UNIVERSITI MALAYSIA KELANTAN

Use of Coconut Water (*Cocos nucifera*) As a Semen Extender

By

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

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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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Date:

I certify that the report of this final year project entitled “Use of Coconut Water (*Cocos nucifera*) As a Semen Extender” by Faqrul Adam bin Zakaria, matric number F14A0059 has been examined and all the correction recommended by examiners have been done for the degree of Bachelor of Applied Science (Animal Husbandry Science) with Honours, Faculty of Agro-Based Industry, Universiti Malaysia Kelantan.

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Use of Coconut Water (*Cocos nucifera*) as a Semen Extender

ABSTRACT

Artificial insemination is a vital genetic improvement in this modern livestock practices and it is just conceivable with established effective extender. Extender is the main key in the conservation of sperm cell. The aim of this study was to use different stages of coconut water as a component in the preparation of the semen extender to replace egg yolk. Three different treatments were used as young coconut water, old coconut water, and egg yolk as control. The semen was collected by Artificial Vagina method and diluted with two different stages (either young or old) of coconut water and stored in a chiller at 4^{o}C. The semen quality of ram was evaluated using Computer Assisted Semen Analysis (CASA). It was observed that egg yolk and young coconut water showed similar (61% vs. 62%) result for progressive motility, but it was differed significantly ($p < 0.05$) with old coconut water (47%). However, there was significant difference ($p < 0.05$) in all treatments for overall motility and egg yolk showed higher motility (88%) followed by young coconut water (81%) and old coconut water (70%). In conclusion, young coconut water may be used as an extender to replace egg yolk in terms of progressive motility.

Keywords: Extender, Semen preservation, Coconut water *(Cocos nucifera),* Semen quality.

Penggunaan Air Kelapa (*Cocos nucifera*) sebagai Penyimpan Sperma

ABSTRAK

Permanian beradas adalah peningkatan genetik yang penting dalam amalan ternakan moden ini dan ia hanya dapat dilihat dengan penyimpan berkesan yang mantap. Penyimpan adalah kunci utama dalam pemuliharaan sel sperma. Tujuan kajian ini adalah untuk menggunakan pelbagai peringkat air kelapa sebagai komponen dalam penyediaan penawar air mani untuk menggantikan kuning telur. Tiga rawatan yang berbeza digunakan sebagai air kelapa muda, air kelapa lama, dan kuning telur sebagai kawalan. Air mani dikumpulkan melalui kaedah Vagina Buatan dan diencerkan dengan dua peringkat yang berbeza (sama ada muda atau tua) air kelapa dan disimpan dalam penyejuk pada 4^{o}C. Kualiti mani bebiri jantan dinilai menggunakan Analisis Semen Bantuan Komputer (CASA). Telah diperhatikan bahawa kuning telur dan air kelapa muda menunjukkan sama (61% vs 62%) untuk motilitas progresif, tetapi ia berbeza dengan nyata ($p < 0.05$) dengan air kelapa tua (47%). Walau bagaimanapun, terdapat perbezaan yang signifikan ($p < 0.05$) dalam semua rawatan untuk motilitas keseluruhan dan kuning telur menunjukkan motilitas yang lebih tinggi (88%) diikuti oleh air kelapa muda (81%) dan air kelapa tua (70%). Sebagai kesimpulan, air kelapa muda boleh digunakan sebagai extender untuk menggantikan kuning telur dari segi motilitas progresif.

Kata kunci: Penyimpan, pemeliharaan sperma, Air kelapa *(Cocos nucifera),* kualiti sperma.

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AI	Artificial Insemination
AV	Artificial Vagina
CASA	Computer Assisted Semen Analysis
BCM	Bovine Cervical Mucus
LDL	Low Density Lipid
ROS	Reactive Oxygen Species
^{o}C	Degree Celcius
mL	Millilitre
%	Percentage

CHAPTER 1

INTRODUCTION

1.0 Research Background

Sperm quality effect on fertility has been documented in many research studies in all over the countries. Mating success is related on both quantity and quality of semen reached to the female (Nebel *et al*., 1994). Males vary not just in fertility at all inseminate dosages, but also in the least amount of sperm per inseminate needed to obtain optimum fertility. Thus, two criteria of a male's semen quality become influential which is compensable, where inflating sperm dosage to the female results in a response in fertility, and that which is incompensable, where differences in fertility in males can no longer be detached by increasing dosage of sperm (Saacke, 2008).

Semen compensable traits are those known to be related with the incapability of sperm to reach the fertilization site. Incompensable traits come out to be related with the incapability of fertilizing sperm to support the process of fertilization and growth of the early embryo. It is now crucial in the evaluation of semen and the male that these two criteria of reproductive insufficiency be reviewed individually. A lot of evidence proved that less sperm amount at the fertilization site (less competition between sperm), due to

either male problems (inseminator) or female origin, may favor fertilization by low competent sperm that may be unable to complete the process of fertilization or support the young embryo. (Saacke *et al*., 1994).

Identification of the factors which affect the spermatic quality of the semen has a major importance in improvement of fertility (Sheweita *et al*., 2005). One of the factors is the effectiveness of the extender used in preservation of semen. Extenders capacity to permit various inseminations from a single ejaculate. In expansion, the extender must give temperature security for sperm and decrease the metabolic rate of sperm cells in cool storage. The extender capacities to allow membrane stabilization in cool temperatures, energy source for sperm metabolism, pH buffering from sperm cell waste, particles for layer and cell adjust, and anti-infection agents to anticipate development of organisms that can lead to disease and compete for nutrients (Weitze *et al*., 1992).

Despite variety past efforts to enhance diluents of semen, only several different additives have been established in the semen extender constitution (Yániz *et al*., 2005). Components of biological like egg yolk or milk in the diluent have not really been productive in the applied use for AI (López *et al*., 2000). The primary components of semi-synthetic diluents for the semen liquid storage (buffers combined with egg yolk and sugars), have altered a bit since introduced (Maxwell and Salamon, 1993).

Coconut water with its numerous approaches, is one of the world's most all-round natural product. It is beneficialas it is nutritious and useful for health. There is growing scientific proof that aids the part of coconut water in both health and medicinal approaches. Traditionally, coconut water is used as a supplement of growth in tissue culture of plant. The wide implementations of coconut water can be rationalized by its special properties (Yong *et al*., 2009).

In this study, coconut water used as a component in the preparation of the semen extender. The variable that used was coconut water from young and old coconut which were at different growth stages. The objective was to measure the effects of the two different stages of coconut water on ram semen quality. By this, the utilization of abundant source coconuts in Malaysia could be applied within this study.

1.1 PROBLEM STATEMENT

The use of coconut water is very wide in a lot of studies, medical, development, and many others. However, little is known about the roles of coconut water as extender in the preservation of semen. Thus, studying the effect of semen quality using the coconut water of different stages, mainly young and old as an extender would be very important as the vary composition and suitability of coconut water in term of its content is very promising. The other concern that lead to this study was the common extender used (egg yolk) has risk of contamination with bacteria and possible endotoxins source which capable of harming the fertility prospective of spermatozoa (Pillet et al., 2011).

Egg yolk might also discourage respiration of spermatozoa or lower their motility rate (Amirat *et al*., 2004). Considering these disadvantages, there was a significant need to replace whole egg yolk by its cryoprotective fraction.

1.2 HYPOTHESIS

The use of young stage coconut water was more suitable and effective compared to old stage coconut water as an extender, which resulted in better quality of ram semen preservation in term of motility and progressive.

1.3 OBJECTIVES

1. To measure the effects of two different stages of coconut water on ram semen quality.

1.4 SCOPE OF STUDY

This research focused on the use of coconut water at two different stages as an extender on ram semen quality.

1.5 SIGNIFICANCE OF STUDY

The main aim of this study was to apply the use of coconut water as an extender ingredient in preserving the semen of ram. The project focused on the coconut water as it was economic and cheaper. Even though there were many other extenders ingredient which are well known their capability in preserving semen, but using coconut water as a new source was considered as an alternative as it was a common fruit, cheap, and easy to get in Malaysia.

The uses of coconut water were well understood at many stages. However, knowledge of its use on preserving semen was still at unsatisfying level. This research involved the quality of the semen in term of motility, progressive, sperm count, and many other factors. Study on the role of coconut water as a natural extender might allow the improvement and maximize the use of coconut water which is abundant in Malaysia.

1.6 LIMITATION OF STUDY

The only limitation in this study was the insufficient of ram and semen analyzer in Universiti Malaysia Kelantan (UMK) that lead to lack of source to collect and evaluate the semen.

CHAPTER 2

LITERATURE REVIEW

2.1 ARTIFICIAL INSEMINATION

Artificial insemination refers to the process of collecting semen from a male animal and place it into the reproductive tract of a female manually. The application of Artificial Insemination were practiced mainly of livestock animals such as cattle, goat, sheep, swine, and many others (Seager *et al*., 1980). There are a lot of benefits those can be cited from the use of Artificial Insemination (Vishwanash, 2003).

2.1.1 HISTORY OF ARTIFICIAL INSEMINATION

Artificial Insemination (AI) was applied to enhance reproduction and genetics of farm animals (Foote, 2002). It made a massive impact worldwide in many species, particularly in dairy cattle. The acknowledgement of Artificial Insemination technology all around the world provided the key for improvement of other technologies, such as

cryopreservation and sexing of sperm, estrous cycle regulation, and embryo harvesting, freezing, culture and transfer, and cloning (Walton, 1993).

So far, successful methods of sire evaluation were broaden. The development history of Artificial Insemination was reviewed, in which the effect on genetic improvement and control of various diseases have been prominent (Roca *et al*., 2006). Other species roughly included are horses, goats, swine, sheep, rabbits, poultry, and endangered species (Brackett *et al*., 1981). In 1980, a survey showed the number of total Artificial Inseminations used globally exceeded 130 million (Bonadonna and Succi, 1980).

Major research and work in Artificial Insemination improvement were recorded, along with the individuals related to these developments. Many of these people helped to enhance as well evolve a new batch of biotechnologists. Many of the sources bring the researchers and readers back to the time before networks of electronic were available, so these former studies will not be found like nowadays. Readers are encouraged to traverse these studies that have provided a springboard for the future (Bonadonna and Succi, 1976).

Until now, Artificial Insemination is still among the most important assisted reproductive technologies. Three main reasons for its application are it is economical, probability of success is high, and simple. The significance of Artificial Insemination will be challenged in the next few times. The remarkable advance made in other assisted reproductive technologies improvement do have the potential to produce offspring rapidly.

2.1.2 APPLICATIONS AND BENEFITS OF ARTIFICIAL INSEMINATION

Artificial insemination is the process of collecting sperm cells from a male animal and depositing them into the reproductive tract of a female manually. The Artificial Insemination approach were practiced mainly in livestock animals such as cattle, goat, sheep, swine, and many others (Seager *et al*., 1980). There are a lot of benefits those can be cited from the use of Artificial Insemination (Vishwanash, 2003).

For instance, it can increase bull usage efficiency. More semen will be deposited from male animal than is theoretically needed to induce pregnancy. Furthermore, natural breeding may cause tension and stress to animals. These two factors both limit the number of natural mating a male can undergo. However, semen that has been collected can be diluted and extended to produce much more doses, about hundreds doses from a single ejaculate (Salisbury *et al*., 1978).

Besides, semen can also be transported easily, making numerous female animals in vary areas to be inseminated at the same moment, and semen can be kept for longer periods of time (Foote, 1978), meaning that males can produce new generations even after their natural reproductive lives end (Foote, 1981).

Besides, it can increase genetic selection potential due to AI allows males to yield more offspring with less amount of males needed. Therefore, it can be chosen only the superior males to be used as parents, thus increasing the selection intensity (Saacke, 1978).

Males often grow to be bigger than females and can ingest relatively more amounts of feed. Also, males are often more stronger, powerful, and potentially ill-mannered and thus needed special housing and handling equipment (Harvey, 1978). Males can also become sensitive and aggressive. These factors mean that having a bull on a farm may be threatening.

More, due to the relatively bigger size of adult males than females, natural mating is more likely to cause accidents and harm to either the cow or the bull than is artificial insemination (Foote, 1980). With the application of Artificial Insemination, the cost and energy of having males can be reduced.

Applying Artificial Insemination can also reduce disease transmission (Bartlett, 1980). Natural mating permits the transmission of venereal diseases between females and males. Some disease caused microorganisms can be transmitted in semen through artificial insemination, but the process of semen collection allows for the screening of disease agents. Collected semen is also regularlyly checked for quality, which can help to prevent problems regarding male infertility (Everett, 1975).

2.2 SEMEN PRESERVATION

A lot of researches have been studied to enhance the livestock production. There are many countries those breed their livestock animals through manual sperm injection strategy (artificial insemination), but the application is not in a vast scale (Cebi Sen *et al*., 2015). The utilization of frozen semen technique (cryopreservation) is limited because of the decreasing of sperm viability during freezing process. There are many advantages in cryopreserving semen, one of which is that the good semen can be stored for a longer time, and filled its need in artificial insemination even after the absence of males.

By utilizing a Tris-based extender, semen has been cryopreserved, however the motility of the sperm is reduced after cryopreservation contrast with the freshly obtained semen (Ramukhithi *et al*., 2011). Good sperm quality will influence the next generation thus guaranteeing that both male and female parent are fertile will ensure a fertile descendants, consequently enhancing the productivity in livestock farming (Shikhet *et al*., 2014). Most regularly utilized extenders in goat semen cryopreservation are egg yolk or non-fat dried skim drain. But, semen diluents containing egg yolk or drain is hurtful to the sperm cells (Çebi Şen *et al*., 2015).

In 1951, the first calf created using cryopreserved semen was introduced (Stewart, 1951). Then, the skill and practice were used and established rapidly. Nowadays the modern industry of cattle globally is based on the utilization of artificial insemination and frozen semen. Quality males can create up to 60 000 doses of semen anually, and by that cryopreservation has granted exploitation of better sires and achieved fast, big-scale improvement of genetics in cattle stocks along with a decrease in sickness transference (Bartlett, 1980).

The chilled semen use for artificial insemination possesses prominent benefit. The strategy and plan must be simple and results must be at least equivalent to natural mating. These states have been achieved and success rates with frozen semen have matched rates from insemination with fresh semen and from natural mating in cattle. But for sheep, pigs and horses the situation is very different (Curry *et al*., 1990).

The situation in the industry of sheep varies in that it is the procedures and skills of artificial insemination that is confining with very low rate of success for trans-cervical insemination (Perez, 1996). When artificial insemination is applied for sheep, it is generally laparoscopic and repeatedtly utilizes frozen semen and good success rates are obtained (Mattner *et al*., 1969). However, surgical insemination is an obvious procedure of veterinary, which is expensive and not supported by welfare grounds.

2.2.1 CRYOPRESERVATION

Sperm cryopreservation and storage presently require fluid nitrogen or ultralow methods of refrigeration to both either long or short term storage which needs routine maintenance and care as well as broad space requirements. Conserving sperms has several purposes, for example, artificial insemination, clinical medication and species conservation (Aboagla and Terada, 2003). The combination of storage temperature, cooling rate, chemical composition of the extender, cryoprotectant concentration, reactive oxygen species (ROS), seminal plasma composition and hygienic control are the main components that effect the life expectancy of spermatozoa. Sperm conservation procedure vary among species of animals due to their natural particularities that change extenders utilization for refrigeration and freezing (Andrabi and Maxwell, 2007).

2.3 SEMEN COLLECTION

Semen collection involves the process of acquiring semen from male animals with the use of diverse procedure and method mainly for the purposes of artificial insemination or medical study. Semen can be collected via artificial vagina (AV) method, electro ejaculation, and manual manipulation (Gordon, 2004).

2.3.1 ARTIFICIAL VAGINA

Artificial vagina (AV) is used to obtain semen from many species counting cattle, sheep, goats, rabbits and even cats. It is compulsory to use of an artificial vagina only if the male conscious, not significantly frightened of people, and trained (Bredderman *et al*., 1964).

An artificial vagina utilizes thermal and mechanical stimulation to trigger ejaculation (Perry, 1995). It consists of a tube with an outer rubber lining that will hold water, into which one end is placed an inner liner that is lubricated (side for penis thrusting) just prior to use. The outer liner is filled and pressurized somewhat with water at around 42-48 degrees Celsius (Salisbury *et al*., 1940). Air is also applied sufficiently into the inner rubber to make sure it will fit the penis of the male once the collection has been made. At another end of the rubber, a collection tube is put to collect the semen.

Females can also be used, but except they are in estrus, they unusually enjoy involving in the process, which can simply cause injury to any of those involved (Hart et al., 1946). Another trouble with using a female as a teaser is that if the artificial vagina missed, the female could be bred or maybe transmit venereal disease to the male (Philpott, 1993). To collect semen, the male is allowed to mount and the penis is diverted into the artificial vagina where he ejaculates. It is best to allow the male to thrust into the artificial vagina rather than trying to slide it onto the male's penis (Evan, 1987).

2.3.2 ELECTROEJACULATION

Electroejaculation is applying a series of short, low-voltage pulses of electrical current to the pelvic nerves which are related to ejaculatory response. It does not cause much distress and can be used on many mammals (Graham et al., 1953). Electroejaculation also does not need animal to mount, and can be applied in the field using a battery-powered unit. It can be used to obtain semen from animals those are physically unable to mount due to musculoskeletal disease or injury (Hamilton et al., 1990).

2.3.3 MANUAL MANIPULATION

A lot of males mainly mammals can be triggered to ejaculate by providing pressure and massage to the penis (McDonnell et al., 1990). After the male becomes induced, a director cone of attached to a collection tube, is moved to the penis to facilitate collecting the semen.

2.4 SEMEN QUALITY AND FERTILITY

Mating success depends on mainly quantity and quantity of semen delivered to the female. Males vary not only in fertility at all inseminate dosages, but also in the lowest sperm number per inseminate required to reach optimum fertility (Saacke *et al*., 1994). Related to this issue, two properties of a male's quality of semen become major those which are compensable, where providing more sperm dosage to the female outcomes in a response in fertility, and that which is incompensable, which differs in fertility among males cannot be improved anymore by increasing sperm amount (Nadir *et al*.,1994). Compensable semen traits are related to the sperm incapability to reach the fertilization site. Incompensable traits known to be associated with the incapability of fertilizing sperm to support the process of fertilization or growth of embryo at early stage.

2.4.1 SEMEN EVALUATION

Fertility of male is related to endocrinology, physiology, and behavior. It starts with spermatogenesis and then to maturation of sperm in the epididymis, ejaculation, sperm transport through the reproductive tract of female, and lastly, ovum penetration. The biological starting point of male infertility unclearly understood even in the most extensively studied of various species (Hall *et al*., 1981). Traditional framework of semen quality include volume and sperm motility, morphology, and concentration. Even so, as a single estimation of fertility, none of these measurements are sufficiently accurate.

When these parameters combined, the fertility assessment accuracy is improved but still incomplete as none of these criteria can predict the ability of sperm to respond effectively in the reproductive tract of female. Truly inclusive semen analysis includes assess of the sperm ability to reach the fertilization site, undergo capacitation and the acrosome reaction, penetrate the zona pellucida, and fuse with the ooplasm and decondense (Bedford, 1981).

2.5 EXTENDER

Extender or diluent is a chemical medium that increases the semen volume extends the reproductive ability of the spermatozoa (Mishra et al., 2011). A good semen extender should contain an energy source for the sperm cell, antimicrobial while maintain the osmotic pressure and pH of the diluents. Semen that has been extended increases the conception rate with a small number of sperm cells (Rehman *et al*., 2013). Although, cryopreserving the semen will reduce the motility, morphology, and fertilizing ability of the sperm cells relatively with time (Mishra *et al*., 2011).

Besides, it contains protective agents that allow survival of sperm exterior the reproductive tract. Lipoproteins, like those contained in egg yolk or milk, protect sperm against cold blow by stabilizing cellular membranes (Cochran, 2001). Metabolizable substrates, such as glucose, provide a plentiful energy source for sperm. Antibiotics are added to extenders to slow or prevent growth of bacteria. The osmotic pressure and pH of extenders can be adjusted to maximize sperm survival.

The pH of semen extenders can probably range from approximately 6.5 to 7.2 without affecting the longevity of sperm viability during storage, but a pH of 6.7 to 6.9 may optimize sperm track linearity (Foote et al., 2002).

The proper selection of antibiotics to include in the semen extender also enhances the viability of stored semen. A combination of amikacin sulfate (1 mg/mL) and potassium penicillin G (1000 U/mL) was shown in one Texas study to be effective for control of bacterial growth and optimization of sperm motility of cool-stored semen. This antibiotic combination has also yielded excellent fertility in breeding trials (Brinsko, 2011). Timentin (ticarcillin disodium–clavulanate potassium; GlaxoSmithKline, New York; 1 mg/mL) and Naxel (ceftiofur sodium; Pfizer Animal Health, Research Triangle Park, NC; 1 mg/mL) have also shown good control of aerobic bacterial growth and maintenance of sperm motility in laboratory conditions.

2.5.1 ENERGY SOURCE

Source of energy is required as it will be utilized by the sperm through process of oxidative phosphorylation, in the mitochondria located at the sperm midpiece. Utilisation of energy helps in the sperm cells motility. Many kinds of sugar like glucose, trehalose, ribose, raffinose, saccharose, and galactose has been used, although fructose is widely used in ruminant extender, where it is less harmful outcomes than other sugars (Rehman et al., 2013). Coconut water will be used as natural energy source in this semen extender as well as a source of natural antioxidant along with fructose.

2.5.2 ANTIMICROBIAL

Antimicrobial properties are added to semen extenders to restraint the microbes' growth that can cause contamination to the semen during collection. However, such a non-therapeutic use of antibiotics is not without problems, since it may lead to the development of antibiotic resistance. Bacteria live very well well in the common extenders, unless antibiotics are used (Almond *et al*., 1994). The semen extenders ingredients also used in media prepared for growing or isolating bacteria.

The proper selection of antibiotics to include in the semen extender enhances the viability of stored semen. A combination of amikacin sulfate (1 mg/mL) and potassium penicillin G (1000 U/mL) was shown in one Texas study to be effective for control of bacterial growth and optimization of sperm motility of cool-stored semen (Hartman, 2011). This antibiotic combination has also yielded excellent fertility in breeding trials. Timentin (ticarcillin disodium–clavulanate potassium; GlaxoSmithKline, New York; 1 mg/mL) and Naxel (ceftiofur sodium; Pfizer Animal Health, Research Triangle Park, NC; 1 mg/mL) have also shown good control of aerobic bacterial growth and maintenance of sperm motility in laboratory conditions (Clark et al., 2000).

2.5.3 CRYOPROTECTANT

Cryoprotectants are chemicals that prevent cellular freezing damage, and their use in cryoprotective media has been integral to maintaining sperm viability during the freeze or thaw process (Critser et al., 1997). Some cryoprotectants work by bringing down the glass change temperature of a solution or material. Along these lines, the cryoprotectant anticipates actual freezing, and the solution keeps up some adaptability in a glassy stage. Numerous cryoprotectants additionally work by forming hydrogen bonds with biological molecules as water molecules are replaced.

Thus, as the cryoprotectant replaces the water molecules, the biological material retains its native physiological structure and function, although they are no longer immersed in an aqueous environment (Imrat *et al*., 2013).

Until now, Egg yolk is still used as primary non penetrating cryoprotectant in semen extenders. Different percentages are used for cryopreservation process so far. However, 20 percent Egg yolk is still used as a standard in most of the underdeveloped countries as it is cheap and readily available (Philips 1939). In egg yolk, Low Density Lipids (LDL) abundance is considered a main cryoprotective agent as it adheres to the cell membrane and protects the cell from cryoshocks. The LDLs are composed of 17-60 nm spherical molecules, with lipid and triglycerides in the core surrounded by protein and phospholipid thin layers. The LDL contains 11-17% proteins and 83-89% lipids (Graham et al., 1987).

The addition of egg yolk increased post thaw motility by solubilizing the cell membrane lipids and binds to the sperm (Moussa et al., 2002), but such a high amount increased the risk of microbial contamination resulting in metritis and transboundary diseases (Watson, 1981).

2.5.4 GLYCEROL

To avoid cryoinjuries to sperm cells, different cryoprotective agents have been used. Glycerol is classified as a penetrating cryoprotectant and most common used for dilution of semen worldwide (Holt et al., 2000). It was found about 15 percent increase in the post thaw quality of semen was observed after addition of glycerol to the egg-yolk based extender (Foote et al., 1949). Glycerol as conventional cryoprotectant prevents the intra-cellular crystallization, but it affects the fertilizing ability of the spermatozoa by an increase in the transient osmotic pressure prior to equilibration around the cell membrane that will increase osmotic pressure and affecting the semen quality parameters (Pommer et al., 2002). A sudden decrease in temperature from +5 °C to -196 °C decreases the membrane integrity and increase permeability (Rehman et al., 2013), but this shock is protected by glycerol in addition to egg yolk lipoproteins. It has been suggested that thermal changes disturb the lipid to lipid and lipid to protein association within the cell membrane which ultimately leads to loss of function (Hammerstedt et al., 1992).

2.5.5 BUFFER

Hydroxymethyl aminomethane (Tris) and citric acid are typical used buffers in various types of diluents used for ruminant semen. Tris containing egg yolk glycerol extender was developed in 1963 and become most popular for fresh and frozen semen (Foote *et al*., 1995). Earlier, phosphate buffer was used with extender but due to limited visibility under the microscope its use was limited (Phillips *et al*., 1939). During processing of semen for cryopreservation, cellular metabolic activities are increased that leads to production of lactic acid (Rigau *et al*., 1996), with some other acids and the extra cellular environment become more acidic resulting in decreased of pH. The decreased pH will reduce cellular activation within spermatozoa and storage life of semen. The simplest buffers used are bicarbonates and sodium citrate. However, they are not temperature stable as compared to Tris which is more stable at high temperature and other different environmental conditions (Gadea, 2003).

2.6 COCONUT WATER

Products of coconut have held a valuable and esteemed place medicine. It is known to be antiblenorrhagic, antibronchitis, febrifugal, and antigingivitic (DebMandal *et al*., 2011). In medicine of Ayurvedic, the oil, milk, cream and liquid from the coconut can be used to treat hair loss, burns and heart problems. Coconut water comprises vitamin B, riboflavin B2, trace amount of thiamine B1, folic acid as well as pyridoxine. It also contains sugars, vitamin C, free amino acids, enzymes (acid phosphatase, catalase,

dehydrogenase, diastase, peroxidase, RNA polymerases) and promoting factors of growth. Young coconut water also contains phytoestrogen and other sex hormone-like properties which can be utilized in hormone replacement therapy, in lowering the risk of dementia and healing wound in postmenopausal women. Cytokinins (e.g. kinetin and trans-zeatin) in coconut water also have significant anti-ageing, anti-carcinogenic, and anti-thrombotic (anti-clot formation) effects.

The main composition of coconut water was primarily carbohydrate, of which sucrose constituted 92% of the total sugar. Vice versa, the chief sugars in the young and the normal-mature water were glucose and fructose, and were higher in the young than in the normal – mature water. A few studies have provedthat the reducing sugar content declines and non-reducing sugar increases as the nut matures (Pue *et al*., 1992). Diminishing content of sugar increases during the development of the fruit until it reaches a maximum at around 6-7 months, then declines (Youngster, 1950). The high sucrose content in the water recommended that the fruits were very mature, or that the abnormality of the fruits during development led to the high content of sucrose in the watery endosperm. Coconut water was found to contain a-tocopherol as well as thiamin,riboflavin and vitamin C. Malic acid, the chief organic acid, was higher in the young compared to normal-mature.

CHAPTER 3

METHODOLOGY

3.1 STUDY AREA

The study was conducted at two different places which were Universiti Malaysia Kelantan (UMK) Jeli campus and Institut Biodiversiti Veterinar Kebangsaan (IBVK). The extender was made in the laboratory of UMK Jeli campus while the semen collection, processing, storing, and analyzing were done in the Institut Biodiversiti Veterinar Kebangsaan.

3.2 MATERIALS

The lists of materials used in this experiment were tris, citric acid, fructose, distilled water, egg yolk, young and old coconut water, ampicillin, streptomycin sulphate, and dimethyl sulfoxide. These materials were used as ingredients to make extender.

3.2.1 APPARATUS AND EQUIPMENT

Apparatus and equipment used in this study were artificial vagina (AV) set, tube jacket, temperature stabilizer, Computer Assisted Semen Analysis (CASA), chiller, thermometer, beaker, test tube, pipette, slides, and cover slides.

3.3 METHODS

3.3.1 SEMEN EXTENDER PREPARATION

To prepare an extender, 3 g of tris (hydroxymethyl) amino methane, 1.7 g of citric acid, 1.25 g of fructose, 15 ml of dimethyl sulfoxide (DMSO), 100 mg of streptomycin sulphate, 100 mg of ampicillin, 20 ml of egg yolk or 20 mL young coconut water or 20 mL old coconut water. These ingredients were put together in a 250 mL beaker and mixed. Distilled water was then filled up to 100 ml. There were three treatments and differences in each extender prepared (total of 3 extenders) which were the use of either egg yolk, young coconut water, or old coconut water. After about 30 minutes, the extenders were then put in a chiller at 4^{o}C temperature. The dilution ratio between semen to extender is 1:9.

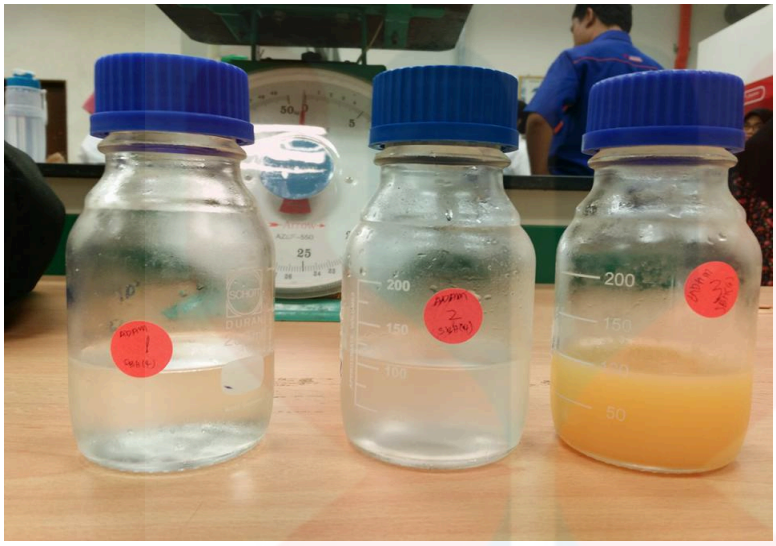

Figure 3.1: Semen extenders

3.3.2 SEMEN COLLECTION

The AV components were put together, and the space located between the rigid casing and the inner rubber liner filled with warm water. AV pressure was also an important factor for obtaining ejaculates of optimum quality. Pressure adjusted by adding air through a valve in the rigid casing until the AV liner protruded slightly from the ends. A small amount of non-spermicidal lubricant applied to the inner liner before the collection performed. Ram to be collected by AV was haltered broken and trained. Once the ram mounted, the penis grasped through the sheath and directed to the opening of the lubricated AV. The ram made seeking motions and thrust into the AV. The thrust should be vigorous to ensure an ejaculate has been collected (Wolfe, 2001).

Figure 3.2: Semen collection via Artificial Vagina method

3.3.3 SEMEN STORAGE AND EVALUATION

After the semen had been collected, it was put into a tube jacket. Three test tubes containing 3 different extenders –egg yolk based, young coconut water based, and old coconut water based, were put into a water bath about 37^{o}C temperature to maintain body temperature. The semen was then pipetted carefully and mixed evenly with the extenders in the test tubes. Then, a sample from each test tube pipetted into a slide. The slide was then covered with a cover slide, and observed under CASA. The percentage of motility and progressive motility of the sample were then taken and recorded. This was the initial result.

After the result was taken, the three test tubes containing sample were put into a 250 ml beaker filled with 100 ml water bath. Then the beaker was put into a chiller at 4^{o}C temperature. The use of beaker filled with water bath was to prevent temperature shock to the sample. After an hour, the water bath removed from the beaker, left only the test tubes containing sample. The test tubes were let in the chiller for another an hour. Then, a sample from each test tube was then put into a slide and observed under CASA once again. The percentage of motility and progressive motility were taken and recorded. The test tubes were then put back in the chiller. After 24 hours, a sample from each test tubes was put into a slide and observed under CASA, and the final result was taken.

Figure 3.3: Semen evaluation using CASA

CHAPTER 4

RESULT AND DISCUSSION

4.1 EFFECT OF EXTENDERS ON FRESH RAM SEMEN QUALITY

Table 4.1: Characteristics of fresh ram semen

Parameter	Value
pH	7.1
Motility (%)	94
Progressive motility (%)	87

Table 4.1 shows the fresh semen characteristics of ram. Semen characteristics included pH, motility, and progressive of the semen. The pH value of the semen was 7.1, which was the normal pH value for the fresh semen. For motility, the percentage was 94% and the progressive motility was 87%. Both of these characteristics were evaluated after the dilution with the extenders and chilling process.

Table 4.2: pH of diluted semen with different extenders (young coconut water, old coconut water, and egg yolk) after 24 hours of chilling at 4°C

Replication number	Young coconut	Old coconut	Egg yolk
1	6.5	6.3	6.7
2	6.5	6.3	6.7
3	6.5	6.3	6.7
Average	6.50±0.00	6.30±0.00	6.70±0.00

Mean± standard deviation

Table 4.2 shows the pH of diluted semen with different extenders including young coconut water, old coconut water, and egg yolk after 24 hours of chilling at 4°C. All of the pH readings showed that it was a decrease in pH value from 7.1 (fresh semen) to 6.5 (young coconut water), 6.3 (old coconut water), and 6.7 (egg yolk). This result showed that the extender might be more alkaline, probably due to the ingredients used, or antibiotics properties in the extenders. However, for young coconut water and egg yolk, they were still higher as (Brinsko, 2011) stated that the pH of semen extender should be in range from approximately 6.5 to 7.2.

Table 4.3: Motility (%) of semen in different diluted extenders (young coconut water, old coconut water, and egg yolk) after 24 hours of chilling at 4^{o}C

Replication number	Young coconut	Old coconut	Egg yolk
1	80	70	91
2	83	67	90
3	81	74	85
Average	81.00±0.1.53	70.33±3.51	88.67±3.21

Mean± standard deviation

Table 4.4: Progressive motility (%) of semen in different diluted extenders (young coconut water, old coconut water, and egg yolk) after 24 hours of chilling at 4^{o}C

Replication number	Young coconut	Old coconut	Egg yolk
1	60	49	61
2	60	43	62
3	63	46	65
Average	61.00±1.73	46.00±3.00	62.67±2.08

Mean± standard deviation

Table 4.3 and 4.4 showed that the percentage motility and progressive motility of semen in different diluted extenders (young coconut water, old coconut water, and egg yolk) after 24 hours of chilling at 4^{o}C. As expected, there would be a percentage decrease in both motility and progressive motility from the initial value (fresh semen). From 94% motility of the fresh semen, it were resulted in average 81.00±0.1.53%) of young coconut water 70.33±3.51% of old coconut water, and 88.67±3.21% of egg yolk. Meanwhile for progressive, from 87% of the fresh semen, it were resulted in average of 61.00±1.73% of young coconut water, 46.00±3.00% of old coconut water, and 62.67±2.08% of egg yolk.

Overall, egg yolk extender showed the best result. This might be due to the pH of the extender that affected the preservation of the semen. The pH of semen extenders should be in range from approximately 6.5 to 7.2 without affecting the longevity of sperm viability during storage, but a pH of 6.7 to 6.9 might optimize sperm track linearity (Brinsko, 2011). Besides, the cryoproctective content in egg yolk also made it better to preserve the semen compared to both young and old coconut water. The protective effect of egg yolk in minimizing cold stress, was related to its content in cholesterol and phospholipids (Bathgate et al., 2006). However, the result taken from young coconut water were also impressive as it is above acceptance level which is above 50% in motility and progressive (Cooper et al., 2010). This was a bit different from old coconut water, which had motility above significant level but the progressive is below the significant level. This might be due to the pH of the extender that was far from the range which was suitable from sperm preservation

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CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

As a conclusion, the extender contained young coconut water showed a better result compared to old coconut water in term of motility and progressive motility. But still, the common ingredient used nowadays which is egg yolk, and also the control in this study was still better than both of old and young coconut water extenders. This might be related to the contents in the ingredients themselves, and also the pH value. According to (Brinsko, 2011), pH of semen extenders should be in range from approximately 6.5 to 7.2. Besides, the cryoproctective content in egg yolk also made it better to preserve the semen compared to both young and old coconut water. The protective effect of egg yolk in minimizing cold stress, was related to its content in cholesterol and phospholipids (Bathgate et al., 2006). Meanwhile, the main obvious difference in between young and old coconut water was the pH value. The pH of young coconut extender was 6.5 and the old coconut extender was 6.3. As mentioned, the pH of semen extenders should be in range of 6.5 to 7.2. Thus, it might influence the result after being analyzed under CASA.

5.2 RECOMMENDATION

For a better improvement in preserving semen, further study on the properties and use of extenders is crucial in order to maintain the quality of the semen for a longer period. The extender should contain energy source for the sperm cell, antimicrobial, suitable pH, cryoprotective agents, and many others. In this study, it was important to practice the right and safe method to obtain a good result. During making the extender, the hygiene was very important, in order to avoid any source of contaminant and spermicide those might kill the sperm cells. Besides, During the semen collection, the preparation of the AV was also crucial. The air must be well filled and the temperature cannot be too hot or too cold, but just in between 37 to 38 ^{o}C to allow the ram to smoothly ejaculate. Safe precaution during handling the ram should also be taken to avoid injuries. Lastly, during chilling and analysing data, avoid any contaminant and spermicide agent those can affect the result or kill the sperm cells instead of maintaining the temperature.

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APPENDIX A

Table A.1 : One-way ANOVA

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Motility	Between Groups	387.556	2	193.778	26.831	.001
	Within Groups	43.333	6	7.222		
	Total	430.889	8			
ProgressiveMotility	Between Groups	480.667	2	240.333	18.647	.003
	Within Groups	77.333	6	12.889		
	Total	558.000	8			

Table A.2: Post Hoc Analysis using Duncan[a] for motility

MOTILITY

Duncan[a]

Treatment	N	Subset for alpha = 0.05		
		1	2	3
TREATMENT 2	3	75.6667		
TREATMENT 1	3		85.0000	
TREATMENT 3	3			91.6667
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table A.3 : Post Hoc Analysis using Duncan[a] for progressive motility

PROGRESSIVE MOTILITY

Duncan[a]

TREATMENT	N	Subset for alpha = 0.05	
		1	2
TREATMENT 2	3	47.3333	
TREATMENT 3	3		61.0000
TREATMENT 1	3		62.6667
Sig.		1.000	.565

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

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