semen in liquid or frozen semen would have egg yolk as a basic ingredient (Bogart et al., 1950). Egg yolk provides an excellent protection for mammalian spermatozoa in protection against cold shock and the lipid-phase transition effect (Aboagla et al., 2004). Egg yolk is also added in animal semen extenders as the main energy source because it is rich in lecithin, proteins, lipoproteins and other complexes (Semenova, 1987). The development of buffers to combine with egg yolk and a reduction in its concentration to 20% (v/v) in mammalian semen extenders are found to be able to improve sperm survivability (Salisbury, 1978).

The conception rate of sheep which were inseminated with sodium citrate glucose-yolk-glycerol extended semen, were about 60 and 90% with frozen thawed and fresh semen respectively. Doctor Louis (2016) believed that egg yolk is toxic to the sheep spermatozoa. An enzyme (phosphotidase) produced by the bulbo-urethral glands of the male sheep can cause the hydrolysis of lecithins in egg yolk to fatty acids to form lysolecithins, that are believed to be a toxin to the spermatozoa. Then the presence of phosphotidase in the seminal plasma of the sheep causes the forbiddance of using any media that contains egg yolk to be used as semen extenders.

Seminal plasma has been blamed as the main limiting factor that affects the sheep semen freezability and fertility. Cleaning of sheep spermatozoa in a physiological solution is believed to improve the sperm motility in fresh ejaculates and post thawed semen.

2.3.3 Effects of Honey in Semen Extender

Honey contains small amounts of antioxidants like chrysin, pinobanksin, vitamin C, catalase, and pinocembrin besides its high concentration of sugar like glucose and fructose (Mato et al, 2003). These properties can contribute to extra longevity of sperms upon long storage.

2.3.4 Semen Extension

A suitable volume of semen which contains sufficient sperms that have high fertilizing ability is the most important target of extending semen. Sperm concentration of the normal ejaculate varies about 2 to 7 × 10^9 / ml. Semen of ram is extended immediately after collection at about body temperature of 37°C and cooled slowly to prevent cold shock that can cause sperm damage. Egg yolk contains lipoprotein and lecithins that are able to protect the sperms from cold shock by adding it into the semen before cooling. Besides, the cooling rate should be controlled to sustain healthy and viable sperm cells besides just avoiding cold shocks. After extension, semen is cooled to 5°C with cooling rate of about 0.5°C / min. Then, glycerol is added to the extenders to protect sperms against the detrimental effects of freezing as it can prevent the increase of the intracellular salt concentration due to the removal of water from the cell during ice formation which is a major cause of sperm damage during freezing.

Glycerol attributes to the salt "buffering" capacity which contributes to the cryoprotection. Thus, electrolytic damage due the freezing water can be minimized. To obtain optimal result, many researches study on the factors like the storing time, temperature, rate of addition, and concentration of glycerol have been conducted.

Some researchers suggest to add the glycerol before cooling (i.e., at 30°C) and others after cooling at 4°C. Since cryo-protective activity of glycerol occurs during the crystallization phase, glycerol is more optimal to be added at 4°C. The composition of glycerol is usually about 5% in extended ram semen (Eaton et al., 1952).

2.4 Sperm Class Analyzer (SCA)

The SCA is computer based sperm analysis software which provides fast, accurate and objectively repeatable results. This level of accuracy would be impossible to attain using traditional (subjective) methods (SCA® CASA System, 2017).

The software generates objective spermograms in a quick and accurate way with a high repeatability factor. The analysis of individual paths and the acquisition of kinetic parameters of sperm motility are extremely reliable and accurate. The morphological analysis in colour carried out by the software intelligently identifies and classifies those spermatozoids with misstructured head and tail (including acrosome). Morphology analysis criteria can be calibrated to use WHO (World Health Organisation), Strict Criteria or even user's personal criteria. The Concentration Module of the SCA® system is specifically designed to determine this parameter accurately using WHO criteria on images of predetermined samples obtained with a Hæmocytometer. Dye Exclusion Test (EOSIN) is used to distinguish the different types of sperms to estimate the proportion of dead and live sperms whereas Hypo-Osmotic Swelling Test (HOS) is used to know the integrity and compliance of the cell membrane of the sperm tail (Pedieos IVF Center, Cyprus, 2015).

2.5 Semen Parameters

Examples of parameters measured in a semen analysis are: sperm count, motility, morphology, volume and viability. A ram may give 0.5 ml to 1.5 ml of semen per ejaculate. Furthermore, daily sperm production per gram of testis in sheeps (efficiency of spermatogenesis) is about 30 million of sperms (Semen Collection, 2017). Seminal quality parameters are recorded to evaluate the effect of chill–thawing procedure on sheep sperm characteristics, and to relate possible changes in sperm parameters to sperm preservation success. Sperm quality parameters (motility, morphology and acrosome) were compared between fresh and chill–thawed samples.

CHAPTER 3

MATERIAL AND METHODS

3.1 Materials

To conduct this research, several materials were needed to set up such as various chemicals, filter papers, latex gloves, a pack of chicken egg yolks, duck egg yolks and quail egg yolks respectively, pure honey and stingless bee honey, fresh sheep semen, distilled water and tubes.

3.1.1 Chemicals and Reagents

Trisaminomethane (Tris), monohydrate citric acid, fructose, penicillin, streptomycin, glycerol and distilled water are prepared in advance to start up the experiment. These chemicals can be obtained in FIAT laboratory in UMK Jeli which was in high purity and quality to be used to make better quality extender afterwards.

3.1.2 Apparatus

AV helps to collect semen from the ram. A piece of 500 ml beaker, micropipette with 100µl tips, and a piece of 10 ml measuring cylinder, electronic scale, 9 falcon tubes (15 ml), glass tubes, glass slides, thermometer, light microscope, platform heater and water bath machine are required in preparation of extender and extension of semen then examination of sheep semen as well.

3.1.3 Equipment

Chiller is used to store the extended semen and fresh semen in controlled cool condition with temperature 0-4°C. Sperm Class Analyzer (SCA) from IBVK was utilized to test and analysed the extended semen and fresh semen as well to record the readings and make comparisons as well.

3.2 Methods

3.2.1 Preparation of Extender

The egg yolks were separated from the egg whites. The yolks were then dried on a filter paper, then punctured, and allowed to drain off into the 10 ml graduated cylinder. No membranes or egg whites were allowed to contaminate the liquid yolks. The egg white was separated completely and that the yolk membrane was blotted dry before tearing to harvest the yolk. Then mixed with a gentle swirling motion until mixture appears homogeneous.

Table 3.1: Materials needed for different extenders

Extender	C	Q	D	C+H	Q+H	D+H	C+SH	Q+SH	D+SH
TRIS (g)	0.121	0.121	0.121	0.121	0.121	0.121	0.121	0.121	0.121
Monohydrate citric acid (g)	0.067	0.067	0.067	0.067	0.067	0.067	0.067	0.067	0.067
Fructose (g)	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Penicillin (g)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Streptomycin (g)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Glycerol (ml)	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35
Egg yolk (ml)	1	1	1	1	1	1	1	1	1
Honey (ml)	-	-	-	0.1	0.1	0.1	0.1	0.1	0.1
Distilled water (ml)	5	5	5	5	5	5	5	5	5

C: chicken egg yolk (control)

Q: quail egg yolk

D: duck egg yolk

H: honey

SH: stingless bee honey

Each set of materials for each extender is prepared to make a 5 ml extender. Use an electronic scale to weigh crystalline materials. All liquid materials are measured out from a 10 ml graduated cylinder and micropipette. In each extender, dilute the crystalline powder in 5 ml distilled water and mixed well. Pour 4 ml of it into a falcon tube. Then replace 0.35 ml of the mixture by 0.35 ml of glycerol. Add in 1 ml of egg yolk to make a complete extender. Replace 0.1 ml of the extender by 0.1 ml of honey to make egg yolk honey extenders. Freeze to -20°C until ready to use. Appropriate extenders are then thawed before use.

3.2.2 Preparation of Semen Sample

Before semen collection, the ewe was synchronized estrus by inserting CIDR into its vagina 15 days before semen collection. After two weeks, the CIDR was removed. At the following day, the ewe was checked for estrus symptoms like swollen and pink vulva, transparent moisture discharge and heat behavior. To ensure the sperm quality is not affected by external factors like sudden temperature drop or contamination, a well-trained ram is summoned to the semen collection site and restrained well, the ewe acts as a teaser and then the semen is collected by using AV and quickly kept in a boiling tube covered with aluminum foil and sun-block protector. The sheep semen was separated into two samples, marked as fresh semen and will-be-extended semen.

3.2.3 Extension of Sheep Semen

The extenders were thawed inside the water bath machine at 37°C. All the glass slides and other glass ware were prepared on the heater platform at 37°C, too. A micropipette was used to measure out 90 µl of continental diluent into a falcon tube then mixed with 10 µl of semen to make a ratio of 9:1 (dilute the semen by 10 times). Immediately drop a small drop of extended semen on the glass slide for microscopic examination. The steps were repeated for other types of extenders. Then the tubes of extended semen are submerged into a beaker with room temperature water before chilled in the chiller at 0°C. There were total of 9 extended semen samples to be stored for 3 periods: 2 hours, 1 day and 2 days.

3.2.4 Analysis of Results

Before analysis of results, both fresh semen and the extended semen were thawed at 37°C for 2 minutes. With a micropipette, a small drop of semen and extended semen was placed on a pre-warmed slide and covered with a cover slip respectively, all the while on a slide warmer at 37°C. The slides were then observed under 400 X, and at least five areas of the smear are used to estimate the percent of motile sperm. All of the slides were estimated to the nearest five percent for total motility. More abnormal sperm will be found near the edge of the slide. It is most probably due to cold shock and drying out of the preparations. In this research, chicken egg yolk extender acts as the control to be compared with other extenders. Examine all the samples with Sperm Class Analyser (SCA) in terms of sperm count, morphology, general motility, concentration, volume and so on. The data was obtained and plotted into tables accordingly to enable further comparison and analysis.

CHAPTER 4

RESULTS

4.1 Characteristics of Fresh Semen

Table 4.1 shows the volume and characteristics of fresh semen. Semen was collected two times and its volume was about 1.64 ml containing 11.5 billion sperms. The sperm concentration was 7.01 billion/ml with 89% general motility and 72% progressive motility. The semen quality was good with high sperm viability up to 96%.

Table 4.1: Volume and characteristics of fresh semen

Parameter	Value
Semen volume (ml/ejaculation)	0.82
Sperm concentration (× 10^9 / ml)	7.01
Sperm general motility (%)	89
Sperm progressive motility (%)	72
Sperm viability (%)	96

Table 4.2 shows the sperm morphology of fresh semen. It was observed that about 67.44% sperm were normal, whereas the rest (32.56%) sperm were abnormal consists of 26.74% of curled tail, 3.49% tailless, 1.17% proximal droplet and 1.16% distal droplet (Figure C.1; see Appendix)

Table 4.2: Sperm morphology of fresh semen

Sperm Morphology	**Value**
Normal (%)	67.44
Abnormal	
Curled tail (%)	26.74
Tailless (%)	3.49
Proximal droplet (%)	1.17
Distal droplet (%)	1.16

4.2 Parameters of Chilled Semen with Different Extenders

Table 4.3 shows the sperm concentration (× 10^9 / ml) of chilled semen with different extenders. Semen was chilled for three periods (two hours, one day and two days). It was observed that there was no specific trend on sperm concentration among three different periods of chilling.

After chilling for two hours, chicken egg yolk extended semen had the highest sperm concentration of 1.293 × 10^9 / ml whereas the quail egg yolk with stingless bee honey extended semen was the lowest of 0.3209 × 10^9 / ml.

After chilling for one day, chicken egg yolk extended semen had the highest sperm concentration of 1.746 × 10^9 / ml whereas the quail egg yolk extended semen was the lowest of 0.5531 × 10^9 / ml.

After chilling for two days, duck egg yolk extended semen had the highest sperm concentration of 0.9512 × 10^9 / ml whereas the quail egg yolk with stingless bee honey extended semen was the lowest of 0.2073 × 10^9 / ml.

Table 4.3: Sperm concentration ($\times 10^9$/ml) of chilled semen with different extenders

Parameter	Semen Extender								
	C	Q	D	C+H	Q+H	D+H	C+SH	Q+SH	D+SH
Chilled semen (after 2 hours)	1.293	0.6571	0.9270	1.127	1.218	0.8840	0.7078	0.3209	0.7411
Chilled semen (after 1 day)	1.746	0.5531	1.421	1.150	1.484	0.8504	0.8758	0.6895	0.6760
Chilled semen (after 2 days)	0.6484	0.9013	0.9512	0.5541	0.2076	0.4401	0.7882	0.2073	0.5410

C: chicken egg yolk (control)

Q: quail egg yolk

D: duck egg yolk

H: honey

SH: stingless bee honey

Table 4.4 shows general motility (%) of chilled sperms with different extenders. Semen was chilled for three periods (two hours, one day and two days). It was observed that there was no specific trend on general motility among three different periods of chilling.

After chilling for two hours, quail egg yolk with honey extended semen had the highest general motility of 86% whereas the quail egg yolk extended semen was the lowest of 27%.

After chilling for one day, duck egg yolk extended semen had the highest general motility of 63% whereas the duck egg yolk with stingless bee honey extended semen was the lowest of 23%.

After chilling for two days, chicken egg yolk extended semen had the highest general motility of 48% whereas the duck egg yolk with stingless bee honey extended semen was the lowest of 6%.

Table 4.4: General motility (%) of chilled sperms with different extenders

Parameter	Semen Extender								
	C	Q	D	C+H	Q+H	D+H	C+SH	Q+SH	D+SH
Chilled semen (after 2 hours)	80	27	48	78	86	72	74	56	72
Chilled semen (after 1 day	45	50	63	31	29	27	28	30	23
Chilled semen (after 2 days)	48	45	29	36	12	37	17	20	6

C: chicken egg yolk (control)

Q: quail egg yolk

D: duck egg yolk

H: honey

SH: stingless bee honey

Table 4.5 shows the progressive motility (%) of chilled sperms with different extenders. Semen was chilled for three periods (two hours, one day and two days). It was observed that there was no specific trend on progressive motility among three different periods of chilling.

After chilling for two hours, quail egg yolk with honey extended semen had the highest progressive motility of 67% whereas the quail egg yolk extended semen was the lowest of 16%.

After chilling for one day, duck egg yolk extended semen had the highest progressive motility of 33% whereas the duck egg yolk with stingless bee honey extended semen was the lowest of 9%.

After chilling for two days, quail egg yolk extended semen had the highest progressive motility of 30% whereas the quail egg yolk with honey extended semen was the lowest of 0%.

Table 4.5: Progressive motility (%) of chilled sperms with different extenders

Parameter	Semen Extender								
	C	Q	D	C+H	Q+H	D+H	C+SH	Q+SH	D+SH
Chilled semen (after 2 hours)	44	16	27	57	67	56	61	44	59
Chilled semen (after 1 day	26	25	33	13	16	16	13	23	9
Chilled semen (after 2 days)	20	30	16	12	0	12	4	19	3

C: chicken egg yolk (control)

Q: quail egg yolk

D: duck egg yolk

H: honey

SH: stingless bee honey

Table 4.6 shows the viability (%) of chilled sperms with different extenders. Semen was chilled for three periods (two hours, one day and two days). It was observed that there was no specific trend on viability among three different periods of chilling.

After chilling for two hours, chicken egg yolk extended semen had the highest viability of 97% whereas the quail egg yolk extended semen was the lowest of 48%.

After chilling for one day, duck egg yolk extended semen had the highest viability of 86% whereas the duck egg yolk with stingless bee honey extended semen was the lowest of 46%.

After chilling for two days, chicken and quail egg yolk extended semen had the highest viability of 67% whereas the duck egg yolk with stingless bee honey extended semen was the lowest of 9%.

Table 4.6: Viability (%) of chilled sperms with different extenders

Parameter	Semen Extender								
	C	Q	D	C+H	Q+H	D+H	C+SH	Q+SH	D+SH
Chilled semen (after 2 hours)	97	48	71	92	93	85	85	69	83
Chilled semen (after 1 day	78	65	86	50	64	50	49	55	46
Chilled semen (after 2 days)	67	67	65	61	37	55	61	23	9

C: chicken egg yolk (control)

Q: quail egg yolk

D: duck egg yolk

H: honey

SH: stingless bee honey

CHAPTER 5

DISCUSSION

5.1 Collection and Extension of Fresh Semen

The Malin ram was summoned to the semen collection site to be collected semen in the morning to minimize stress to the male donor and gave extra time for laboratory work. According to Nel-Themaat et al. (2006) there will be higher motility in the second ejaculate compared to the first, thus in this research the ram was collected two times with 24 hours interval. The first ejaculation was tested with low motility although the concentration of sperms was very high. Thus at the following day, the ram was collected semen again and the result is better with higher motility as stated in result part. Gündoğan (2007) stated that the season, ram's age and breed as well as the frequency of ejaculation can affect the semen parameters like volume, concentration, motility, normal and abnormal sperm. To ensure the uniformity and higher credibility result, the ram named Jack from IBVK which is three years old, healthy and only collected semen by using artificial vagina in the morning at around 9 o'clock. Kaya et al. (2002) also discouraged to collect semen from the same ram in short time because of the motility of sperms is decreased with increasing the number of ejaculations. Jack was collected semen two times with interval of 15 minutes.

Before extension, the semen in the tube was protected by aluminum foil all the time because the spermatozoa are sensitive to sunlight (Louis C. Nuti, 2016). In IBVK, the semen protector was used which was specially designed to prevent any cold shock and direct sunlight penetration (Figure B.3; see Appendix) throughout the transportation from semen collection site to the laboratory. The extenders were thawed in the water bath machine whereas all apparatus to be used are warmed on

the heater platform (Figure B.4 and B.5; see Appendix) with temperature about 35°C to 37°C to analyze the parameters as needed.

5.2 Characteristics and Morphology of Fresh Semen

This Malin ram gave 1.64 ml semen after two ejaculations and it contained 11.5 billion sperms with sperm concentration of 7.01 billion sperms per ml. It gave about 0.82 ml per ejaculation. The collected semen was about 2.5 times more than ordinary Malin sheep semen which is only 0.32 ml (Musaddin et al, 1993). The collected semen had extremely high motility of 89% which is higher than 30% to 70% as stated in Sheep Production Handbook (2002). The percentage of normal sperms was 67.44% which is also higher than 30% to 50% as stated in the book. Normal sperms were distinguished under observation of microscopic sperm morphology using nigrosine eosin stain technique (Figure C.1; see Appendix)

5.3 General Comparison among the Performance of Different Extenders in Chilling Semen for Two Hours

Comparison between the chilled semen for two hours with different extenders, quail egg yolk with honey is the best in terms of concentration, general motility, progressive motility and viability. From Table 4.4 and 4.5, it is obvious to find quail egg yolk with honey shows best result in terms of motility which is 86% general motility and 67% progressive motility. Its sperm concentration and viability were also very high at 1.218×10^9 / ml and 93% respectively. However, the chilled semen with only quail egg yolk extender shows worst result in terms of general motility, progressive motility and viability with 27%, 16% and 48% respectively. Its sperm concentration was also low with only 0.6571×10^9 / ml.

From Table 4.4, 4.5 and 4.6, strong relationship between general motility, progressive motility and viability can be observed. If the sperm viability is high, the general motility and progressive motility would be high as well. This is due to the nature of incomplete activation of sperms after released from male genital tracts and they will undergo further modification when entering female reproductive tracts (Leyla, 2015). Higher sperm viability will more likely to have higher motility and progressive motility as well since the survival rate of sperms is high.

Besides, since extended semen would be packaged into 0.25 ml straw for AI, these extended semen should have target sperm concentration of 1.0×10^9 / ml to 1.6×10^9 / ml (depends on ram breed) for better chance of impregnating the ewes (Ingrid et al, 2015), then chicken egg yolk and quail egg yolk with honey extenders will be the ideal extenders to chill semen for two hours.

5.3.1 Effects of Sugar Content and Viscosity of Different Extenders

Quail egg yolk extender shows worst results at early stage of chilling might due to its less nutritive properties compared to other egg yolks (Hannah, 2012), quail egg yolk has no sugar which is essential as the energy source for sperms to stay motile. The sperms in the extender have to depend on the fructose added during preparation of extenders as stated in Method 3.2.1. Thus the extender that contains quail egg yolk with honey or stingless bee honey performs better.

Duck egg yolk extender also performed less effective without addition of honey or stingless bee honey. Although the sugar content in duck egg yolk is the highest among three egg yolks (Figure A.1, A.2 and A.3; see Appendix), duck egg yolk has the highest viscosity, too. Addition of honey or stingless bee honey helps to lower the viscosity and enable the sperms to have better motility.

5.4 General Comparison among the Performance of Different Extenders in Chilling Semen for One Day

From Table 4.4, 4.5 and 4.6, overall decreasing in terms of general motility, progressive motility and viability can be observed in all chilled semen except for quail egg yolk and duck egg yolk extended semen. Both of them show increasing in stated semen parameters. This makes duck egg yolk extended semen best with performance of 63% general motility, 33% progressive motility, 86% viability and sperm concentration of 1.421×10^9 / ml. However, duck egg yolk with stingless bee honey shows worst performance of 23%, 9% and 46% respectively.

5.4.1 Effects of Cholesterols in Different Egg Yolks Extenders

According to Pace (1974), low-density lipoproteins (LDLs) in the egg yolk is the major factor that contributes to sperms protection. After years of research, Quinn et al. (1980) suggested that LDL helps to form protective film after binding with sperm membrane. Foulkes et al. (1980) even believed that LDL can help to replacing the lost phospholipids of the sperm membrane surface. Thus, the higher amount of cholesterol in quail egg yolk and duck egg yolk than chicken egg yolk (Figure A.1, A.2 and A.3; see Appendix) shall offer more LDL to protect the sperms for longer period.

5.4.2 Effects of Honey and Stingless Bee Honey in Different Extenders

However the addition of honey and stingless bee honey make a massive drop in all terms of semen parameters indicating honey and stingless bee honey are not suitable to be used as supplement to chill the semen for more than one day. It might be caused by inappropriate properties of honey that kill the sperms. The pH of honey ranges about 3.4 to 6.1 with average of 3.9 (Rusty B., 2011), it decreases the semen

pH and at the same time the semen itself also producing lactic acid that causes the shift of pH (Louis, 2016). Therefore, the longer time of chilling, the lower the pH value of semen, the less the viability of sperms will be since the sperms survive optimally at the neutral pH condition.

5.5 General Comparison among the Performance of Different Extenders in Chilling Semen for Two Days

From Table 4, 5, 6 and 7, the performance of all extenders decrease in terms of all semen parameters. However, chicken egg yolk and quail egg yolk extenders still performed in acceptable results. Quail egg yolk extended semen had good result of 45% general motility, 30% progressive motility, 67% viability and sperm concentration of 0.9013×10^9 / ml. Duck egg yolk with stingless bee honey extender performed worst result of 6%, 3%, 9% and 0.5410×10^9 / ml respectively.

5.5.1 The Relationship between Cholesterol and Honey that affects the Performance of Different Extenders

Honey contains very high concentration of sugar, especially fructose and glucose which can contribute to antimicrobial actions due to its high osmolality that can inhibit the microbes' growth (Lusby et al, 2005). Although honey provides sufficient amount of sugar to be used as the energy source for sperms and contains various antioxidants and minor amount of minerals (Kris, 2013), the antioxidants can cause decreasing of LDL in cholesterol and found the ability to elevate HDL slightly (Al-Waili NS., 2004). Decreasing of LDL is damaging the sperms as the sperms are no more protected by the film formed by LDL when binding to the sperm membrane surface.

Besides, when the chilling period is longer, the glucose in honey starts to precipitate into solid granules (Figure C.4; see Appendix). Honey is a super cooled liquid, it will not freeze at very low temperatures but its viscosity increases as well when temperature is getting colder. Honey becomes thick and condensed when the temperature is dropping (Muhammad et al, 2014). This makes honey is not suitable for chilling as the temperature is maintained at 0°C to 4°C and it precipitates easily causing massive drop in sperm motility.

Honey might be suitable to be added as supplement in the extenders that propose to cryopreserve the sperms since it has good amount of sugar and antioxidants to inhibit microbial growth and provide sufficient energy for the sperms. According to Hu et al. (2008), the content of sucrose in honey is proved to be cryoprotective as well.

CHAPTER 6

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

From this study, quail egg yolk was found to be the most appropriate ingredient to replace chicken egg yolk in sheep semen extender in term of chilling for more than one day. It may due to higher amount of cholesterol in quail egg yolk which the LDL is essential to protect the sperm cells from damage as it forms protective film when binding to the sperm membrane surface.

Supplementation of honey in sheep semen extenders was better in chilling semen of less than one day, especially added to quail egg yolk extender due to its lack of sugar property. Honey provides high amount of sugar which is good as the energy source to the sperms and provide antimicrobial growth environment. Due to its low pH at about 3.9 and super cooled liquid properties, honey is not suitable to supply in the chilling semen that is more than one day and with temperature of 0°C to 4°C. Honey forms acidic condition and precipitates that kill sperms and influence badly of sperm motility.

This study has successfully shown that different egg yolks can affect the chilled semen quality due to the varied nutritive values like sugar, cholesterol, protein and so on. Besides, this study has found out that honey has direct influence to the semen quality as well in terms of storage duration and egg yolk types. Supplementation of honey was found to improve the lack of sugar in quail egg yolk as well as improving the semen quality in short chilling period.

6.2 Recommendation

To improve this field of study, other related studies such as the effects of skimmed milk, soya beans milk and fruits on the post thawing semen quality can be studied due to their high protein content and high vitamins properties. These supplements can be tested in different combinations to find out the better portions to obtain better semen quality results.

Besides, from this study there would be a better improvement by studying on the effects of different concentration of honey and egg yolk to find out the better portions. Various concentrations might show different level of effectiveness at different semen storage durations and method of storage. Due to lack of time and resources, this study was conducted with only one concentration and three periods of storage duration with one method: chilling.

A better result which is more reliable and precise shall be obtained by practicing more tests on semen parameters like pH of semen and sperm movement velocity (mass movement). Moreover, the extenders should be tested with different breeds of animal to find the best-suited. However, every study should have a constant and should be focus on certain variables that are more likely to contribute to this field.

To minimize the difficulties that are potentially encountered in this study, a site that is well-equipped and available should be ready upon the kick-start of study. All the facilities such as CASA, semen collection site, semen processing site and other equipment are extremely important to ensure the smoothness of research conduction. Semen collection shall be conducted by experienced crew as it is a high skill requiring process. All semen processing procedures need to be done with extreme care and patience because dealing with cells is not easy and very sensitive.

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APPENDIX A:

Egg Nutrition Facts

Table A.1: The nutrients found in egg yolk

Nutrient	Yolk	% Total in Yolk
Protein	2.7g	43%
Fat	4.5g	99%
Calcium	21.9 mg	90.5%
Magnesium	0.85 mg	19.2%
Iron	0.4 mg	93.8%
Phosphorus	66.3 mg	93%
Potassium	18.5 mg	25.6%
Sodium	8.2 mg	13%
Zinc	0.4 mg	99.8%
Copper	0.013 mg	62%
Manganese	0.009 mg	69.2%
Selenium	9.5 mcg	59%
Thiamin	0.03 mg	96.8%
Riboflavin	0.09 mg	48.3%
Niacin	0.004 mg	9.3%
Pantothenic acid.	0.51 mg	89%
B6	0.059 mg	96.7%
Folate	24.8 mcg	95%
B12	0.331 mcg	91.7%
Vitamin A	245 IU	100%
Vitamin E	0.684 mg	100%
Vitamin D	18.3 IU	100%
Vitamin K	0.119 IU	100%
DHA and AA	94 mg	100%
Carotenoids	21 mcg	100%

Figure A.1: The nutrients found in chicken egg

Nutrition Facts

Serving Size 1 egg (50g)

Amount Per Serving

Calories 78

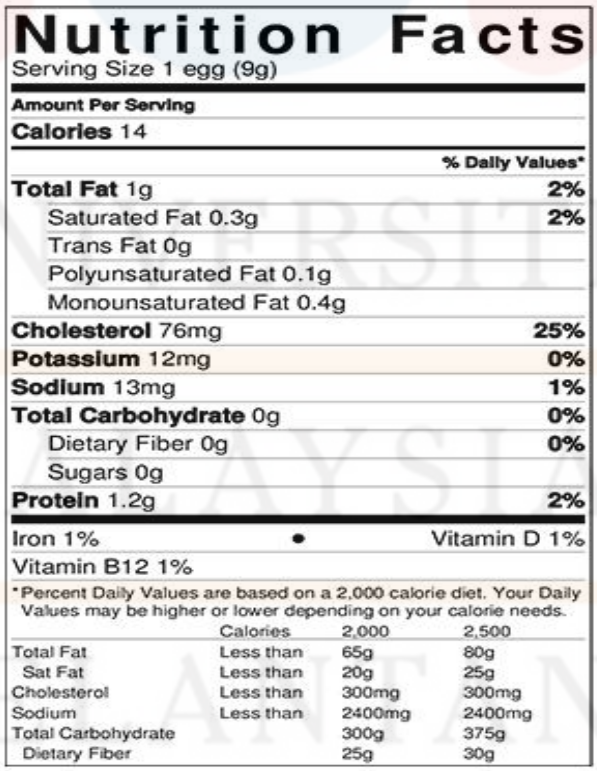
	% Daily Values*
Total Fat 5g	**8%**
Saturated Fat 1.6g	**8%**
Trans Fat 0g	
Polyunsaturated Fat 0.7g	
Monounsaturated Fat 2g	
Cholesterol 211mg	**70%**
Potassium 63mg	**2%**
Sodium 70mg	**3%**
Total Carbohydrate 0.6g	**0%**
Dietary Fiber 0g	**0%**
Sugars 0.6g	
Protein 6g	**12%**

Vitamin A 5%	•	Calcium 2%
Iron 3%	•	Vitamin D 11%
Vitamin B6 5%	•	Vitamin B12 10%
Magnesium 1%		

*Percent Daily Values are based on a 2,000 calorie diet. Your Daily Values may be higher or lower depending on your calorie needs.

	Calories	2,000	2,500
Total Fat	Less than	65g	80g
Sat Fat	Less than	20g	25g
Cholesterol	Less than	300mg	300mg
Sodium	Less than	2400mg	2400mg
Total Carbohydrate		300g	375g
Dietary Fiber		25g	30g

Figure A.2: The nutrients found in quail egg



Nutrition Facts

Serving Size 1 egg (9g)

Amount Per Serving

Calories 14

	% Daily Values*
Total Fat 1g	**2%**
Saturated Fat 0.3g	**2%**
Trans Fat 0g	
Polyunsaturated Fat 0.1g	
Monounsaturated Fat 0.4g	
Cholesterol 76mg	**25%**
Potassium 12mg	**0%**
Sodium 13mg	**1%**
Total Carbohydrate 0g	**0%**
Dietary Fiber 0g	**0%**
Sugars 0g	
Protein 1.2g	**2%**

Iron 1%	•	Vitamin D 1%
Vitamin B12 1%		

*Percent Daily Values are based on a 2,000 calorie diet. Your Daily Values may be higher or lower depending on your calorie needs.

	Calories	2,000	2,500
Total Fat	Less than	65g	80g
Sat Fat	Less than	20g	25g
Cholesterol	Less than	300mg	300mg
Sodium	Less than	2400mg	2400mg
Total Carbohydrate		300g	375g
Dietary Fiber		25g	30g

Figure A.3: The nutrients found in duck egg

Nutrition Facts

Serving Size 1 egg (70g)

Amount Per Serving

Calories 130	Calories from Fat 87
	% Daily Values*
Total Fat 9.64g	**15%**
Saturated Fat 2.58g	**13%**
Trans Fat 0g	
Cholesterol 619mg	**206%**
Potassium 155.4mg	**4%**
Sodium 102mg	**4%**
Total Carbohydrate 1.01g	**0%**
Dietary Fiber 0g	**0%**
Sugars 0.65g	
Protein 8.97g	**18%**

Vitamin A 9% • Calcium 4%

Iron 15%

*Percent Daily Values are based on a 2,000 calorie diet. Your Daily Values may be higher or lower depending on your calorie needs.

	Calories	2,000	2,500
Total Fat	Less than	65g	80g
Sat Fat	Less than	20g	25g
Cholesterol	Less than	300mg	300mg
Sodium	Less than	2400mg	2400mg
Total Carbohydrate		300g	375g
Dietary Fiber		25g	30g

APPENDIX B:

Semen Collection and Processing

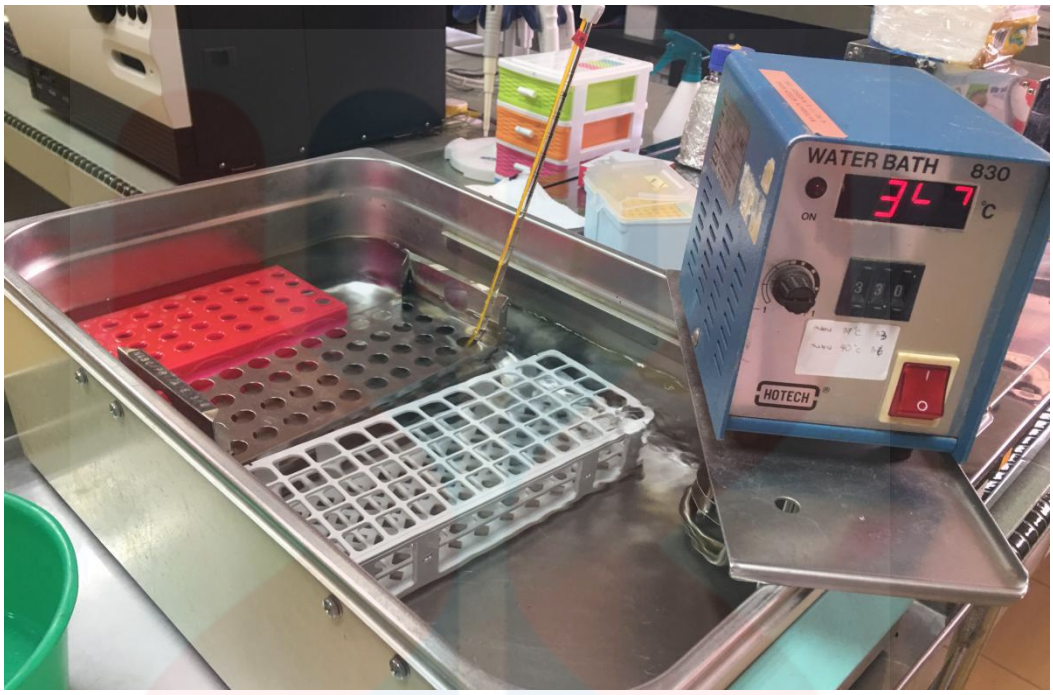
Figure B.1: The male sperm donor with teaser

Figure B.2: Artificial vagina

Figure B.3: Protection of collected semen

Figure B.4: Heater platform

Figure B.5: Water bath machine



APPENDIX C:

Morphology and Live Image of Sperms

Figure C.1: Morphology of Sperms

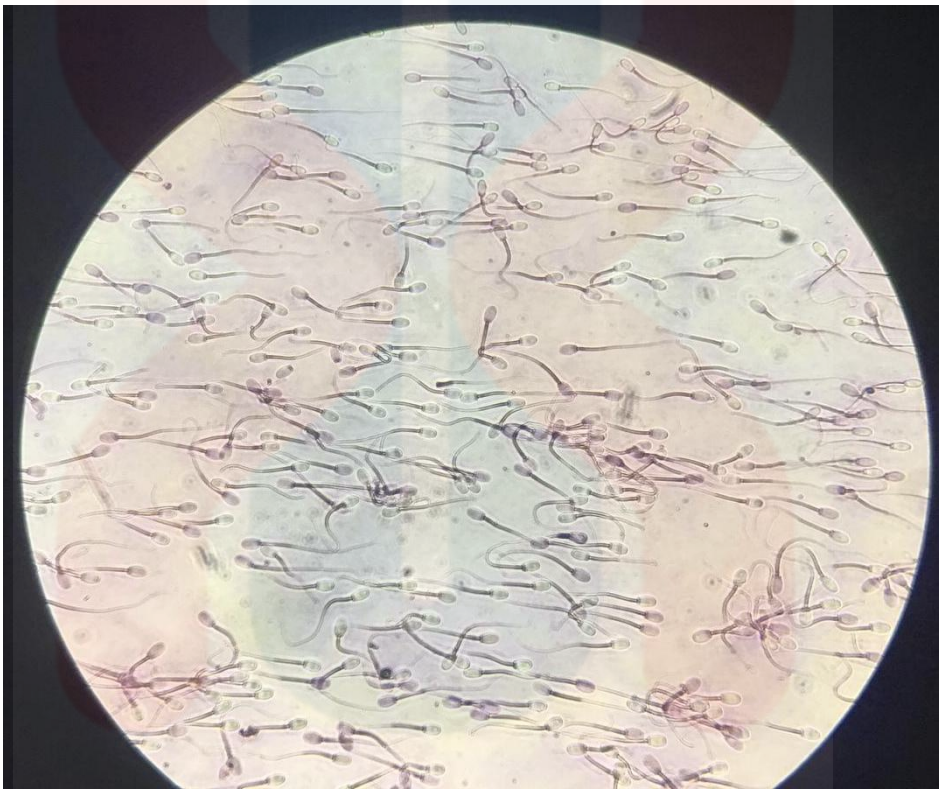


Figure C.2: Live Image of Active Sperms

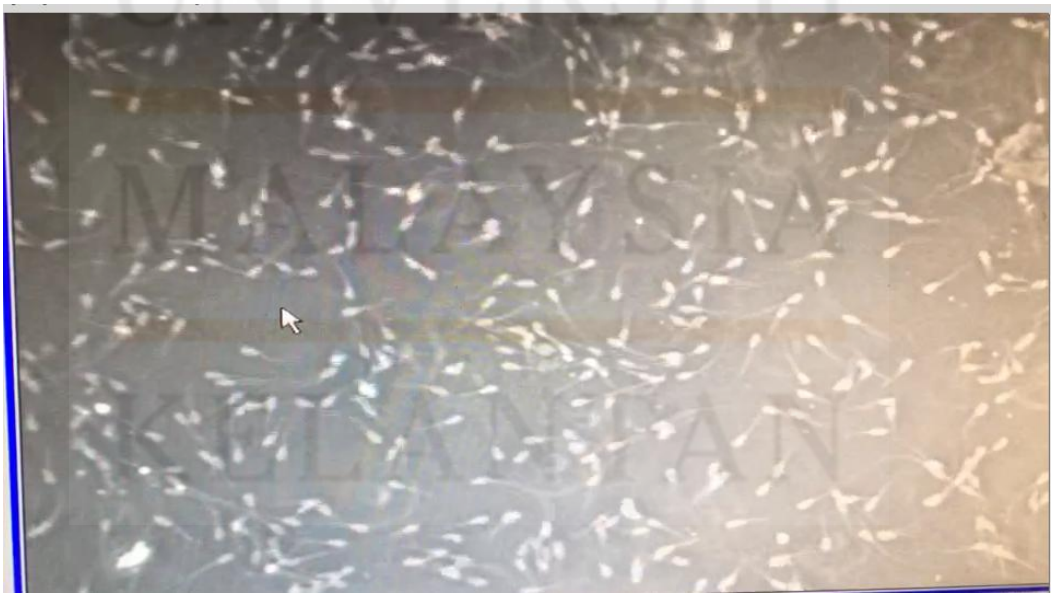


Figure C.3: Live Image of Static Sperms

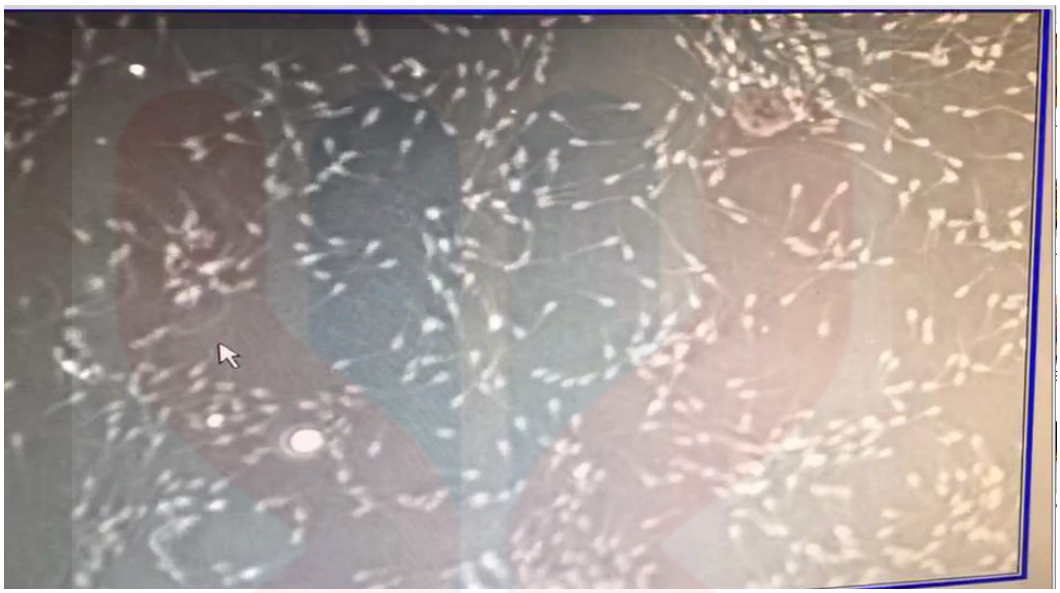


Figure C.4: Granules in semen

APPENDIX D:

Figures

Figure D.1: Semen extenders

Figure D.2: Filter the egg yolk

Figure D.3: Measurement

Figure D.4: Chemicals used in preparation of extender