Effect of Different Dilution Rate of Guava Juice Extender on Malin Ram Spermatozoa at 4°C and Room Temperature

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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I certify that the report of this final year project entitled " _____" by_____, matric number _____has been examined and all the correction recommended by examiners have been done for the degree of Bachelor of Applied Science (Agriculture Technology) with Honours, Faculty of Agro-Based Industry, Universiti Malaysia Kelantan.

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Supervisor

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Date:

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ABSTRACT

Semen extender is vital in animal reproduction especially in artificial insemination. The ingredients used in semen extender vary throughout the world; nevertheless there is a lack of local fruits usage as an alternative ingredient in Malaysia. Tropical fruits used as alternative ingredients in semen extender are gaining more attention recently. This study was conducted to assess the effect of the guava juice extender on the sperm motility, progressive motility, path velocity (VAP) and progressive velocity (VSL) at 4°C and room temperature (RT). Guava juice extender was prepared by adding 20 ml of the supernatant with standard extender. Semen collected was diluted with control extender at 1:9, and with guava extenders of dilution 1:5, 1:10, 1:15 and 1:20. Each group of 4°C and RT (n=5), where each sample was supplemented with 0.1 ml of Malin ram semen. The samples were assessed with Computer Assisted Semen Analyser (CASA) at 0 hour, after 4-5 hours and after 16-17 hours. The sperm characteristics were analysed through ANOVA. The results showed that sperm manage to survive in the guava juice extender although, after 16-17 hours chilled, dilution 1:5 extender was significantly high in motility (75.7%), progressive motility (37.0%), VAP (75.9 µm/s), and VSL (55.0 µm/s). Dilution 1:15 was significantly high in terms of VAP (79.9 µm/s) and VSL (63.0 µm/s), after 16-17 hours storage in room temperature. Dilution 1:5 was significantly high in motility (25.7%) and progressive motility (3.7%) after 16-17 hours of storage at room temperature. In conclusion, dilution 1:5 of guava juice can be used as an alternative in semen extender for ram in chilled storage.

Keywords: Malin ram, chilled stored, room temperature, guava juice extender, semen

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ABSTRAK

Pencair air mani amat penting di dalam pembiakan haiwan terutamanya di dalam permanian beradas. Bahan-bahan yang digunakan di dalam pencair air mani adalah berbeza-beza, namun terdapat sedikit kekurangan di dalam penggunaan buah-buahan tempatan sebagai alternatif di dalam pencair air mani. Pengunaan buah-buahan tropika sebagai bahan alternatif di dalam pencair air mani semakin mendapat perhatian di dalam kajian yang terkini. Kajian ini dijalankan untuk menaksir pergerakan, pergerakan progresif, halaju laluan dan halaju progresif sperma pada suhu bilik dan suhu 4°C. Penyediaan pencair jus guava bermula dengan penambahan 20 ml supernatan dicampur bersama bahan lain. Air mani biri jantan dikumpul dengan bantuan faraj tiruan, di mana air mani itu dicairkan pada nisbah 1:9 untuk pencair kawalan dan nisbah 1:5, 1:10, 1:15 serta 1:20 untuk pencair air mani yang mengandungi jus jambu batu. Kedua-dua kumpulan suhu bilik dan suhu 4°C (n=5), di mana setiap sampel ditambah dengan 0.1 ml air mani. Semua sampel di analisa dengan Computer Assisted Semen Analyser (CASA) pada 0 jam, selepas 4-5 jam dan selepas 16-17 jam untuk kedua-dua suhu. Ciri-ciri sperma di analisa dengan bantuan perisian ANOVA. Hasilnya sperma berjaya hidup di dalam pencair jus jambu batu walaubagaimanapun, selepas 16-17jam disimpan pada suhu 4°C, pencairan 1:5 memberikan nilai purata yang paling tinggi dari segi pergerakan (75.7%), pergerakan progresif (37.0%), halaju laluan (75.9 µm/s) dan halaju progresif (55.0 µm/s). Pencair 1:15 memberikan nilai purata tertinggi dari segi halaju laluan (79.9 µm/s) dan halaju progresif (63.0 µm/s) selepas 16-17 jam disimpan pada suhu bilik, manakala, 1:5 memberikan nilai purata tertinggi dari segi pergerakan (25.7%) dan pergerakan progresif (3.7%) selepas 16-17 jam disimpan pada suhu bilik. Konklusinya, pencair jambu batu pada 1:5 boleh digunakan sebagai alternatif pencair air mani untuk biri-biri disimpan pada suhu sejuk.

Kata kunci: biri Malin, simpan sejuk, suhu bilik, pencair jus jambu batu, air mani

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LIST OF ABBREVIATIONS AND SYMBOLS

AI	Artificial Insemination
AV	Artificial Vagina
CASA	Computer Assisted Sperm Analyser
DMSO	Dimethyl Sulfoxide
RT	Room Temperature
SEM	Standard Mean of Error
VAP	Path Velocity
VSL	Progressive Velocity
rpm	Rotation per minute

CHAPTER 1

INTRODUCTION

1.1 General Background

Artificial insemination (AI) is the deposition of semen into the uterus mechanically (Rehman, Zhao, Shah, Qureshi, & Wang, 2013). Salamon & Maxwell (2000) cited that the research of AI, exist since the beginning from the last century which leads to applying this practice onto the livestock. They also stated that by early 1930s, AI of fresh ejaculates and semen mixed with diluents were applied in mass breeding sheep project, led by Milanov, when the First World War ended within what used to be Soviet Union region. Further research was conducted on semen storing, when the need to prolong the high quality sperm life, due to the sperm traveling from the location of semen collection to the farms situated further away for conception (Salamon & Maxwell, 2000).

Prolonging the life of the sperm can be accomplished through decreasing their metabolic activity where the researchers discovered that the semen can be stored by (i) cryopreservation, which is lower than 0°C and (ii) chilling, (0-5°C or 10-15°C) (Salamon & Maxwell, 2000). Cryopreservation of semen is the storing of diluted semen at cryogenic temperature, far prolonging the sperm life while maintaining fertility at a standard level after thawing. Extensive researches resulted in the improvement of the procedures involving a wider range of cell types such as spermatozoa and ovum, and even embryos (Di Santo, Tarozzi, Nadalini, & Borini, 2012).

According to Di Santo et al. (2012), cryopreservation techniques are divided into two: (i) slow freezing technique and (ii) rapid freezing technique. They also stated that slow freezing technique can be applied manually or automatically with the help of semi-programmable freezer in an amount of time (2-4 hours). According to them, manually, in slow freezing the temperatures of the diluted semen were reduced and adding cryoprotectant at the same time following the protocol, which will then be immersed in liquid nitrogen after ample equilibration of temperature. Alas, with successful result in freezing the sperm at cryogenic temperatures by this way, their fertility are affected, which leads to the invention and extensive researches on programmable freezer (Di Santo et al., 2012).

The slow freezing of semen involves a few steps before plunging the straws into the liquid nitrogen at -196°C. The diluted semen was filled into straws with uniform amount of cryoprotectant in each and every one of them and left for 10 minutes at 4°C (Di Santo et al., 2012). They also specified that for 15 minutes the straws were positioned above the liquid nitrogen (-80°C) horizontally, approximately at 15-20 cm on the liquid nitrogen, and finally the straws were plunged in liquid nitrogen. Unfortunately, this method has its downside, for instance, low conception rate and “the temperature drop curve cannot be controlled” (Di Santo et al., 2012).

Chilled storage of semen is a substitute to cryopreservation in storing the diluted semen. Usually chilling of semen can be done by storing the extended semen between the temperatures of 0-15°C with the help of cooling devices. Chilled storage has an advantage over the fact that it will not injure the sperm, hence affecting the fertility during freezing and thawing of the diluted semen (Paulenz, Söderquist, Pérez-Pé, & Andersen Berg, 2002).

There is no significant drop in motility after 12 hours of storing the human semen at 20°C, though the same thing could not be said on semen stored at 4°C and

37°C (Appel, Evans & Blandy, 1977). Besides that, this research is not precise due to the fact that they cannot warm up the semen stored at 4°C with the lack of materials.

1.2 Problem Statement

The aim of this research was to determine whether *Psidium guajava* Linn can be used as an alternative ingredient in semen extender. There is only one article that uses guava as a natural antioxidant in the semen extender, constricting further information to be extracted regarding this research.

1.3 Hypothesis

H_o: Guava juice extender will give affects to the ram semen characteristics.

H_1: Guava juice extender will not give affects to the ram semen characteristics.

1.4 Objectives

1. To assess the different dilution ratio of the guava juice extender on the sperm characteristics at 4°C and room temperature.
2. To determine the characteristics of the sperm at 4°C and room temperature in guava juice extender.
3. To assess the diluted semen characteristics at 0 hour, after 4-5 hours and after 16-17 hours.

1.5 Scope Of Study

This study involved guava juice and extender preparation, semen collection, semen dilution and semen evaluation. Guava juice was prepared based on (Daramola et al., 2016), extracted juice was centrifuged at 3000 rpm, and the supernatant was mixed in the extender. Preparation of extender for both control and samples were based on the formula from Salamon & Maxwell (2000). Semen collected with the aid of artificial vagina (AV) and trained sheep. Semen collected was diluted with extenders, where ten extenders is divided into two groups, which are extenders kept at room temperature and extenders stored at 4°C. Each group consists of control extender, guava juice extenders at 1:5, 1:10, 1:15 and 1:20 ratio. In addition, the diluted semen was evaluated under Computer Assisted Semen Analysis (CASA), where semen parameters of sperm motility, progressive motility, path velocity (VAP) and progressive velocity (VSL) were evaluated under room temperature and after chilled storage at 4°C.

1.6 Significance Of Study

Guava is a very nutritious fruit, where it can be found cultivated within Malaysia itself as it is a subtropical and tropical fruit (Yusof, 2003). If guava is proven to be a source of alternatives in semen extender, this may reduce the cost of semen extender involved in storing semen, since guava is a local product of Malaysia. Hopefully, this research may encourage more local farmers to practice semen collection and storing as one of the way to generate an income in their farm.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This chapter will discuss about the literature review regarding the researches that had been conducted in relation to this scope of study. Using the past researches information extracted from sources such as scholarly journals, books and reviewed articles, which act as a guide in this research. This literature review will be comprised of topics such as Malin sheep, ram semen, *Psidium guajava* Linn, extender, energy source, antimicrobial, salt, cryoprotectant, semen collection, chilling of semen, and sperm evaluation.

2.2 *Psidium guajava* Linn

According to Kadam, Kaushik & Kumar (2012), guava is also known as "superfruits", packed with nutrition, with a high content of ascorbic acid, where it is a powerful fruit that can fight off free radicals and oxidation that are the main reason of many degenerative diseases. They also stated that guava can be made into anything such as beverages, flavouring agents, and chocolate bars. Believed to be originated from Central America and the southern part of Mexico, *Psidium guajava* Linn is claimed to be the fourth most important fruit in terms of area and production after mango, banana, and citrus and India is the biggest producer of guava (Kadam et al., 2012).

Since the early 17th century, India has been cultivating guava where it gradually became an importance commercial crop. Guava is quite hardy, prolific bearer and highly remunerative even without much care. The vitamin C in guava

makes absorption of vitamin E much more effective in reducing the oxidation of the LDL cholesterol and increasing the (good) HDL cholesterol (Kadam et al., 2012). The fibres in guavas promote digestion and ease bowel movements. The high content of vitamin A in guava plays an important role in maintaining the quality and health of eye-sight, skin, teeth, bones and the mucous membranes. In this context, guava has excellent digestive and nutritive value, pleasant flavour, high palatability and availability in abundance at a moderate price. The fresh fruit has limited shelf life, therefore, it is necessary to utilise the fruit for making different products to increase its availability over an extended period and to stabilise the price during the glut season (Kadam et al., 2012). Guava can be consumed fresh or can be processed into juice, nectar, pulp, jam, jelly, slices in syrup, fruit bar or dehydrated products, as well as being used as an additive to other fruit juices or pulps (Kadam et al., 2012).

2.3 Malin Sheep

Malin sheep are natives in Malaysia. According to Mastura, Salleh, Mohd-Hafiz, A., & Mohd-Hafizal (2014), Malin sheep can be found mainly in Kelantan, Terengganu, Pahang and Negeri Sembilan aside from other north-eastern and central part of Peninsular Malaysia, these wool type sheep consist of white, greyish brown, whitish grey and light brown in colour. They furthermore states that only the males possess horns and the weight for adult males may range from 25-30 kg, on the other hand, the weight of adult females are ranging from 20-25 kg. Compared to other foreign breeds this breed is more able to withstand against diseases (Mastura et al., 2014).

2.4 Ram Semen

While ovum is one of the largest cells in the body, sperm is known to be one of the smallest cells. Sperm carries the paternal genes for reproduction. The shape of the sperm is streamlined for speedy and efficient conception; sperm are actively motile consisting of head and tail. The tail of the sperm comprised of neck, mid piece, principal piece and end piece. Basically, the tail helps the sperm to propel forwards to the egg and the head contains the genes, mitochondria and enzymes for fertilisation (Colledge, 2013). In addition, sperm morphology is highly dependent on the body as well as the testicular health, where the testicles are highly sensitive to the intrinsic and extrinsic environment (Menkveld, Holleboom, & Rhemrev, 2011).

2.5 Extender

Extender or diluent is a chemical medium that increases the semen volume extends the reproductive ability of the spermatozoa (Mishra, Alam, Khandokar, Mazumder, & Munsi, 2011). Good semen extenders should contain an energy source for the sperm cell; antimicrobial while maintaining 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 fertilising ability of the sperm cells relatively with time (Mishra et al., 2011).

2.6 Energy source

The energy source is needed as it will be utilised by the sperm through oxidative phosphorylation process, in the mitochondria located at the midpiece of the sperm. Energy utilisation helps in the motility of the sperm cells. Many kinds of sugar like glucose, trehalose, ribose, raffinose, saccharose, and galactose has been used, although fructose is widely used in the ruminant extender, where it is less harmful outcomes than other sugars (Rehman et al., 2013). Mitochondrial and plasma membranes surrounded the different compartment of the sperm, where the membranes need not to retain any damage as it will reduce the sperm motility (O'Connell, McClure, & Lewis, 2002). Guava, *Psidium guajava* Linn will be used as natural energy source in this semen extender as well as a source of natural antioxidant.

2.7 Antimicrobial

Usually, freshly acquired semen from physically fit male animals is free of any microorganism, though along the semen collecting process, with the presence of fructose in the extender at room temperature of 20°C will stimulate bacterial growth. Bacteria contamination will mitigate the nutrients in the extender resulting decreasing the pH which affects the motility and viability of spermatozoa. Penicillin will be used in this study as a microbial inhibitor based on Salamon & Maxwell (2000).

2.8 Salt

Hydroxymethyle aminomethane (Tris) and citric acid are the common buffer used in ruminant semen extender. Tris containing egg yolk glycerol extender was developed in 1963 and become most popular for both fresh and frozen semen. Earlier, phosphate buffer was used with extender but due to limited visibility under the

microscope its use was limited. During processing of semen for cryopreservation, cellular metabolic activities are increased that leads to production of lactic acid and some other acids and the extracellular environment become more acidic resulting in decreased pH. The decreased pH will reduce cellular activates within spermatozoa and storage life of semen. The simplest buffers used are bicarbonates and sodium citrate. This experiment will be using sodium citrate as buffer according to Salamon & Maxwell (2000).

2.9 Cryoprotectant

Generally, cryoprotectant is like a shield for the sperm against cryodamage from the crystallisation of ice (Di Santo et al., 2012). According to Barbas & Mascarenhas (2008) sperm cryopreservation extenders consist of a non-permeating cryoprotectant like milk and egg yolk and/or permeating cryoprotectants like glycerol, ethylene glycol, or dimethyl sulfoxide. They also stated that non-permeating cryoprotectant does not cross plasma membrane and only acts as extracellular, thus, non-permeating cryoprotectant may alter plasma membrane, or act as a solute, lowering the freezing temperature of the medium and decreasing the extracellular ice formation. Glycerol is frequently used as a cryoprotectant for freezing ram semen. Furthermore, Barbas & Mascarenhas (2008) also states that glycerol or dimethyl sulfoxide can induce osmotic stress and toxic effects to spermatozoa, but the extent of the damage varies according to the species and depends on its concentration in the extender solution.

Several experiments were done to calculate the optimal level of glycerol in semen extenders, combining cooling and freezing rates, extender composition and type of glycerol addition. During slow freezing of conventional method involving mostly hypertonic extenders, glycerol is used approximately beween 6-8%, where a

higher amount may damages the cell and affecting the survival rate, and the optimum results is between 4-6% use of glycerol with 10–100°C/min of freezing rate (Barbas & Mascarenhas, 2008).

2.10 Semen Collection

Semen can be collected with the help of electro-ejaculation or artificial vagina, though in terms of domestic males, artificial vagina is much more preferable even though training is needed on the animals involved in this process, while electro-ejaculator is used onto the non-trained animals including wild species (Bopape, Lehloenya, Chokoe, & Nedambale, 2015). Bopape et al. (2015) also states that electro-ejaculation is a stressful procedure to the animals and semen collected from electro-ejaculating is less resilient to cold shock compared to semen collected through AV.

2.11 Chilling of Semen

Chilling is a process of storing the extended semen at 4-5 °C in appropriate expanse of a short time usually under 72 hours (Kheradmand, Babaei, & Abshenas, 2006). It is one of the ways to prolong and maintain the quality and characteristics of the sperm at a standard amount which are approve to achieve a high fertilisation rate. Chilling the sperm helps in decreasing their metabolic activity, thus, prolonging their viability and motility (Sumadiasa, Susilawati, Ciptadi, & Isnaini, 2015). However, this method of storing has its downside where it cannot be stored longer than 72 hours, though this amount of time varies according to different species, extenders and protocols among other reasons (Varisli, Scott, Agca, & Agca, 2013). Studies also confirmed that there is a rapid decline in mainly sperm motility

and sperm morphological integrity with increasing amount of time in storage (Salamon & Maxwell, 2000).

2.12 Sperm Evaluation

Computer assisted semen analysis (CASA) helps in providing diagnosis of the semen quality based on the amount of sperm activity (Bopape et al., 2015). Recently, more research on semen assessments of many aspects are aided with the help of CASA, especially when evaluating the sperm motility as it is the major sign of the sperm fertility (Kozdrowski, Dubiel, Bielas, & Dzięcioł, 2007). Over the manual counting and assessment, as an alternative CASA comes with more advantages though improper use of it, may affect the results, hence trained personnel in handling this machine is compulsory (Schleh & Leoni, 2013). The diluted semen was assessed by the parameters of motility, progressive motility, path velocity (VAP) and progressive velocity (VSL) at fresh ejaculates, after 4-5 hours and 16-17 hours at 4°C and room temperature.

CHAPTER 3

MATERIALS AND METHODS

3.1 Animals

In this research, the sperm used was collected from a total of three male sheep. The sheep were grown under semi-intensive farming. The rams were separated from the ewes and lambs, and they had their own section of space. The ram used in this research were all light brown coated sheep of approximately three years old from the breed Malin. The male had been trained to do a scheduled semen collection since it was mature enough to produce semen. The ram can only ejaculate two times per day, and collection can only be done twice a week, to maintain high quality semen production. Semen collection was done at the evening or preferably in the morning. The rams were fed twice and given clean water daily with the addition supplement of mineral block.

3.2 Semen Collection

The semen was collected manually with the use of AV (Artificial Vagina). The materials involved in the semen collection were sterilised first and then the AV was constructed. The semen ejaculated was collected into the collecting tubes, attached at the base of the silicone cone of the AV. The collecting tubes were covered with a cloth specially designed to fit the tubes to reduce rapid loss of heat in the semen. Hot water was poured into the AV through the valve filling the inner liner. Air was blown into the AV using mouth, inflating the inner liner leaving a small space enough for the penis to go through. KY jelly was applied at the mouth of the AV and in the AV.

A trained female teaser was constricted, and the ram was brought out to walk a few rounds around the teaser as to stimulate the interest on to the teaser. When the rams were allowed to mount, the first thrice mounting were prevented as to avoid any fake ejaculation and build up the frustration. Soon when the ram mount the teaser and the penis comes out, a gloved hand supported the base of penis directing it towards the AV. The semen collectors have to maintain and make sure the penis stays in the AV until a jerk can be felt, signalling that the ram ejaculated. The collector tube was extracted and put inside a specially designed Styrofoam container that is carved in the middle to perfect fit for the semen collector tube that has been covered with aluminium foil at the mouth of the tube. The semen tube was brought to the laboratory immediately. To avoid contamination in the laboratory, the staff collecting the semen cannot be allowed in the laboratory. The semen collector tubes were passed through the window of the laboratory.

3.3 Extender Preparation

The guava juice preparation was based on Daramola et al. (2016). The guava was thoroughly cleaned with distilled water and pat dried. Then it was cut into smaller pieces before it was crushed by mortar and pestle. The juice was extracted by squeezing and filtering it through the sieve and centrifuged at 3000 rpm for 20 minutes. The supernatant of the guava juice was taken as one of the ingredients and mixed into the extender.

3.4 Semen Dilution

The sample used in this experiment was divided into two groups which were samples for room temperature and samples for chilled storage at 4°C, consisting five samples each group. The extender that act as control used in this experiment is extracted from Salamon & Maxwell (2000) where it involves with the mix of 2.37 grams of sodium citrate, 0.50 gram of glucose, 15 ml of egg yolk, 100000 i.u. (0.5988 gram) of penicillin, 100 mg (0.1 gram) of streptomycin and distilled water up to 100 ml of extender. Meanwhile, the experimental extender consisted of 20 ml guava juice, 2.37 grams of sodium citrate, 15 ml of dimethyl sulfoxide and 0.5988 gram of penicillin based on Salamon & Maxwell (2000).

The apparatus needed in semen dilution were heated on the pre warm stage between 36-37°C. The extender were distributed into the test tubes accordingly, and then placed in the water bath. The acquired semen was immediately diluted at the ratio of 1:5, 1:10, 1:15, and 1:20 in guava juice extender and 1:9 for control. Each sample was added with 0.1 ml of ram sperm respectively. The diluted semen was then put in the water bath, waiting to be evaluated.

3.5 Semen Evaluation

A drop of the sample using the micropipette was put on to the microscope slide and covered with a cover slip. Each slide contained three different samples to reduce the unnecessary wastage of microscope slides. The slide was placed under the microscope and evaluated using the CASA (Computer Assisted Sperm Analysis). The sperm motility, progressive motility, path velocity and progressive velocity were assessed, over 3 fields, under the 10x magnification.

3.6 Statistical Analysis

The diluted semen characteristics were analysed using three-way analysis of variance (ANOVA) in SPSS Software. The data were expressed as Mean ± SEM with 95% confidence interval ($p < 0.05$).

CHAPTER 4

RESULTS

4.1 Assessment of the Diluted Semen at 4°C

Table 4.1 shows the mean value and standard error of the mean (SEM) of sperm motility, progressive motility, path velocity (VAP) and progressive velocity (VSL) of the semen in different dilutions of fresh ejaculate 4°C. Where it shows that the extender with guava dilutions of 1:20 had the highest mean value which was 86.0% in motility, 61.3% in progressive motility, 80.0 µm/s in VAP and 62.3 µm/s in VSL.

Table 4.1 Sperm motility, progressive motility, VAP and VSL in different dilutions of fresh ejaculate at 4°C

Extender	Motility (%)	Progressive motility (%)	Path velocity (µm/s)	Progressive velocity (µm/s)
Control	$75.3^{c} \pm 2.6$	$60.0^{d} \pm 1.0$	$88.4^{e} \pm 1.3$	$82.0^{d} \pm 0.8$
1:5	$77.0^{c} \pm 2.5$	$31.3^{c} \pm 1.7$	$62.3^{c} \pm 1.0$	$46.7^{b} \pm 1.3$
1:10	$17.7^{a} \pm 1.5$	$2.3^{a} \pm 0.3$	$45.6^{a} \pm 1.3$	$37.3^{a} \pm 1.9$
1:15	$34.7^{b} \pm 3.2$	$14.0^{b} \pm 2.9$	$50.3^{b} \pm 0.6$	$42.2^{b} \pm 0.7$
1:20	$86.0^{d} \pm 1.5$*	$61.3^{d} \pm 0.9$*	$80.0^{d} \pm 1.6$*	$62.3^{c} \pm 1.5$*

*****Highest mean value between the dilutions consisting of guava juice only.

^{a,b,c,d}Values in the same column with different superscripts differ significantly ($P<0.05$).

SEM = Standard Error of mean; VAP = path velocity; VSL = progressive velocity

Table 4.2 shows the mean value and SEM of sperm motility, progressive motility, VAP and VSL of the semen in different dilutions after 4-5 hours chilled at 4°C. Where it shows that the extender with guava dilutions of 1:20 had the highest mean value than the other samples for motility at 78%, progressive motility at 56.7%, VAP at 84.5 µm/s and VSL at 64.2 µm/s.

Table 4.2 Sperm motility, progressive motility, VAP and VSL in different dilutions of guava juice extender after 4-5 hours chilled at 4°C

Extender	Motility (%)	Progressive motility (%)	Path velocity (µm/s)	Progressive velocity (µm/s)
Control	$71.7^{b} \pm 2.0$	$58.3^{c} \pm 1.2$	$93.7^{d} \pm 1.7$	$84.7^{e} \pm 1.2$
1:5	$77.3^{a} \pm 1.8$	$24.0^{b} \pm 1.2$	$56.7^{b} \pm 1.5$	$42.7^{c} \pm 1.6$
1:10	$34.3^{a} \pm 4.0$	$4.3^{a} \pm 0.9$	$47.6^{a} \pm 1.7$	$37.0^{b} \pm 1.9$
1:15	$30.7^{b} \pm 1.5$	$4.0^{a} \pm 0.0$	$44.2^{a} \pm 1.0$	$31.6^{a} \pm 1.4$
1:20	$78.0^{b} \pm 10.0$*	$56.7^{c} \pm 1.5$*	$84.5^{c} \pm 0.7$*	$64.2^{d} \pm 0.9$*

*Highest mean value between the dilutions consisting of guava juice only.

^{a,b,c,d,e}Values in the same column with different superscripts differ significantly ($P<0.05$).

SEM = Standard Error of mean; VAP = path velocity; VSL = progressive velocity

Table 4.3 shows Table 4.2 shows the mean value and SEM of sperm motility, progressive motility, VAP and VSL of the semen in different dilutions after 16-17 hours chilled at 4°C. Where it shows that the extender with guava dilutions of 1:5 had the highest mean value in terms of motility at 75.7%, progressive motility at 37%, VAP at 75.9 µm/s and VSL at 55 µm/s, than the other samples.

Table 4.3 Sperm motility, progressive motility, VAP and VSL in different dilutions of guava juice extender after 16-17 hours chilled at 4°C

Extender	Motility (%)	Progressive motility (%)	Path velocity (µm/s)	Progressive velocity (µm/s)
Control	$73.0^{c} \pm 6.0$	$60.7^{c} \pm 4.4$	$87.6^{c} \pm 2.4$	$78.5^{c} \pm 3.6$
1:5	$75.7^{c} \pm 1.2$*	$37.0^{b} \pm 0.6$*	$75.9^{bc} \pm 1.0$*	$55.0^{b} \pm 0.2$*
1:10	$1.3^{a} \pm 0.3$	$0^{a} \pm 0$	$22.7^{a} \pm 11.4$	$16.3^{a} \pm 8.2$
1:15	$3.3^{a} \pm 0.9$	$0.7^{a} \pm 0.3$	$38.6^{a} \pm 0.5$	$26.5^{a} \pm 0.5$
1:20	$54.3^{b} \pm 2.6$	$30.3^{b} \pm 1.2$	$68.6^{b} \pm 0.3$	$51.6^{b} \pm 0.2$

*Highest mean value between the dilutions consisting of guava juice only.

^{a,b,c,d,e}Values in the same column with different superscripts differ significantly ($P<0.05$).

SEM = Standard Error of mean; VAP = path velocity; VSL = progressive velocity

Figure 4.1 shows a bar chart of the percentage of sperm motility against different dilutions in fresh ejaculate, after 4-5 hours chilled and after 16-17 hours chilled at 4°C. SEM bars also displayed at the top of each bars. From the bar chart, it shows that 1:20 dilution has a significant different in terms of motility initially though it decreases over time. .

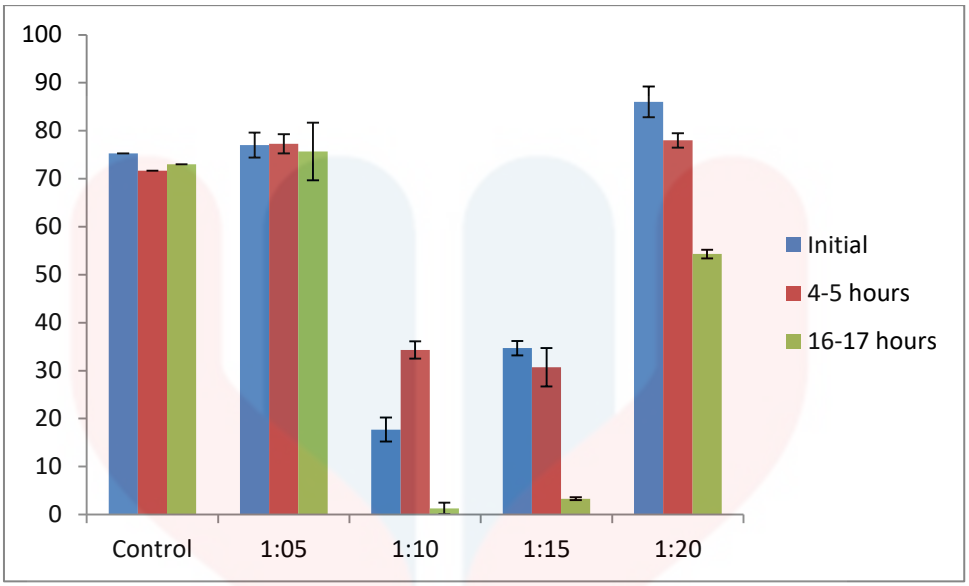

Figure 4.1 The percentage of sperm motility from fresh ejaculate, after 4-5 hours and after 16-17 hours chilled at 4°C

From Figure 4.2 it shows the bar chart of the percentage of sperm progressive motility against different dilutions in fresh ejaculate, after 4-5 hours chilled and after 16-17 hours chilled at 4°C. SEM bars also displayed at the top of each bars. Among the samples 1:15 and 1:20 show a significance fluctuation over the hours.

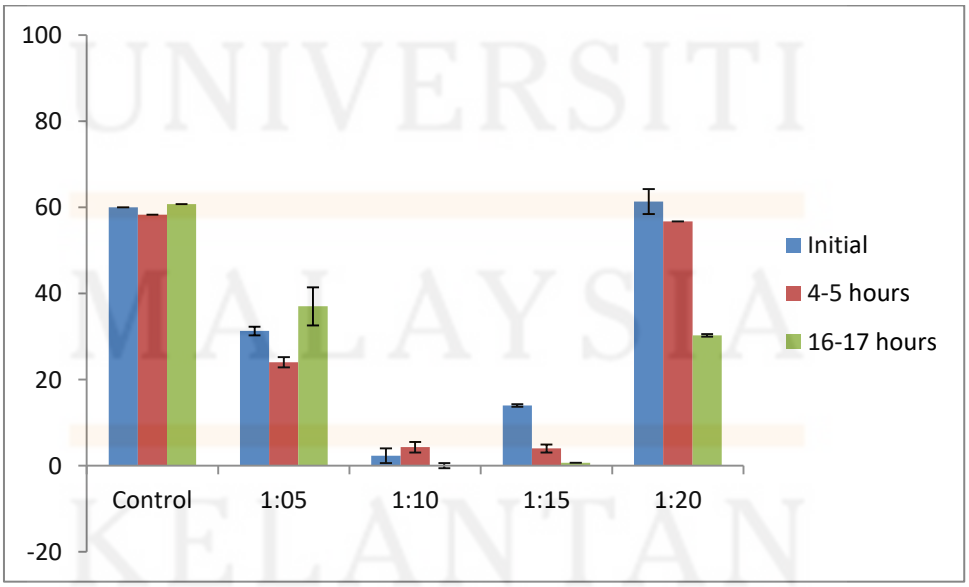

Figure 4.2 The percentage of sperm progressive motility from fresh ejaculate, after 4-5 hours and after 16-17 hours chilled at 4°C

Figure 4.3 shows the bar chart of the mean value in μm/s of sperm path velocity against different dilutions in fresh ejaculate, after 4-5 hours chilled and after 16-17 hours chilled at 4°C. SEM bars also displayed at the top of each bars. The bar chart of dilution 1:15 shows a steady decrease in its VAP while other samples show inconsistency of VAP.

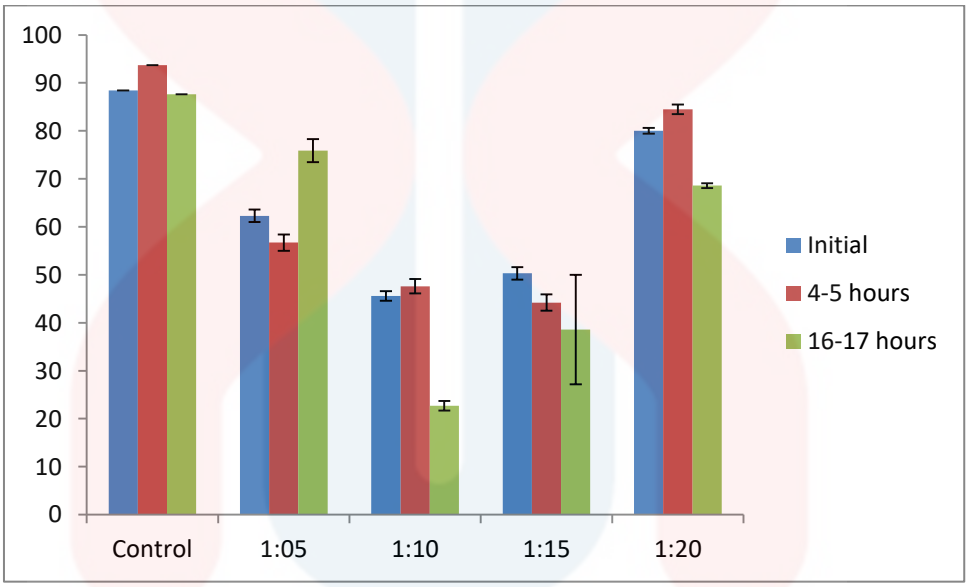

Figure 4.3 The path velocity in μm/s from fresh ejaculate, after 4-5 hours and after 16-17 hours chilled at 4°C

Figure 4.4 shows the bar chart of the mean value in μm/s of sperm progressive velocity against different dilutions in fresh ejaculate, after 4-5 hours chilled and after 16-17 hours chilled at 4°C. SEM bars also displayed at the top of each bars. The bar chart of dilution 1:10 and 1:15 also show a decreased level in its VSL while other samples show inconsistency.

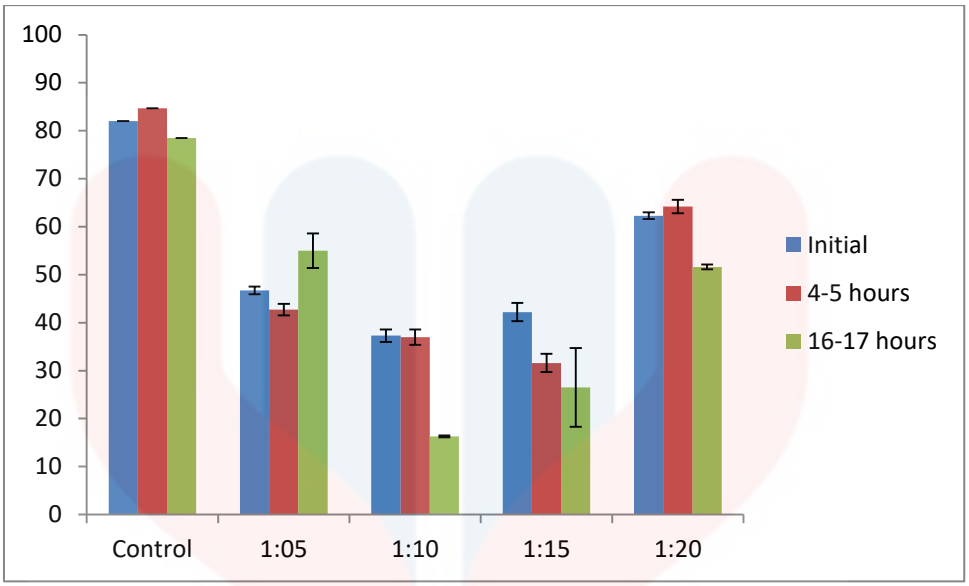

Figure 4.4 The sperm progressive velocity in µm/s from fresh ejaculate, after 4-5 hours and after 16-17 hours chilled at 4°C

Table 4.4 shows the highest dilution ratio of sperm motility, progressive motility, VAP, VSL at fresh ejaculate, after 4-5 hours and after 16-17 hours chilled at 4°C. From the table, dilution 1:20 shows the highest mean value initially and after 4-5 hours chilled though it took a down turn in all aspects after 16-17 hours chilled storage. The dilution 1:5 out to be the highest mean value in all of the parameters.

Table 4.4 Highest dilution ratio of sperm motility, progressive motility, VAP, VSL at fresh ejaculate, after 4-5 hours and after 16-17 hours chilled at 4°C

	Motility	Progressive Motility	Path Velocity	Progressive Velocity
Initial	1:20	1:20	1:20	1:20
4-5 hours	1:20	1:20	1:20	1:20
16-17 hours	1:5	1:5	1:5	1:5

VAP = path velocity; VSL = progressive velocity

Table 4.5 shows the lowest dilution ratio of sperm motility, progressive motility, VAP, VSL at fresh ejaculate, after 4-5 hours and after 16-17 hours chilled at 4°C. Initially, dilution 1:10 was the lowest mean value in all of in all the motility, progressive motility, VAP and VSL, then it changed to dilution 1:15 after 4-5 hours chilled storage. Dilution 1:10 became the lowest mean value again in all aspects except progressive motility.

Table 4.5 Lowest dilution ratio of sperm motility, progressive motility, VAP, VSL at fresh ejaculate, after 4-5 hours and after 16-17 hours chilled at 4°C

	Motility	Progressive Motility	Path Velocity	Progressive Velocity
Initial	1:10	1:10	1:10	1:10
4-5 hours	1:15	1:15	1:15	1:15
16-17 hours	1:10	1:15	1:10	1:10

VAP = path velocity; VSL = progressive velocity

4.2 Assessment of the Diluted Semen at Room Temperature

Table 4.6 shows the mean value and standard error of the mean (SEM) of sperm motility, progressive motility, path velocity (VAP) and progressive velocity (VSL) of the semen in different dilutions of fresh ejaculate at room temperature. Where it shows that the extender with guava dilutions of 1:10 has the highest mean value in sperm motility at 88% and progressive motility at 48%. 1:15 dilution has the highest mean value in terms of sperm path velocity at 68.5 μm/s and progressive velocity at 53.4 μm/s.

Table 4.6 Sperm motility, progressive motility, VAP and VSL in different dilutions of guava juice extender of fresh ejaculate at room temperature

Extender	Motility (%)	Progressive motility (%)	Path velocity (μm/s)	Progressive velocity (μm/s)
Control	$74.3^{b} \pm 1.2$	$58.3^{d} \pm 1.7$	$84.2^{c} \pm 0.7$	$78.0^{c} \pm 0.9$
1:5	$76.7^{b} \pm 1.5$	$38.0^{b} \pm 2.3$	$66.8^{b} \pm 1.3$	$51.5^{b} \pm 1.0$
1:10	$88.0^{c} \pm 0.6$*	$48.0^{c} \pm 4.6$*	$63.2^{a} \pm 0.6$	$47.0^{a} \pm 0.4$
1:15	$77.3^{b} \pm 2.3$	$42.3^{bc} \pm 2.8$	$68.5^{b} \pm 0.6$*	$53.4^{b} \pm 1.3$*
1:20	$63.0^{a} \pm 3.1$	$28.0^{a} \pm 1.5$	$65.9^{b} \pm 0.4$	$46.2^{a} \pm 0.6$

*Highest mean value between the dilutions consisting of guava juice only.

^{a,b,c,d,e}Values in the same column with different superscripts differ significantly ($P<0.05$).

SEM = Standard Error of mean; VAP = path velocity; VSL = progressive velocity

Table 4.7 shows the mean value and standard error of the mean (SEM) of sperm motility, progressive motility, VAP and VSL of the semen in different dilutions after 4-5 hours at room temperature. Where it shows that the extender with guava dilution of 1:15 has the highest mean value in sperm progressive motility at 57.3%, VAP at 77.4 μm/s and VSL at 65.8 μm/s while, 1:5 dilution has the highest percentage mean value at 72.7% in sperm motility between the extenders with guava juice.

Table 4.7 Sperm motility, progressive motility, VAP and VSL in different dilutions of guava juice extender after 4-5 hours at room temperature

Extender	Motility (%)	Progressive motility (%)	Path velocity (μm/s)	Progressive velocity (μm/s)
Control	$96.3^{d} \pm 0.3$	$78.0^{d} \pm 0.6$	$107.2^{d} \pm 2.2$	$93.2^{d} \pm 1.0$
1:5	$72.7^{c} \pm 0.9$*	$35.0^{b} \pm 0.6$	$72.2^{b} \pm 1.1$	$53.9^{b} \pm 1.3$
1:10	$63.7^{b} \pm 2.2$	$31.3^{b} \pm 2.4$	$73.4^{bc} \pm 1.4$	$55.9^{b} \pm 0.5$
1:15	$67.3^{b} \pm 1.2$	$57.3^{c} \pm 0.9$*	$77.4^{c} \pm 0.8$*	$65.8^{c} \pm 0.5$*
1:20	$44.3^{a} \pm 1.2$	$10.3^{a} \pm 0.3$	$52.5^{a} \pm 1.1$	$40.6^{a} \pm 0.4$

*Highest mean value between the dilutions consisting of guava juice only.

^{a,b,c,d,e}Values in the same column with different superscripts differ significantly ($P<0.05$).

SEM = Standard Error of mean; VAP = path velocity; VSL = progressive velocity

Table 4.8 shows the mean value and standard error of the mean (SEM) of sperm motility, progressive motility, VAP and VSL of the semen in different dilutions after 16-17 hours at room temperature. Dilution of 1:5 shows the highest percentage

of the mean at sperm motility at 25.7% and progressive motility 3.7%, on the other hand, 1:15 dilution is the highest mean value in μm/s in both VAP at 79.9 μm/s and VSL at 63 μm/s.

Table 4.8 Sperm motility, progressive motility, VAP and VSL in different dilutions of guava juice extender after 16-17 hours at room temperature

Extender	Motility (%)	Progressive motility (%)	Path velocity (μm/s)	Progressive velocity (μm/s)
Control	$74.3^{c} \pm 1.2$	$58.3^{c} \pm 1.7$	$94.3^{d} \pm 2.8$	$82.9^{c} \pm 2.6$
1:5	$25.7^{b} \pm 2.2$*	$3.7^{b} \pm 0.3$*	$47.1^{b} \pm 1.0$	$33.0^{a} \pm 1.3$
1:10	$4.3^{a} \pm 0.3$	$1.0^{a} \pm 0$	$79.8^{c} \pm 0.2$	$63.0^{b} \pm 0.3$
1:15	$4.0^{a} \pm 0.6$	$1.0^{a} \pm 0$	$79.9^{c} \pm 0.6$*	$63.0^{b} \pm 1.3$*
1:20	$2.7^{a} \pm 0.3$	$1.0^{a} \pm 0$	$41.3^{a} \pm 1.9$	$34.2^{a} \pm 2.1$

*Highest mean value between the dilutions consisting of guava juice only.

^{a,b,c,d,e}Values in the same column with different superscripts differ significantly ($P<0.05$).

SEM = Standard Error of mean; VAP = path velocity; VSL = progressive velocity

Figure 4.5 shows the percentage of the mean value and standard error of the mean (SEM) of sperm motility from fresh ejaculate, after 4-5 hours and after 16-17 hours at room temperature. It can be seen that the all the dilutions of guava juice shows a decrease in sperm motility over the hours but the control extender shows a significant rise before it fluctuates.

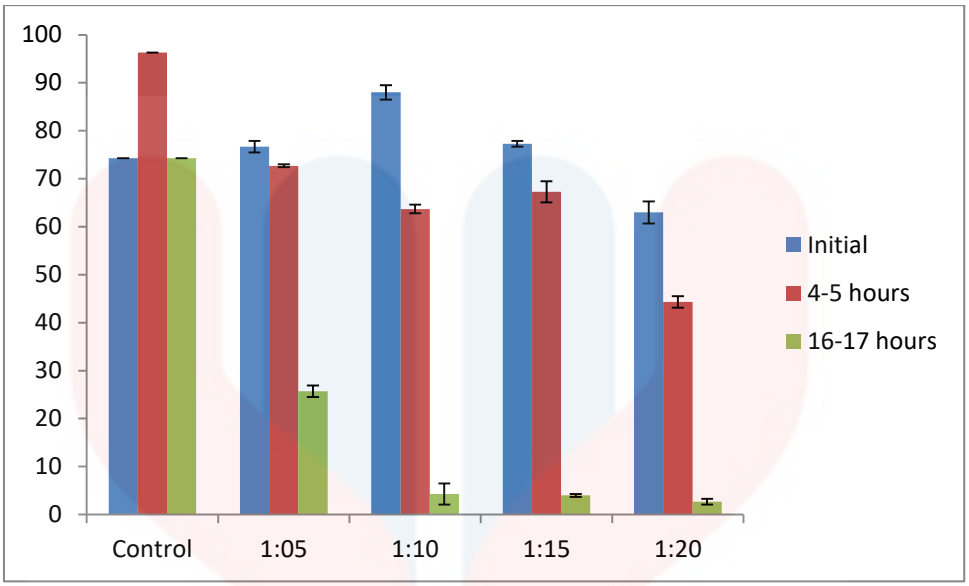

Figure 4.5 The percentage of sperm motility from fresh ejaculate, after 4-5 hours and after 16-17 hours at room temperature

Figure 4.6 shows the percentage of the mean value and standard error of the mean (SEM) of sperm progressive motility from fresh ejaculate, after 4-5 hours and after 16-17 hours at room temperature. A significant drop in all of the guava juice dilutions can be seen for the sperm progressive motility.

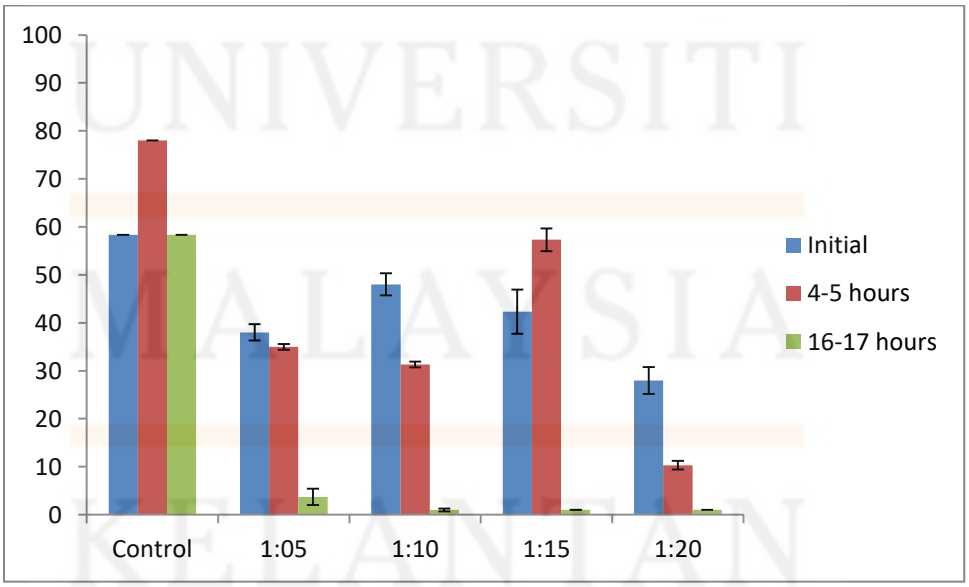

Figure 4.6 The percentage of progressive motility from fresh ejaculate, after 4-5 hours and after 16-17 hours at room temperature

Table 4.7 shows the mean value in μm/s and the standard error of the mean (SEM) of sperm VAP from fresh ejaculate, after 4-5 hours and after 16-17 hours at room temperature. From the bar chart it can be seen a distinctive difference between the extender of the control and the extender of the guava samples.

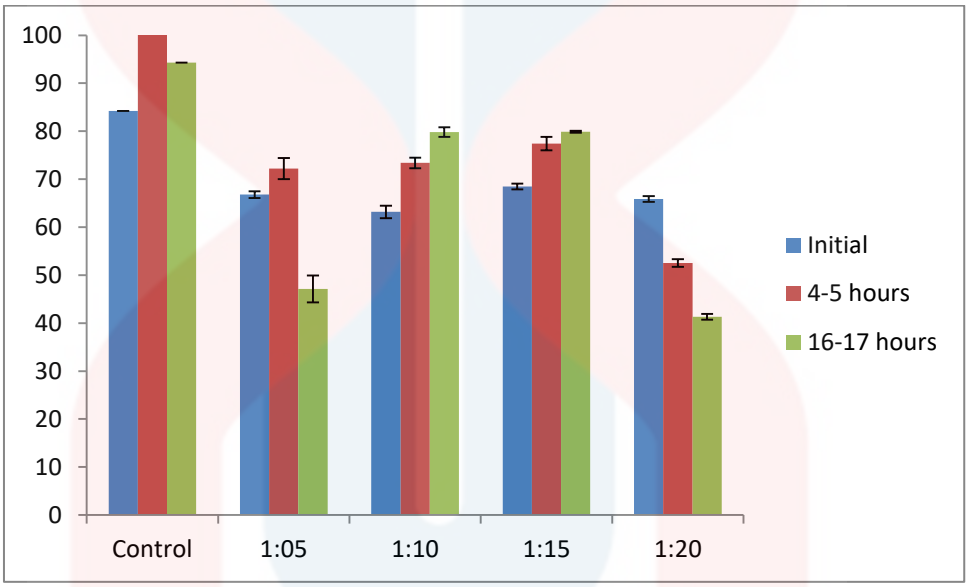

Figure 4.7 The sperm path velocity in μm/s from fresh ejaculate, after 4-5 hours and after 16-17 hours at room temperature

Table 4.8 shows the mean value in μm/s and the standard error of the mean (SEM) of sperm VSL from fresh ejaculate, after 4-5 hours and after 16-17 hours at room temperature. A huge difference can also be seen between the control and the guava juice extender samples.

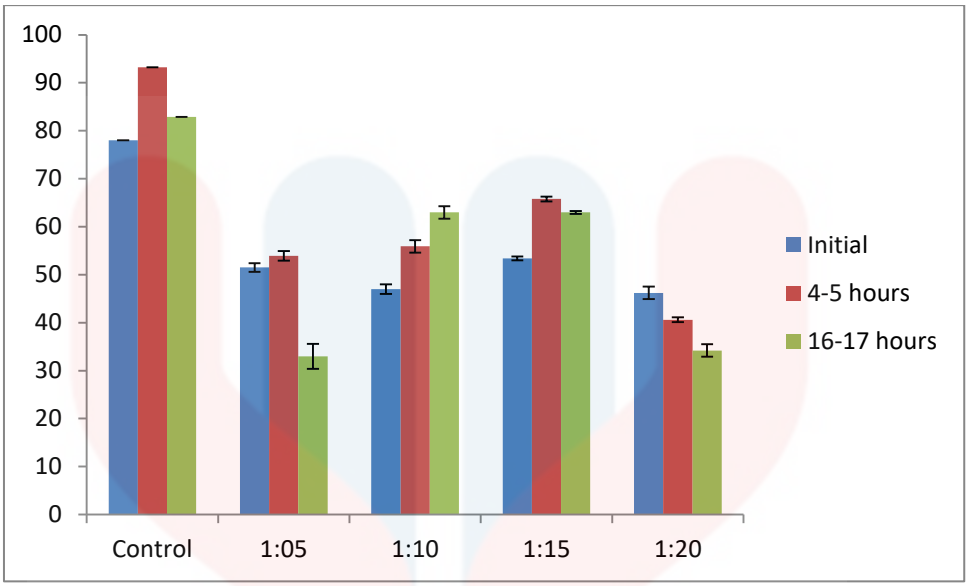

Figure 4.8 The sperm progressive velocity in µm/s from fresh ejaculate, after 4-5 hours and after 16-17 hours at room temperature

Table 4.9 shows the highest dilution ratio of sperm motility, progressive motility, VAP, VSL at fresh ejaculate, after 4-5 hours and after 16-17 hours chilled at room temperature. 1:10 dilution was the highest mean value in all aspects assessed in this research at fresh ejaculate. It changed place with dilution 1:15 after 4-5 hours. The dilution 1:10 became the highest mean values in motility, VAP and VSL except for progressive motility.

Table 4.9 Highest dilution ratio of sperm motility, progressive motility, VAP, VSL at fresh ejaculate, after 4-5 hours and after 16-17 hours chilled at room temperature

	Motility	Progressive motility	VAP	VSL
Fresh ejaculate	1:10	1:10	1:15	1:15
4-5 hours	1:5	1:15	1:15	1:15
16-17 hours	1:5	1:5	1:15	1:15

VAP = path velocity; VSL = progressive velocity

Table 4.10 shows the lowest dilution ratio of sperm motility, progressive motility, VAP, VSL at fresh ejaculate, after 4-5 hours and after 16-17 hours chilled at room temperature. Initially, like Table 4.9, the 1:10 dilution was the lowest mean value in every parameter, after 4-5 hours 1:5 dilution takes the lowest place. Eventually 1:10 turns back into the lowest mean values in sperm motility, VAP and VSL.

Table 4.10 Lowest dilution ratio of sperm motility, progressive motility, VAP, VSL at fresh ejaculate, after 4-5 hours and after 16-17 hours chilled at room temperature

	Motility	Progressive motility	VAP	VSL
Fresh ejaculate	1:20	1:20	1:10	1:20
4-5 hours	1:20	1:20	1:20	1:20
16-17 hours	1:20	1:10, 1:15, 1:20	1:20	1:5

VAP = path velocity; VSL = progressive velocity

CHAPTER 5

DISCUSSION

Over the years more ingredients such as fruits and dairy products are tested to be one of the ingredients in the extenders as to suit the economical limitations and geographical barriers. This research used the egg yolk based extenders as the control and guava juice at different rate of dilutions. From the results it can be seen that the control or egg yolk extender are among the higher if not the highest mean value in terms of motility, progressive motility, path velocity (VAP), and progressive velocity (VSL) compared to the extenders with guava juice. This may be due to the fact that the egg yolk extenders formula used are of the established mix of ingredients. Where extensive researches have been proven and conducted on improving the egg yolk based extender which helps in maintaining the sperm activity even after chilled stored. On the other hand, the guava juice extenders ingredients were composed based on Salamon & Maxwell (2000) mixed ingredients, which is also used in making the control extenders. Specific amount of guava juice best complimenting the other three ingredients involved in the extender for diluting ram sperm is yet to be proven from other various sources of scholarly journal. Various authors suggested that sperm of different species react differently to the extenders, frozen storage and liquid or chilled storage (Varisli et al., 2013).

Sperm diluted with extenders and kept at room temperature (RT) is a way to test the survival time of the sperm without the reduction of temperature needed to decrease the metabolic activity as to maintain its quality. Survival of the semen during the field work without any chilling or freezing may contribute in estimating the ample time before the need to reach the laboratory. According to Gibb &Aitken (2016), this way of storage will reduce the uses of ingredients that protect the sperm from any

cryodamage or stabilising the cell membrane. Unfortunately, there are some drawbacks in this type of storing the semen such as a surge of microbial growth and number due to the nutritious substance in the extender (Gibb & Aitken, 2016).

Based on the Table 4.4 there is a rise of the sperm progressive motility kept at 4°C, this may be due to the fact the reduction in metabolic activity of the sperm according to (Paulenz et al., 2002) revived the energy of slow motile sperm, or maybe they were energized after their “sleep” state. In the Table 4.9 there is a fluctuation of sperm progressive motility where it might be because of the dilution in 1:20 is thinner where, the ingredients that keeping it to survive such as energy source was less in amount than needed. On the other hand, inconsistent result of the extender may be due to human error from semen collection to mixing the extender. There is a vast reason to why these result shows inconsistency, but as sperm are very sensitive cells, proper protocols should be followed in handling semen. Adding another storing hour between 4-5 hours to 16-17 hours for both groups would also help in counteracting this inconsistency patterns and determine the best dilutions for all the parameters in both RT and 4°C.

The three-way ANOVA shows that there is a significant difference between dilution rates, storage temperature and storage time interaction effect in this experiment. This means that the dilution rate of guava juice extender, storage temperature and storage time does affect the characteristics of the sperm.

CHAPTER 6

CONCLUSION

6.1 Conclusion

This study of chilled stored sperm in guava juice extender shows that after 16-17 hours of chilling the sperm in dilution 1:15 guava juice had the highest mean value in sperm motility (75.7%), progressive motility (37.0%), VAP (75.9 μm/s) and VSL (55.0 μm/s). In comparison, the control extender which contained egg yolk, gives a higher mean value in terms of progressive motility (60.7%), VAP (87.6 μm/s) and VSL (78.5 μm/s), except for sperm motility (73.0%) after 16-17 hours chilled storage. Some improvement on guava juice extender ingredients and maybe handling may produce results that are up to par with control extender.

On the other hand, dilution 1:15 gave the highest mean value after 16-17 hours of storing in ambient temperature in terms of sperm VAP (79.9 μm/s) and VSL (63.0 μm/s), while control extender produces higher mean value for sperm VAP (94.3 μm/s) and VSL (82.9 μm/s). Dilution 1:5 had the highest mean value in terms of sperm motility (25.7%) and progressive motility (3.7%) while control extender also produces a significantly higher mean value in terms of sperm motility (74.3%) and progressive motility (58.3%) after 16-17 hours of room temperature storage. The major difference between control extender and guava juice extender in terms of motility and progressive motility shows that the control extender is significantly better at maintaining the sperm characteristics than guava juice extender.

The guava juice extender of dilution 1:5 can be used as an alternative extender in chilled storage for ram, although semen containing guava juice can be used for chilled storing semen as the ram sperm survived the extender although

further research regarding the use of guava juice in the extenders should be conducted to improve the formula of this extender. Comparing the results between control extender and guava juice extender, it clearly shows that guava juice as an alternative ingredient provide results that are very noticeable which means that the null hypothesis is accepted.

6.2 Recommendation

Adding another storing hour in between 4-5 hours to 16-17 hours for both groups as it can give a more concrete and definitive result is my recommendation. Semen analysis can also be assessed every two hours or so to monitor the sperm and detecting any significant drop in their survival rate and characteristics.

Further study on the use of guava juice in ram extender should be done as there is only one scholarly journal that use guava as an alternative extender and it was used on bulls' semen. Hopefully further research can provide any scientifically detailed prove on how the guava helps in maintaining the semen quality and characteristics during storage. In addition, further study on ram semen diluted and stored at room temperature should also be done, for there is lack of studies in extended ram semen as well as diluted ram semen stored in room temperature.

Adding more parameters on the diluted semen with guava juice extender to justify which dilution gives the best result to the sperm characteristics. The addition of parameters may affect the sperm characteristics.

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APPENDIX

Media recipe

Table A.1 Control extender recipe

Components	Amount
Sodium citrate	2.37 g
Glucose	0.50 g
Egg yolk	15 ml
Penicillin	100,000 i.u.
Streptomycin	100 mg
Distilled water	Up to 100 ml

Table A.2 Guava juice extender recipe

Components	Amounts
Guava juice	20 ml
Dimethyl sulfoxide	15 ml
Sodium citrate	2.37 grams
Distilled water	Up to 100 ml

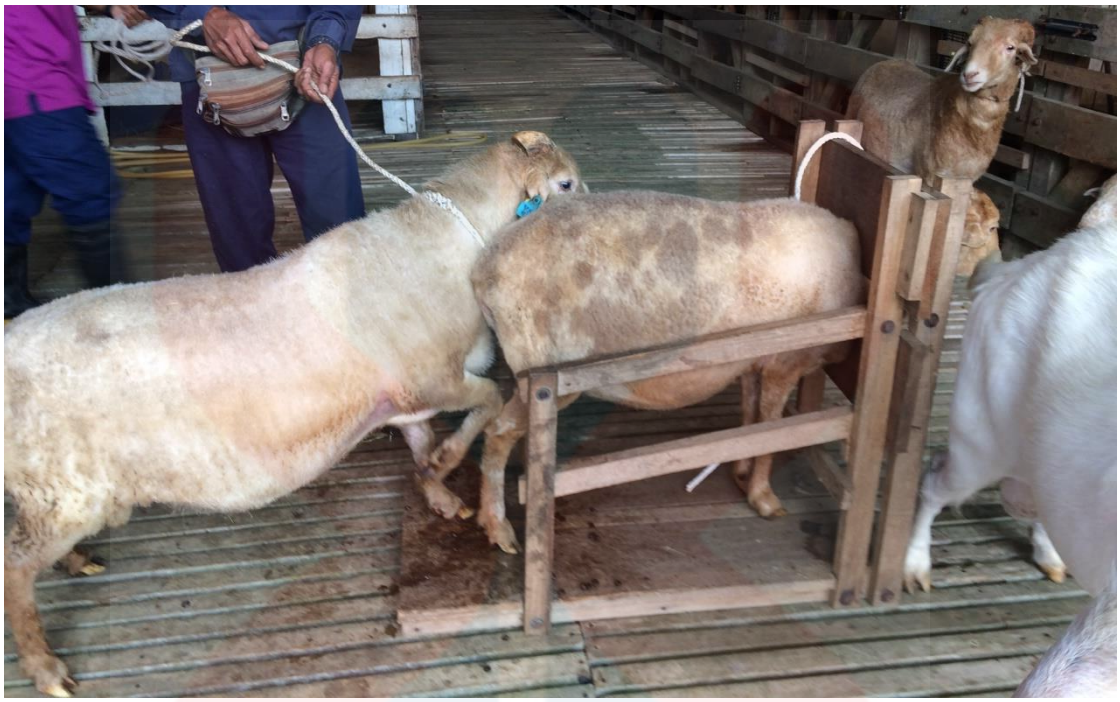

Figure A.1 Ram mounting the teaser

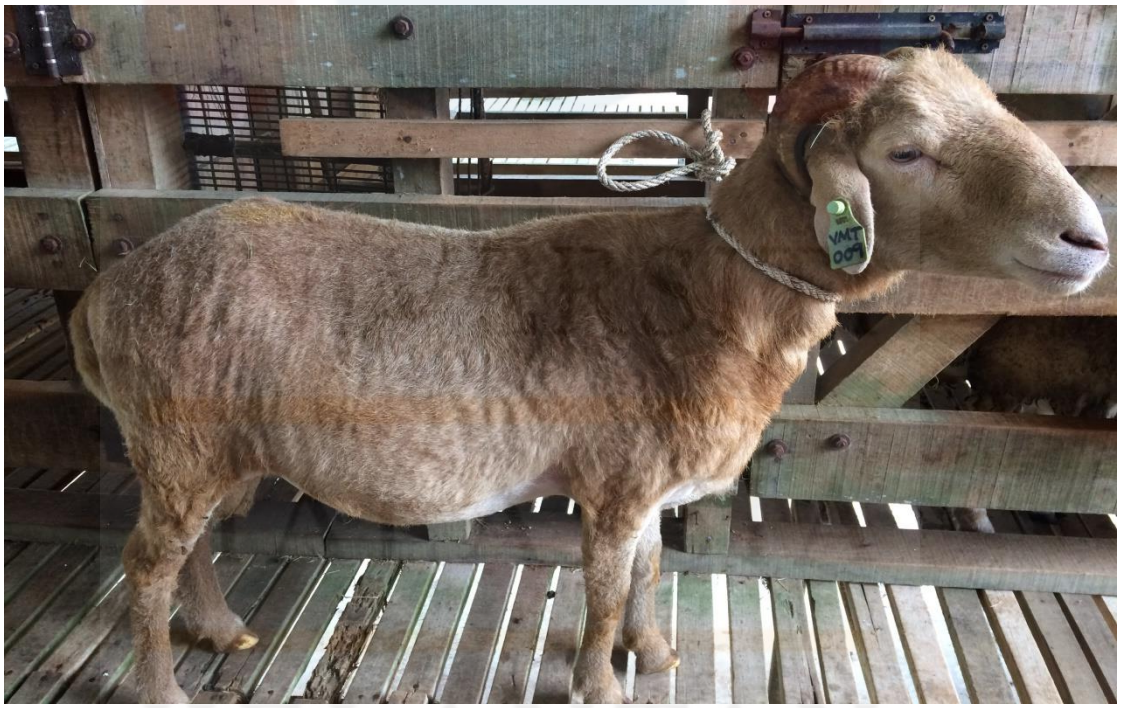

Figure A.2 Mature Malin ram used in semen collection