Effect of Royal Jelly as Supplementation in Feed and Extender on Sperm Quality of Rabbits

by

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Kesan Royal Jelly sebagai Suplemen dalam Makanan dan Extender terhadap Kualiti sperma Arnab

ABSTRAK

Latar Belakang: Royal Jelly (RJ) digunakan sebagai makanan tambahan untuk meningkatkan pengeluaran arnab di Malaysia sambil mengkaji kesannya terhadap kesuburan arnab jantan. Objektif: Untuk menilai kesan RJ pada kualiti sperma melalui pemberian oral dan extender dan untuk menyiasat kesan RJ pada histologi testis arnab. Bahan dan Kaedah: Dua puluh arnab jantan dibahagikan kepada empat kumpulan, setiap satu terdiri daripada lima ekor. Setiap pejantan dalam kumpulan kawalan (C), T1, T2, dan T3 telah diberi suap dengan 0mg, 100 mg, 200 mg, dan 300 mg RJ/kg berat badan masing-masing tiga kali seminggu, untuk tempoh 8 minggu. Penilaian sperma dilakukan setiap dua minggu sekali. Sampel dikumpulkan dan dibahagikan kepada dua extender: extender kawalan (TCFY) dan extender RJ (TCFY dengan 10mg RJ). Ciri-ciri fizikal sperma, sperma hidup, pergerakan sperma, pergerakan aktif sperma, keabnormalan sperma, dan diameter tubul seminiferus dinilai menggunakan mikroskop kompaun. Keputusan: Penjantan dalam T1 menunjukkan peratusan yang lebih tinggi ($p<0.05$) pada sperma hidup, pergerakan sperma, dan pergerakan aktif sperma berbanding dengan T2 dan T3 dalam kedua-dua extender. Walau bagaimanapun, tidak terdapat perbezaan yang signifikan ($p>0.05$) dalam sperma abnormal antara semua kumpulan. Seterusnya, extender RJ menunjukkan perbezaan ketara dalam sperma hidup daripada extender kawalan (masing-masing, 58.10±1.37 dan 46.52±1.79). Tambahan pula, tidak terdapat perbezaan yang signifikan ($p>0.05$) dalam berat testis antara kumpulan tetapi diameter tubul seminiferus menunjukkan positif yang signifikan ($p<0.05$) dalam T1 berbanding kumpulan kawalan (masing-masing, 246.03±1.92 dan 194.72±1.66). Kepekatan RJ yang tinggi mempunyai kesan negatif terhadap kualiti sperma. Ini mungkin disebabkan oleh kepekatan RJ yang tinggi. Komponennya boleh mengakibatkan ketidakseimbangan dalam tindak balas redoks disebabkan oleh aktiviti antioksidan yang berlebihan. Kesimpulan: Ini menyimpulkan bahawa RJ terbukti meningkatkan sperma hidup dan pergerakan sperma, tetapi dos RJ sangat penting kerana ia mempunyai beberapa kesan ke atas kualiti sperma.

Kata kunci: Royal Jelly, Ciri-ciri sperma, Extender, Testis

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ABSTRACT

Background: Royal jelly (RJ) was applied as a supplement to boost rabbit production in Malaysia while examining its impact on buck fertility. *Aims and objectives*: To evaluate the effect of RJ on sperm quality by oral administration and extender and to investigate the effect of RJ on the histology of rabbit's testis. *Material and Methods*: Twenty male rabbits were divided into four groups, each consisting of five bucks. Bucks in the Control (C), T1, T2, and T3 groups were supplemented with 0mg, 100 mg, 200 mg, and 300 mg of RJ/kg body weight three times a week, respectively, for 8-weeks of period. A sperm evaluation was performed once every two weeks. The sample was pooled and divided into two extenders: a control extender (TCFY) and an RJ extender (TCFY with 10mg of RJ). Sperm characteristics, sperm viability, sperm motility, sperm progressive motility, sperm abnormality, and seminiferous tubule diameter were evaluated using a compound microscope. *Results*: Bucks in T1 showed significantly higher ($p<0.05$) sperm viability, sperm motility, and sperm progressiveness compared with T2 and T3 in both the control and RJ extender. However, there was no significant difference ($p>0.05$) in abnormal sperm between all groups. Next, RJ extenders showed a significant difference in viability sperm than control extenders (58.10±1.37 and 46.52±1.79, respectively). Furthermore, there was no significant difference ($p>0.05$) in testis weight between groups, but seminiferous tubule diameter showed a significantly positive ($p<0.05$) in T1 compared to control groups (246.03±1.92 and 194.72±1.66, respectively). A high concentration of RJ has a negative effect on sperm quality. This may be due to the high concentration of RJ. Its components may result in an imbalance in redox reactions due to their excessive antioxidative activity. *Conclusion*: This concluded that RJ proves to improve sperm viability and sperm motility, but the dose of RJ was very important because it has several effects on sperm quality.

Keywords: Royal Jelly, Sperm Characteristic, Extender, Testis

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LIST OF ABREVIATIONS

ART	Assisted Reproductive Technology
IVF	*In vitro* fertilization
AI	Artificial Insemination
RJ	Royal jelly
pH	Acidity or basicity
B6	Pyridoxine
B5	Pantothenic acid
mg/100 g	Milligram per 100 grams
LH	Luteinizing hormone
FSH	Follicle stimulating hormone
ODD	Oral drug delivery
Vit E-Se	Vitamin E and Selenium
MDA	Malondialdehyde
NZW	New zealand white
BSA	Bovine serum albumin
NaCl	Sodium chloride
KCl	Potassium chloride
PMAI	Plasma membrane and acrosomal integrity
TIL7	0.600 $mg.mL^{-1}$ of tilmicosin
TILK	Control groups (without tilmicosin)
mg/mL	Milligrams per millilitre
$10^6 mL^{-1}$	Cells per volume
TIL1	0.300 $mg.mL^{-1}$ of tilmicosin
DNA	Deoxyribonucleic acid

AV	Artificial vagina
Kg	Kilogram
UMK	University Malaysia Kelantan
C	Control groups – no royal jelly
T1	Treatment 1 – 100 milligrams of royal jelly
T2	Treatment 2 – 200 milligrams of royal jelly
T3	Treatment 3 – 300 milligrams of royal jelly
Mg	Milligram
mL/kg	Millilitre per kilogram
TCFY	Tris-citric acid-fructose-egg yolk
G	Gram
mL	Millilitres
TCFYRJ	Tris-citric acid-fructose-egg yolk with 10 milligrams of royal jelly
µL	Microliter
Mm	Millimetre
FFPE	Formalin-fixed paraffin- embedded
µm	Micrometres
H&E	Haematoxylin and eosin
ANOVA	Analysis of variance
SPSS	Statistical Package for the social science
SEM	Standard error of mean
ABP	Androgen binding protein
T	Testosterone
ROS	Reactive oxygen species
GSH	Glutathione

GST	Glutathione S-transferase
GSTT1	Glutathione S-transferase theta 1
PG	Pregabalin drug
mg/day	Milligram per day
AMP	Adenosine monophosphate
cAMP	Cyclic adenosine monophosphate
mg/kg	Milligram per kilogram
BW	Body weight
mg/6mL	Milligram per 6 millilitres
TEY	Tris egg yolk
TEY-1	Tris egg yolk with 1% of royal jelly
TEY-0.25	Tris egg yolk with 0.25% of royal jelly
TEY-0.50	Tris egg yolk with 0.50% of royal jelly
µg	Microgram
mg/kg/day	Milligram per kilogram per day
BTB	Blood testes barrier
GnRH	Gonadotropin-releasing hormone
HCG	Human chorionic gonadotropin
PMSG	Pregnant mare serum gonadotropin
SC	Sperm count
VS	Viability sperm
MS	Motility sperm
PM	Progressive motility
AB	Abnormal sperm
TW	Testis weight
STD	Seminiferous tubule diameter

LIST OF SYMBOLS

°C	Degree Celsius
%	Percentage
°	Angle degree
<	Less than to
>	More than to
±	Plus or minus
/	Divided by

CHAPTER 1

INTRODUCTION

1.1 Background Research

In early 2020- 2021, farmers faced the difficulty recovering their losses during two-year lockdowns that required prompt action to guarantee that food supply and demand were balanced (Hashim *et al*., 2023). Malaysia has had to discover alternative food sources due to the current issue with the shortage of white meat, particularly from the chicken sector (Saidon *et al*., 2023). In order to meet the growing demand for high-protein white meat, the cuniculture or rabbit farming industry had to grow its stock (Hashim *et al*., 2023). But the cost of commercial feed was still very high due to manufacturer overhead costs due to the small volume, and rabbit meat was still new to Malaysians (Gunalan *et al*., 2019).

According to Ali *et al*. (2023), self-sufficiency has revealed declining and stagnant patterns caused by a huge discrepancy between domestic output and the market demand for meat products. Many tactics and initiatives are put in place to increase the amount of beef and mutton produced locally, but the results are still negligible (Ali *et al*., 2023). Since chicken is the most often consumed meat and there is a significant demand for chicken eggs in Malaysian diets, chickens make up a large portion of the country's livestock population (Zayadi, 2021). When compared to non-ruminant farms, the number of farms housing ruminant animals, such as goats and cattle, is substantially higher. This is due to the fact that large-scale enterprises dominate the non-ruminant farming sector,

whereas ruminant producers are typically smallholders. Malaysia has currently attained a comfortable self-sufficiency ratio of over 100% for chicken meat and over 90% for swine meat, but less than 30% for ruminant meat. (Zayadi, 2021).

Assited Reproductive Technology (ART) such as artificial insemination (AI) and *in vitro* fertilization (IVF) improved the reproductive performance of rabbits. It can assist the farmer in selecting the ideal genetics for rabbits to yield premium meat by selecting the high-quality breed for mating. There are a few breeds of rabbit that can naturally produce a high amount of meat, such as the New Zealand White, Rex, and Flemish Giant. Farmers can increase the production of rabbit without using natural mating. It can save on the cost of buying a male rabbit and its maintenance. AI can impregnate a great number of does on the same day with only a buck (Lebas *et al*., 1997). It can be used to increase the production of the rabbit industry. The process of collecting sperm cells is called semen collection. AI in the livestock industry was recommended to increase the productivity of the animal. AI in rabbits was not fully developed in Malaysia. It was because of the lack of experience and less knowledge on AI in rabbit.

Freshly diluted semen was used to perform AI in animals, but the pregnancy rate of the female was low. This was due to the lack of energy, nutrients, and temperature shock for the sperm cell (Gadea, 2003). Then, semen extenders were formulated to supply the nutrient-needed energy of the sperm, protect the sperm against temperature shock, control the pH, and prevent the growth of bacteria (Gadea, 2003). There was a risk during semen transportation because the semen will be exposed to light and different temperatures. During this process, an additional supplement can increase the percentage of live sperm in the extender by supplying extra nutrients for the sperm. Semen extenders and

storage conditions need to be improved to increase storage time and maintain their function (Di Iorio *et al*., 2014).

There are a few methods to increase the lifespan of the sperm, such as oral supplementation and an additional supplement in semen extender. The semen extender also proved to improve the semen quality of the animal. It was reported that Oral administration of royal jelly (RJ) and bee honey improved body weight and produced better semen quality than control treatment in rabbits (Khadr *et al*., 2015). Other than that, some researchers stated that oral administration of royal jelly increases the sperm function parameters of male mice (Ibrahim *et al*., 2017).

Although the semen extender's role was to supply nutrients to the sperm, it also extends the lifespan of the sperm. Then, the semen storage was done either to give long-term storage or short-term storage for the sperm. *Mohamed et al*. (2012) reported that the best way to store sperm was at 25℃ during transportation in order to preserve the sperm's characteristics. Bresciani *et al*. (2016) stated that fresh or chilled semen can be a great tool to avoid economic losses because it was cost-efficient and cheaper than cryopreservation.

The aim of this study is to investigate the effect of Royal Jelly on sperm quality through oral supplementation and as semen extender to improve semen quality of rabbits such as sperm motility, viability, and abnormal; to investigate the effect of royal jelly on the histology of rabbit testis and to investigate the correlation between sperm quality with histology of testis in rabbit supplemented with Royal Jelly.

1.2 Problem Statement

The pandemic lockdowns caused a sharp rise in the rabbit population, which made it difficult for the farmers to sell their product (Hashim *et al*., 2022). Malaysia, a developing nation, was not exempt from the problems related to food security (Saidon *et al.*, 2023). Compared to other animals, poultry make up the largest portion of Malaysia's population because it is the most popular sort of meat consumed there. According to Zayadi (2021), poultry meat is favoured by a varied range of Malaysian ethnic groups because it is not forbidden by religion like beef or pork. The present problem of the scarcity of white meat, especially from the chicken industry, has forced Malaysia to find other food sources (Saidon *et al*., 2023).

Hanim *et al*. (2023) state that the cuniculture, or rabbit farming, industry had to increase its stock in order to fulfil the growing demand for high-protein white meat. But the rabbit industry is still quite young, especially in Malaysia. The market for rabbit meat has not grown because buyers are worried about keeping rabbits as pets rather than eating them (Hashim *et al*., 2022). Although the production of meat and pet rabbits has increased, Malaysia has not yet established ART for this species. ART such as semen collection and artificial insemination are common practices in the livestock sector, particularly in the beef industry. Agricultural organisations in the area, including the cooperative, have not promoted rabbit meat to increase its popular acceptance (Mohd Aminuddin *et al*., 2023). According to Mohd Aminuddin *et al*. (2023), the rabbit meat niche has the potential to positively impact the local economy and well-being of Cenderong Balai, Hilir Perak, and other cuniculture industry participants in Malaysia.

There are a few methods to increase sperm quality in animals. For example, using supplements for the animal to increase their reproduction, such as oral supplementation and semen extenders. Khadr *et al*. (2015) concluded that RJ or/and bee honey given through oral administration can increase the male New Zealand white rabbit's body weight, testis weight, testis index, and epididymis weight. Both oral supplements and extenders can improve the semen quality of the animal, but which method has the best effect on the sperm quality, and what was the effect of the royal jelly to the testicle in rabbits? The aim of this study was to determine the optimum concentration of Royal Jelly on sperm quality in male rabbits; to investigate the effect of royal jelly on histology feature of testis in rabbits; and to investigate the correlation between sperm quality with histology of testis in rabbit supplemented with Royal Jelly.

1.3 Hypothesis

It was hypothesised that Royal Jelly can affect the sperm quality and the histology of testis in male rabbits. The current study also hypothesised that there was a significant effect on sperm quality and histology of testis in different concentration of RJ. It was also expected to have a significant difference on the correlation between sperm quality with histology of testis in rabbit supplemented with RJ.

1.4 Objective of Study

The objectives of this study were: -

1. To determine the effect of optimum concentration of royal jelly on sperm quality in male rabbits.
2. To investigate the effect of different concentrations of royal jelly on histology feature of testis in rabbits.
3. To investigate the correlation between sperm quality with histology of testis in rabbit supplemented with royal jelly.

1.5 Significance of Study

The effects of Royal Jelly supplementation in feed and extender on sperm quality of rabbits was not fully understood. No formal studies on the effect of Royal Jelly on different method between oral administration and extender has been undertaken thus far. In this study, we compared the effects of Royal Jelly supplemented in feed and extender on sperm quality of rabbits.

1.6 Scope of Study

This research was mainly focus on Principle of assisted reproductive technology such as sperm quality. The research was conducted to see the effect of Royal Jelly on the quality of the sperm and histology of the testis.

CHAPTER 2

LITERATURE REVIEW

2.1 Royal Jelly

Apis mellifera L. was the scientific name of the Royal Jelly. A young nurse worker bees' hypopharyngeal glands secrete royal jelly (RJ), a viscous, incredibly nutritious milky white, creamy substance that was fed to the queen, queen larvae, and other young larvae (Maghsoudlou *et al*., 2019). The RJ was made up entirely of 67% water, 12.5% crude protein, 11% simple sugars (monosaccharides), and a comparatively high quantity of fatty acids (5%) (Ahmadnia *et al*., 2015). Kunugi and Ali (2019) stated that fresh RJ consists of 60-70% water with pH usually ranges between 3.6 and 4.2 while, sugars constitute of 7.5–15% of RJ content. About 90% of the total sugar fraction of RJ was fructose and glucose, while sucrose accounts for 0.8–3.6% (Kunugi & Mohammed Ali, 2019). Additionally, RJ contains B-plex vitamins, pyridoxine (B6), pantothenic acid (B5), acetylcholine, vitamins A, C, D, and B, minerals, enzymes, hormones, and antimicrobial components (Al-Sanafi *et al*., 2007). The most rich vitamin in RJ is pantothenic acid (52.8 mg/100 g), followed by niacin (42.42 mg/100 g) (Kunugi & Mohammed Ali, 2019).

Al-sanafi and Abdulla (2007) reported that RJ can significantly improve semen motility and testosterone level which are important for motility of the sperm. The RJ consumption can increase the seminal parameters especially the motility and sperm count (Ahmadnia *et al*., 2015). The addition of RJ to semen

extenders has a variety of effects on the quality of the sperm, as well as the ability to keep sperm viability, motility, and fertility. It also interacts with the activity of the hormones such as luteinizing hormone (LH), follicle stimulating hormone (FSH), and testosteron (Abdelnour *et al*., 2020). Some researchers conclude that RJ can probably be treated as treatment to improve fertility (Ghanbari *et al*., 2018). El-Sherbiny (2014) conducted research on the impact of RJ and bee honey added to semen extender on rabbit semen fertility capacity at room temperature. Research has shown that the addition of RJ and bee honey can preserved the fertility capability and semen quality, including progressive motility, viability, and abnormal spermatozoa, for at least two days when the semen was stored at room temperature (El-Sherbiny, 2014).

The RJ improved its likelihood of having a significant impact on fertility by promoting the growth of bee larval cells and maintaining ovulation capacity. It was assumed that the polyunsaturated fatty acids and phospholipids in RJ serve as a potential physiological mechanism for this effect, acting to protect the sperm cell membrane from oxidative damage, interact with testosterone receptors, promote cell growth, and elevate testosterone levels (Tasdogan *et al*., 2020). Figure 2.1 showed the example of Royal Jelly.

Figure 2.1: Liquid royal jelly

2.2 Rabbit Production in Malaysia

Oryctolagus cuniculus is the scientific name of a rabbit. It was the most common domestic rabbit in the world. There were a few common domestic rabbits used in Malaysia, such as the New Zealand White, Flemish Giant, and Rex rabbit. This breed was usually used for meat production, while the Holland lop, lionhead, and Netherland dwarf rabbit were usually kept as pets. According to Hashim *et al*. (2023), five rabbit farmers were found to be over the age of 46, two were found to be between the ages of 36 and 35, and just one was found to be between the ages of 26 and 35. 62.5% of those who farm rabbits do so to support their income, while 37.5% do it full-time and only raise the New Zealand White (100%).

In 1988, small-scale farmers and business owners in Malaysia were urged to start raising rabbits for both export and domestic use. Farmers were currently aware of this prospect and the intriguing characteristics of the animal, but rabbit industry still unfamiliar in the Malaysia market (Gunalan *et al*., 2019).

Hashim *et al*. (2023) stated that in Malaysia, the agriculture industry has lost appeal as a new food source. It's time to make improvements to the menu because a sizable portion of the population views rabbits as cute pets. Even though there are numerous rabbit breeders in the nation, the market for broilers has not been particularly significant, according to data and surveys. Other parts of the world have seen greater economic growth in the rabbit meat sector than Malaysia. However, because there were so many rabbit farmers, the sector required additional assistance from the appropriate organisations to grow. Alternatively put, rabbit meat demands additional performance (Hashim *et al*., 2022).

2.3 Effect of Royal Jelly on Human and Animal Reproduction

In cases of male infertility, Royal Jelly also markedly increased semen motility. This effect can be attributed to an increase in testosterone levels, which are what cause sperm motility (Al-Sanafi *et al*., 2007). According to Xu *et al*. (2011), the intragastric administration of RJ can stimulate the development of male reproductive organs, such as the hypothalamus and testicles. While, in testicular tissues of rats exposed to high levels of heat stress, the RJ therapy can raise blood testosterone levels, improve DNA integrity, suppress oxidative stress, decrease lipid peroxidation, and boost total antioxidant capacity (Mahdivand *et al*., 2021). According to Ghanbari *et al*. (2015), RJ enhanced testicular weight, sperm count, viability, motility, morphology, DNA integrity, chromatin quality, blood testosterone, and testicular tissue MDA levels in diabetic rats. Royal jelly also protects male reproductive system damage induced by doxorubicin administration in mice (Pourzamani *et al*., 2022). When given to male rabbits

under stress due to heat, royal jelly can effectively prevent "summer infertility" by increasing the sperm motility, total sperm output, ejaculated volume, testosterone levels, and seminal fructose levels. It can also reduce the concentrations of abnormal and dead sperm (Elnagar, 2010). When given to female rats, royal jelly may have a repro-protective effect simply because to its anti-oxidant and anti-apoptotic qualities (Khodabandeh *et al*., 2021).

2.4 Semen Extender in Animal

Semen extenders are chemical agents used in artificial insemination (AI) to preserve, lengthen, and shield sperm cells from shock during the production, storage, and transportation of semen (Raheja *et al*., 2018). The general goal of the semen extender was to increase the volume of a single ejaculate, obtain more semen, as well as increase spermatozoa vitality while being stored *(Bresciani et al*., 2016). The function of the semen extender is to provide the sperm cell with the energy-requiring nutrient glucose, to enable protection against temperature shock with BSA, to regulate the medium's pH with bicarbonate, tris, and hepes and osmosis pressure with NaCl and KCl, and to stop microbial growth (antibiotics) (Gadea, 2003). Tris and citrate were the two components of extenders that are most frequently used for freezing bovine semen (Raheja *et al*., 2018). Formula semen extender enhanced sperm dose viability for an extended period of time, which was helpful for long-distance transport (Di Iorio *et al*., 2014).

2.5 Method of Semen Storage

Semen was a seminal fluid produced by the sexual systems and contains spermatozoa. The spermatozoa are cells that a male mammal releases in the semen during the ejaculation. The sperm was extremely sensitive to temperature variations, so the changes in the environment can have an impact on the sperm's quality (Mohamed *et al*., 2012). The semen was froze in order to be stored or transported over great distances. For frozen preservation, the sperm were placed in a nitrogen tank filled with liquid nitrogen that was extremely cold (-196°C). This process called semen cryopreservation. According to study by Mohamed *et al.* (2012), 25°C was the ideal temperature for sperm storage during transportation, as opposed to 0°C and 37°C, in order to preserve the sperm's characteristics. For AI, semen can be stored at either 4°C for 72 hours or 23°C for 24 hours yields the same level of productivity as fresh semen and also have other alternative method to store the semen at 4℃ and room temperature (23°C) as suggested by Wusiman *et al.* (2012).

One technique for storing sperm was the chilled process. That was cooling the sperm at 4°C temperature. According to Rijsselaere *et al*. (2011), in AI chilled and frozen-thawed semen can generate interest among dog breeders, veterinarians, and experimental research centres globally. Because it was more advantageous and cost-effective than transporting an entire mammal, chilled and frozen semen has evolved into a means of transporting genetic material internationally (Rijsselaere *et al.*, 2011). In comparison to using cryopreserved semen for short distance transportation, chilled semen was obtainable and cost-effective (Rijsselaere *et al.*, 2011). Dromedary camels' chilled semen caused poor fertility (<50%) and no pregnancies were achieved with frozen-thawed

semen (Al-Bulushi *et al.*, 2019). This research showed that the location of insemination and the sperm doses used during insemination can have an impact on the pregnancy rate. However, for motility measurements, the spermatozoa motility was greater for extenders stored at 5°C than for extenders stored at room temperature (Udeh & Oghenesode, 2011). Udeh and Oghenesode (2011) stated that sperm motility in goat-milk prolonged semen kept at 5°C can be sustained for up to 96 hours (4 days) after ejaculation.

2.6 Semen and Sperms Characteristics

Semen quality, which consists of motility, viability and abnormalities. The success of AI depends on the quality of the semen. Each buck has unique traits that may influenced the size of the individual's sperm due to pathological or environmental variables (Safaa *et al*., 2008). Additionally, some researchers claimed that different rabbits produce sperm at various rates (Ambriz *et al.*, 2002).

To have excellent sperm quality, sperm motility was one of the most crucial sperm characteristics. The potential to move was known as motility. Typically, the term "sperm motility" referred to sperm movements. Table 2.1 showed the detailed about sperm motility based on previous research.

Table 2.1: Summary of sperm motility

Author	Title	Source	Findings
Heba-T-Allah *et al.* (2018)	Using Rabbits as a Model: Artificial Insemination as a Tool to Increase Productive and Reproductive Traits.	In International Journal of Agriculture Innovations and Research	Sperm that traveled from one location to another in a mostly straight route was said to be progressively motile.
Murphy *et al.* (2018)	Optimizing storage temperature of liquid bovine semen diluted in INRA96.	Journal of Dairy Science	Semen stored at constant 15°C had a greater total motility score than semen stored at 5–28°C ($P<0.05$). In terms of the progressive motility, the semen with the greater progressive motility score was kept at 5° - 15°C as opposed to 5° - 28°C ($P<0.01$).
Olurode and Ajala (2016)	Effects of storage temperature and extension media on motility of caprine spermatozoa.	Sokoto Journal of Veterinary Sciences	The sperm showed significantly greater motility ($P<0.01$) at 5°C (refregerator temperature) than at 25°C (room temperature) in caprine spermatozoa. The sperm motility of liquid semen that has been stored can drastically diminish as a result of the gradual loss of nutrients like potassium, sodium, and plasma protein. This nutrient was essential for the sperm's elevated metabolic needs during its journey through the female genital tract.
Olurode and Ajala (2016)	Effects of storage temperature and extension media on motility of caprine spermatozoa.	Sokoto Journal of Veterinary Sciences	Storing liquid sperm at a refrigerator temperature slows the metabolic process, which allowed the sperm cells to use nutrients like fructose.

Author	Title	Source	Findings
Slanina *et al*. (2016)	Impact of tilmicosin on the rabbit spermatozoa motility and viability	Journal of Microbiology, Biotechnology and Food Sciences	The spermatozoa diluted with antibiotic (T1L7) solution gave significantly higher (P<0.001) progressive motility (81.19% vs. 66.12%) than the sample without antibiotic (T1LK), respecrtively. Rabbit spermatozoa's motility and progressive motility were significantly improved by tilmicosin at this dose of 0.350 mg/mL.

In general, sperm viability referred to the percentage of viable sperm in the semen sample. Using stains that can enter cells with broken membranes, the proportion of live, dead, and defective spermatozoa was calculated. Table 2.2 showed about sperm viability based on previous research.

Table 2.2: Summary of sperm viability

Author	Title	Source	Findings
Slanina *et al*. (2016)	Impact of tilmicosin on the rabbit spermatozoa motility and viability	Journal of Microbiology, Biotechnology and Food Sciences	The solution of T1L1 (0.300 mg/mL) displayed the best viability values (P<0.05) when compared to the control sample. Even the highest concentration of tilmicosin sample did not give any negative effect on viability of rabbit sperm.
Di lorio *et al*. (2014)	Comparison of different extenders on the preservability of rabbit semen stored at 5°C for 72 hours	Italian Journal of Animal Science	The cortalap extenders had higher viability values (P<0.05) than other extenders when stored for 4, 24, 47, and 72 hours. Additionally, 90% of the population's sperm showed signs of viability, motility, and acrosome integrity.

Author	Title	Source	Findings
El-Hanoun *et al*. (2014)	Impact of royal jelly to improve reproductive performance of male rabits under hot summer conditions.	World Rabbit Science	Normal live sperm did not exhibit the eosin stain and appeared white in color, but dead sperm displayed the eosin stain and displayed a reddish tint due to lack of membrane integrity.
Udeh and Oghenesode (2011)	Effects of Type of Extender and Storage Conditions on the Motility of Goat Spermatozoa.	International Journal of Animal and Veterinary Advances,	Chilled liquid goat semen with sodium citrate was used to keep the highest viability of the sperm cells.

The structure and shape of a spermatozoa were referred to as sperm morphology. In identify potential fertility, morphology may be even more crucial than sperm count or motility. Teratozoospermia was also known as abnormal sperm morphology. Sperm that were shaped differently cannot fertilise an egg. For adequate fertility, sperm should be about 60% of the usual size and shape. Table 2.3 explained about sperm abnormality based on previous research. While Figure 2.2 showed the example of abnormal sperm morphology.

Table 2.3: Summary of sperm abnormality

Author	Title	Source	Findings
Heba-T-Allah *et al*. (2018)	Using Rabbits as a Model: Artificial Insemination as a Tool to Increase Productive and Reproductive Traits.	In International Journal of Agriculture Innovations and Research	Faulty spermatogenesis or spermiogenesis was the cause of abnormal sperm production. This occurred because of hereditary conditions, illnesses, inadequate nutrition, and other environmental factors.
Ewuola and Akinyemi (2017)	Semen characteristics and seminal oxidative status of four breeds of rabbit in Southwest, Nigeria	The Journal of Basic and Applied Zoology	The incidence of undesirable sperm was higher in the summer compared to other seasons in the male ejaculates of Bouscat White and NZW, but there was no breed-specific difference.
El-Hanoun *et al*. (2014)	Impact of royal jelly to improve reproductive performance of male rabits under hot summer conditions.	World Rabbit Science	Abnormal sperm have flawed in the head, midpiece, or tail, such as an oversized or malformed head or a crooked or double tail.
Reproductive facts.org, (2014)	*Sperm morphology (Shape): Does it affect fertility?*	American Society for Reproduction Medicine.	A typical sperm features an oval, smooth head that was 5 to 6 micrometres long and 2.5 to 3.5 micrometres wide (smaller than the size of a needle tip), as well as a well-defined cap (acrosome) that covers 40% to 70% of the sperm head. Other abnormalities of the semen, such as low sperm count or motility, were linked to increase quantities of irregularly shaped sperm.

Author	Title	Source	Findings
Enciso *et al.* (2011)	Major morphological sperm abnormalities in the bull are related to sperm DNA damage.	Theriogenology	Major sperm abnormalities that may be genetically based or the outcome of an apoptotic mechanism that failed to complete appear to be directly linked to the presence of severely damaged DNA molecules.
Siddique *et al*. (2011)	Spermatozoal abnormalities and sperm dna fragmentation vis-à-vis male infretility	Wayamba Journal of Animal Science	Ejaculates from Holstein bulls born locally were collected during the hot summer season, and they were of much inferior quality than those taken in the winter and spring. Primary anomalies were most prevalent in hot temperatures, mild in spring, and least prevalent in winter.
Bodnár *et al*. (2000)	Comparative evaluation of abnormal spermatozoa in Pannon White, New Zealand White and Angora rabbit semen (short communication).	Archives Animal Breeding	The NZW bucks' sperm abnormalities and semen pH were strongly influenced by the season. Significant correlations were found between the pH of the sperm and the percentage of main and secondary sperm abnormalities.

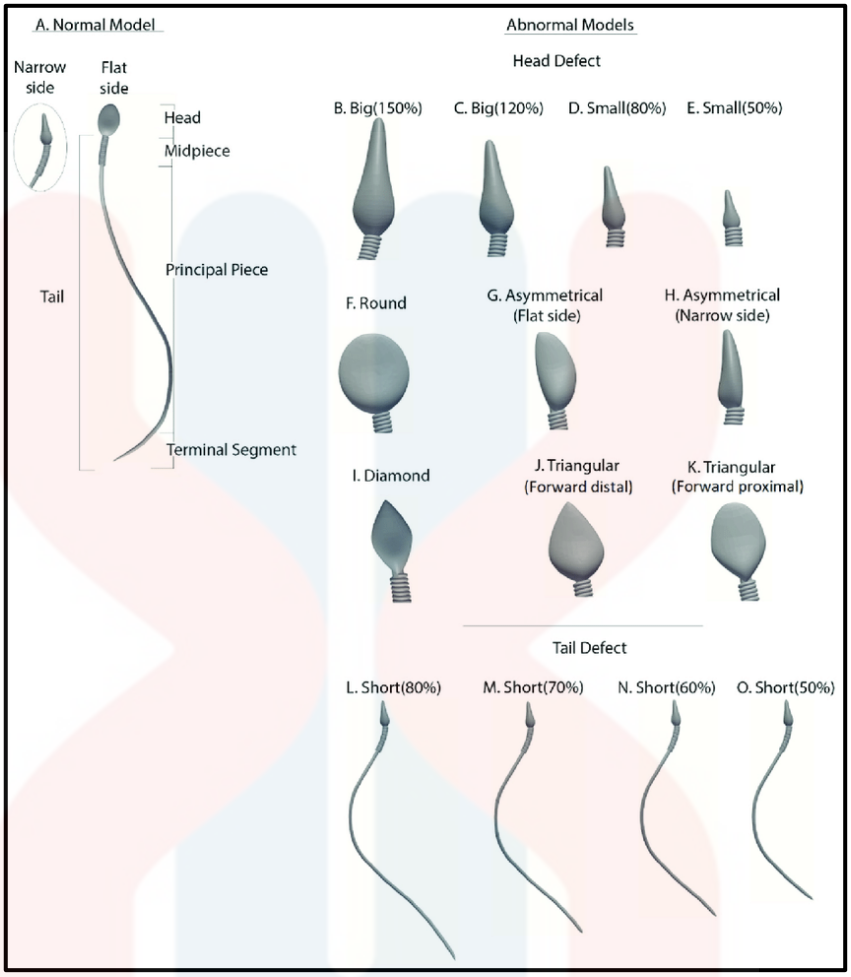

Figure 2.2: Abnormal sperm morphology (Nassir *et al*., 2022)

2.7 Semen Collection in Rabbit

Male's scrotum contained the oval-shaped testicles. About two months old the testicles descend in male rabbits. According to Lebas *et al*. (1997), spermatogenesis in rabbits begins between days 40 and 50. However, the testicular ducts started to function around day 84. Semen collection was carried out to obtain semen from male animals, which was then either kept or inseminated into female animals. An artificial vagina (AV) kept at 45–46°C and a teaser doe were used to collect ejaculates. After removing the gel mass, the volume of each ejaculate was measured (El-Hanoun *et al*., 2014). Semen sampling was also done to assess an animal's sperm quality. Semen that was

gathered using an AV that contain a warm liquid (approximately 45°C) (Castellini *et al*., 2005).

The semen collecting procedure involved an AV. In order to gather rabbit sperm, an affordable AV was developed. According to Naughton *et al*. (2003), rabbit semen could be collected reliably and successfully using an affordable AV. After each semen collection, the AV needs to be cleansed and sterilised by soaking it in a warm detergent solution and rinsing it with water and alcohol (Bredderman *et al*., 1964). When compared to the winter and spring, semen on the summer contains more spermatozoa with damaged heads and midpieces (Siddique *et al.*, 2011).

2.8 Histology of Testis

The biological field of histology studied the structure and organization of plant and animal tissues in relation to their unique activities. The mediastinum testis, a conical mass of connective tissue, projects into the testis from the tunica albuginea, a thick capsule that surrounds the testis. Androgens and spermatozoa are both produced by a pair of testes. The fluid components of semen were generated by several accessory glands. The sperm were kept in long tubes until they were delivered to the penis. Spermatogenic cells and Sertoli cells were present in the seminiferous tubules' germinal (seminiferous epithelium). Spermatogenesis takes place in the testis, which contains a group of u-shaped seminiferous tubules. Sertoli cells, that help germ cells in various phases of development, line the seminiferous tubules. The basement membrane of tubules was surrounded by a layer of contractile myoid cells (Lendvai, 2019). The

process of spermatogenesis involved the division of spermatogenic cells through mitosis and meiosis to create gametes that develop into sperm. The initial cells of spermatogenesis were called spermatogonia. (spermatogenesis in rabbit)

There were two different kinds of spermatogonia: type A and type B. The nucleus of type A spermatogonia was spherical and had one or two nucleoli in addition to very small chromatin granules. They were stem cells that can divide to produce both type A and type B spermatogonia in fresh generations. The round nuclei of type B spermatogonia have a single nucleolus and chromatin granules of various sizes that frequently adhere to the nuclear membrane. Type B spermatogonia can divided repeatedly, but they were not stem cells, and their ultimate mitosis always produces primary spermatocytes, secondary spermatocytes, and spermatids (Lendvai, 2019). There were significant variations between species in the testicular interstitial space, which included every area between the seminiferous tubules (Foley, 2001). According to Uyar *et al*. (2019), testes play a crucial role in the production of semen as well as the early identification of bulls with high reproductive potential for use as breeders. Because it was obvious that semen quality and testicle quality were closely related (Uyar *et al*., 2019). Figure 2.3 showed the stage of spermatogenesis in seminiferous tubules and Figure 2.4 showed the seminiferous tubules of rabbit's testis with 20X magnification.

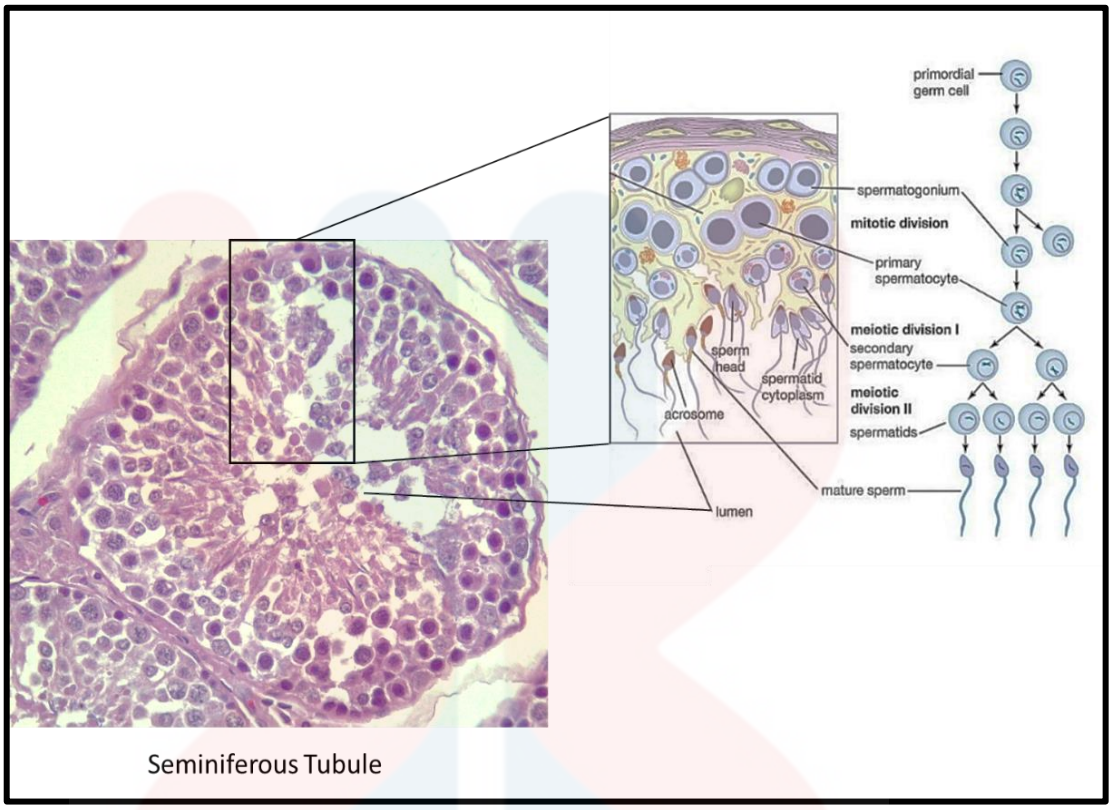

Figure 2.3: Stage of spermatogenesis in seminiferous tubules

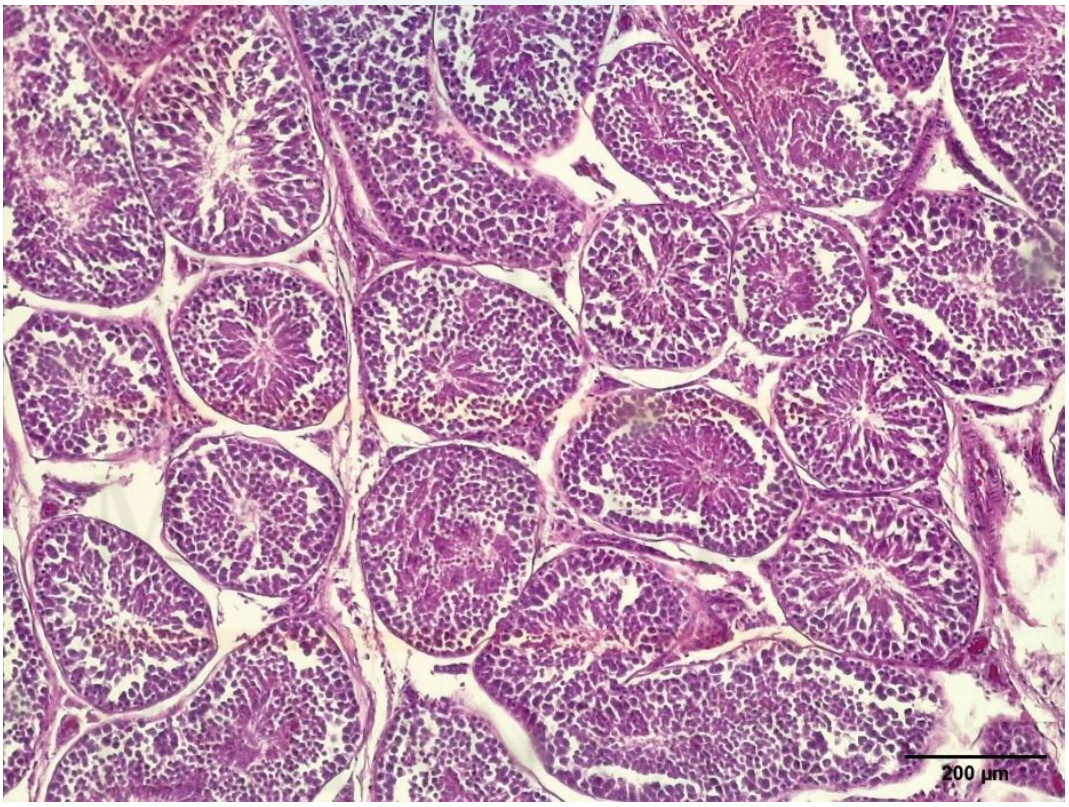

Figure 2.4: Seminiferous tubules of rabbit's testis

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Experimental Animal

Total of twenty (20) male rabbits was used in the study. Different breeds were used in the study, which was Angora, Broken New Zealand, New Zealand white, and Flemish Giant. The average body weight of the bucks was 2.80 – 3.10 kg. The age of bucks was 6 – 12 months. The study was conducted at Rabbit House, Jeli Campus, UMK. The rabbits were fed with commercial feed diet and fresh water. Each group consists of 5 bucks. There were 4 groups in the study which was control group contains no royal jelly (C), Treatment 1 contain 100 mg of RJ + 0.5 mL/kg of body weight (T1), treatment 2 contains 200 mg of RJ + 0.5mL/kg of body weight (T2) and treatment 3 contain 300 mg of RJ + 0.5 mL/kg of body weight (T3) (Khadr *et al*., 2015). All the treatment was given with a tap water. Oral administration was given once a day, three times a week for 8 weeks and 2 weeks of adaptation period. Animal ethic number for this research was UMK/FIAT/ACUE/FYP/2/2020.

3.2 Preparation of Extender

There were two groups of extenders: control and royal jelly extenders. The control extender was prepared using the formula tris-citric acid-fructose-egg yolk (TCFY). TCFY consists of tris, citric acid, fructose, egg yolk, and penicillin. All the ingredients were dissolved in 100 mL of distilled water. The

ingredients were mixed with a magnetic stirrer for 10 minutes. Next, the TCFY were filtered using filter paper and a filter syringe. For RJ extender, 6 mL of TCFY were diluted with 10mg of RJ (El-Sherbiny, 2014). 6% of glycerol was prepared by mixing 0.6 mL of glycerol with 9.4 mL of distilled water. The ratio of 10 mL of extender was a mix of 5 mL of 6% glycerol, 4 mL of TCFY (control) or 4 mL of TCFYRJ (royal jelly), and 1 mL of semen. A total of 10 mL of each semen extender was prepared for each treatment group.

3.3 Semen Collection

In the study, the semen collection was performed using an AV that was designed especially for rabbits (Bredderman *et al*., 1964). The temperature of the AV was around 45–49°C. The semen collection was performed during the day between 7:00 and 10:00 a.m. The collection was carried out every two weeks for eight weeks. Figure 3.1 showed the picture of artificial vagina of rabbit.

Figure 3.1: Artificial vagina of rabbit

3.4 Physical Evaluation

3.4.1 Semen Volume

Semen volume was measured using a 1 mL syringe. The gel mass was removed for more accurate results (El-Hanoun *et al*., 2014).

3.4.2 Semen pH

Semen pH was measured using universal pH paper. The colour of the paper will indicate the pH of the semen (Khadr *et al*., 2015).

3.5 Sperm Evaluation

3.5.1 Assessment of Sperm Count

Sperm concentration was calculated using a haemocytometer (WHO, 2010). The fresh semen was mixed with semen dilution fluid. Semen dilution fluid consists of 500 mL of distilled water, 5 mL of 35–40% formaldehyde, and 25g of sodium bicarbonate (NaHCO3). About 380 µL of semen dilution fluid and 20 µL of diluted semen were mixed using a pipette. To allow capillary action to draw the cell suspension out, the mixture was gradually filled into both chambers underneath the coverslip. 10X objective on a compound microscope was set on centre of the haemocytometer's grid lines. Sperm cells were counted manually in one set of 16 squares. Continue counting until all four sets of 16

corners have been calculated. The measurement analysis was according to the following formula: -

Average of sperm count in all 4 sets X semen dilution X 10000 (10^4)

= Sperm cell X 10^6 /mL

3.5.2 Assessment of Sperm Viability

Semen viability was analysed using the eosin-nigrosine stain. An eosin-nigrosine stain was performed to differentiate the live and dead sperm. Normal live sperm did not show the eosin stain and were white in colour, whereas dead sperm showed the stain and were reddish in colour because of the absence of membrane integrity (El-Hanoun *et al*., 2014). About 50 mL of distilled water and 0.5g of eosin powder were mixed in a beaker to make 1% of the eosin stain. To ensure that all the eosin powder thoroughly dissolved in distilled water, the mixture was mix uniformly using a glass rod. A drop of diluted semen was placed on a glass slide. Next, add two drops (100µl) of 10% nigrosine along with a drop (50µl) of 1% eosin to the same slide. Another clean slide was taken and smeared. The smear was left to dry naturally. Under a compound microscope (Biological Compound Microscope, View Solution Inc) with a 40X objective, the slide was examined. Three measurements were made, each with a count of 100–200 sperm (Ng *et al*., 2022).

3.5.3 Assessment of Sperm Motility

About 3 µL of diluted semen was put on the warm glass slide. A coverslip was put on the warm slide. The glass slide was observed under a

compound microscope (Biological Compound Microscope, View Solution Inc) with a 40X objective. Three measurements were made, each with a count of 100–200 sperm (Ng *et al*., 2022). The sperm was graded based on its movement. There were four grades of sperm motility: -

1. Grade A: Rapid progressive motility, which sperm can swim fast in a straight line.
2. Grade B: Slow or Sluggish or Non-linear progressive motility, which the sperm move forward but in a curved or crooked motion.
3. Grade C: Non-progressive motility or vibrate, which move their tail but do not move forward.
4. Grade D: immotile which fail to move at all.

3.5.4 Assessment of Sperm Morphological Abnormality of Defects

Sperm abnormalities were observed by using the eosin-nigrosine stain. Eosin-nigrosine staining was performed to differentiate the sperm morphology. About 50 mL of distilled water and 0.5g of eosin powder were mixed in a beaker to make 1% of the eosin stain. To ensure that all the eosin powder thoroughly dissolved in distilled water, the mixture was mix uniformly using a glass rod. A drop of diluted semen was placed on a glass slide. Next, add two drops of 10% nigrosine along with a drop of 1% eosin to the same slide. They then mixed and waited for 10 seconds. Another clean slide was taken and smeared. The smear was left to dry naturally. Under a compound microscope (Biological Compound Microscope, View Solution Inc) with a 40X objective, the slide was examined. Three measurements were made, each with a count of 100–200 sperm (Ng *et al*., 2022). Figure 3.2 was the example of defect sperm morphology.

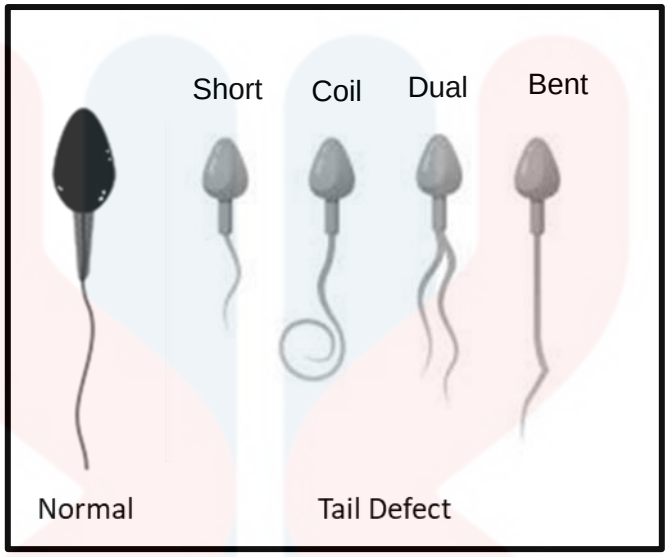

Figure 3.2: Sperm Morphological Defects

3.6 Histology of Testis

Two rabbits were slaughtered from each group and the testis was taken for the preservation process. The testicles of the slaughtered bucks were each preserved using 10% buffered formalin. The sample was brought to the histology lab on the Pengkalan Chepa Campus, UMK for histology standard operating procedure after being preserved. The specimen was handled for histology by being cut into small pieces. The tissue must be sliced to a maximum thickness of 3 mm. The specimen was then put on a cassette with a label. The tissue processing and embedding process was then carried out overnight on the tissue cassette using an automated tissue processor (Leica TP1020 Automatic Benchtop Tissue Processor, Semi-Enclosed).

Following tissue preparation and embedding, tissue sectioning was carried out using Leica EG1150 Modular Tissue Embedding Center. To ensure

that the wax was cool enough for sectioning to generate a thin, smooth, and consistent thickness of tissue section ribbon, FFPE tissue blocks were put on a –5°C cold plate for 15 minutes. Then, tissue sectioning was carried out by using Leica Biosystems RM2245. The blade and blade holder's angle were fixed to 5°. To prevent significant tissue loss, the tissue block was positioned in the object clamp in the same orientation as the last tissue block. The tissue block was thinned out to a thickness of 10 to 30 µm so that sectioning could provide a representative section. The tissue block's surface was polished with a few light strokes to remove the rough trimming. The slides for staining will be obtained using a subsequent segment with a thickness of 3–4 µm. Following that, the cohesive ribbon will float on the spotless 45°C tissue flotation. Until they were sufficiently flattened, sections were left floating on the water. A slide was slowly dipped into the water bath to allow attachment of the tissue section. The slide was set up on the wooden support.

Cryostat sample handling was the next stage (ECOCELL Oven with Natural Air Convection, MMM Medcenter). For urgent biopsies, continuing surgery, neurohistology samples, demonstrating fibrofatty tissue, and donor transplant samples, frozen sections were used in cryostat sample handling. The specimen was kept in the cryochamber overnight with 30% sucrose. The auto-Stainer equipment was then used to execute the Harris haematoxylin and eosin (H&E) staining procedure (Leica ST5010 Autostainer XL). Finally, mounting, decalcification, and Masson trichrome were carried out. Figure 3.3 showed the flowchart of the histology of testis on step by step how to stain the sample.

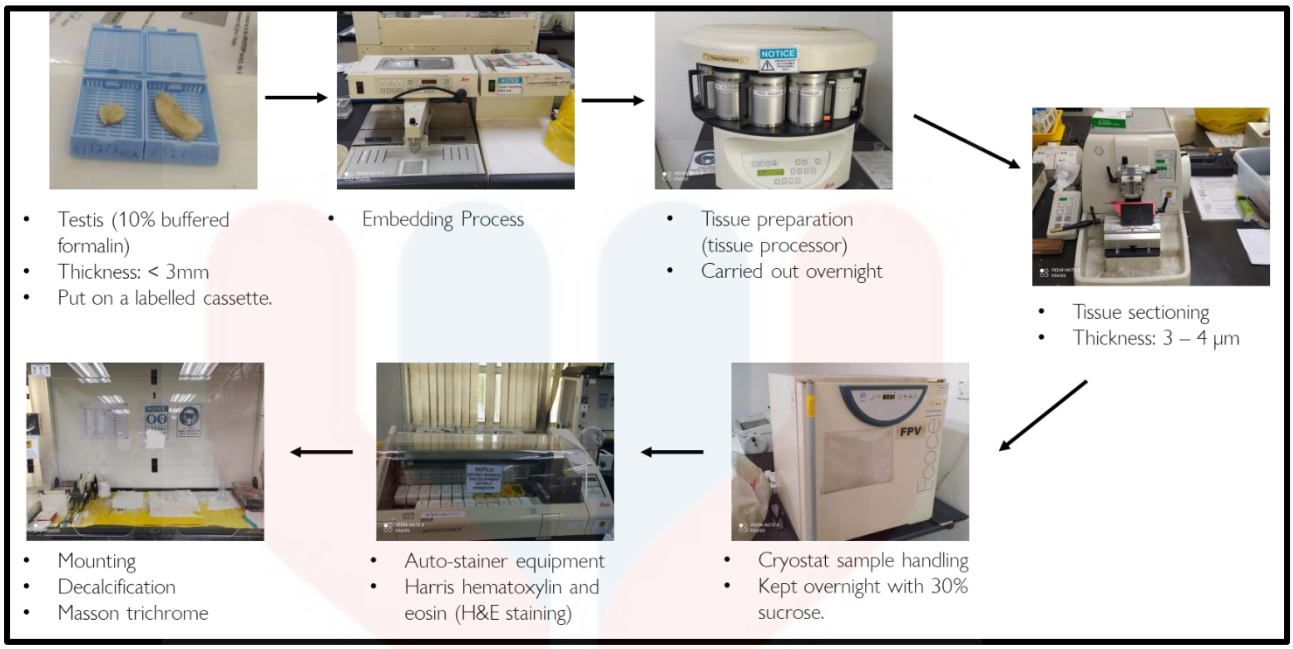

Figure 3.3: Procedure for histology of testis

3.7 Seminiferous Tubule Diameter

Two slides were prepared from each sample. Seminiferous tubule was taken on selected slide to measure the diameter. The image of seminiferous tubule was capture using a compound microscope for 20X magnification. Seminiferous tubule diameter was measured by using ImageJ software. Total of 30 seminiferous tubules was measured on each sample. The seminiferous tubule was calculated as the average of the each sample by groups (Princewill *et al.*, 2009).

3.8 Experimental Design

There were 20 bucks selected for the research. This buck was divided into 4 groups of oral administration consisting of 0 mg, 100 mg, 200 mg, and 300 mg of RJ. Each group consists of 5 bucks. The bucks have been given a 2-

week adaptation period before the oral administration. Semen collection was performed in each buck every 2 weeks for 8 weeks. Semen characteristics such as sperm concentration, pH, and semen volume were observed in each buck. Every collection, the sample was pooled and divided into 2 groups of extenders: control extenders and RJ extenders. Sperm quality, such as sperm viability, sperm motility, and sperm abnormalities, was observed in each extender. The evaluation was made after 2 hours in chilled temperatures. In week 8, two bucks from each group of oral administration were selected to be slaughtered. The testicle was taken and preserved for histology. Testicle characteristics such as testicle weight and seminiferous tubule diameter were recorded. Figure 3.4 showed the flow of the experimental design.

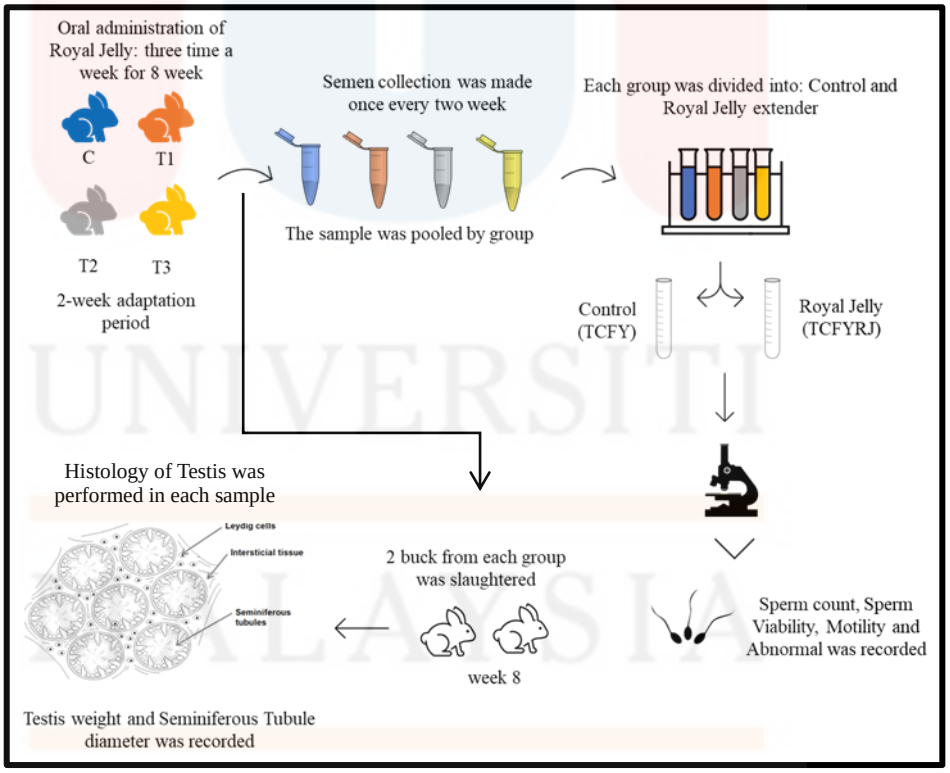

Figure 3.4: Experimental design of the research

3.9 Statistical Analysis

Percentage data of semen, sperm quality, and histology of the testis were analyzed using one-way ANOVA in SPSS version. The comparison of control and RJ extender was analyzed by independent t-test in SPSS. Sperm quality and histology were also analyzed by Spearman correlation in SPSS. To compare the differences between the values, a p-value of $p<0.05$ was considered significant. The data were expressed as mean ± standard error of mean (SEM).

CHAPTER 4

FINDINGS AND DISCUSSION

4.1 Effect of Royal Jelly on Semen Quality

Table 4.1 shows the physical characteristic of rabbit semen. The current results showed that there was a non-significant difference ($p>0.05$) in pH, semen volume, and total sperm count between groups. The control group showed the highest pH and total sperm count compared to other groups (7.2±0.15 and 85.28±23.47 X 10^6, respectively). While T2 showed the highest semen volume between groups (1.07±0.13 ml). According to Figure 4.1, there was an insignificant change ($p>0.05$) throughout the 8 weeks. Furthermore, there was a non-significant difference ($p>0.05$) between groups for the overall mean, but T1 showed a positive change in the overall total sperm count (70.06±11.34 X 10^6).

Table 4.1: Physical Characteristic of Rabbit Semen

Parameters	Experimental group (Mean ± SEM)			
	C	T1	T2	T3
	n = 5	n = 5	n = 5	n= 5
pH	7.2±0.15[a]	7.19±0.18[a]	6.84±0.18[a]	6.65±0.21[a]
Semen volume (ml)	0.89±0.09[a]	0.93±0.09[a]	1.07±0.13[a]	0.81±0.11[a]
Total sperm count (X 10^6)	85.28±23.47[a]	70.06±11.34[a]	55.16±9.47[a]	37.19±9.02[a]

SEM, Standard error of Mean. [ab] Means with different superscripts in a same row differ significantly ($p<0.05$). C = Control Group, T1 = 100mg of Royal Jelly, T2= 200mg of Royal Jelly, T3 = 300mg of Royal Jelly

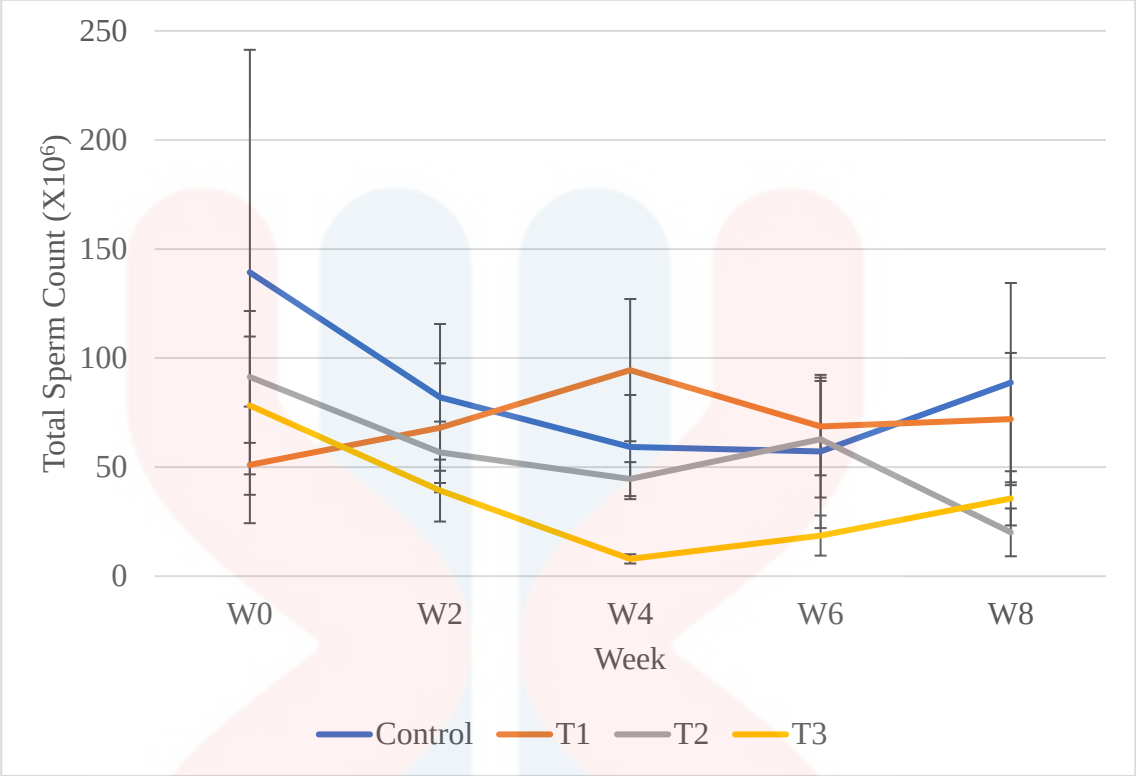

Figure 4.1: Effect of royal jelly on total sperm counts for 8 weeks. Error bars, Standard error of mean

Based on the table, there was no significant difference between groups in pH, semen volume, or sperm count. The findings showed that control and T1 were neutral, while T2 and T3 were slightly acidic. According to Heba-T-Allah *et al.* (2018), semen extenders should function as a buffer against excessive acidity or alkalinity since the pH of the semen tends to be alkaline, which serves as an indication of the proper condition of accessory secretion and the viability of spermatozoa. Akpa *et al.* (2012) research concluded, that regarding age, breed, and sire, the bucks exhibited notable variations in semen traits and body weight. In this research, different breeds and ages were used, which may be the cause of the changes in semen pH due to the variation of the buck's breed. A similar pattern of results was obtained in El-Hanoun *et al.* (2014) that most semen properties, with the exception of volume and pH, were significantly ($p<0.05$) increased through Chinese RJ.

The present findings showed T2 and T1 have a higher semen volume than control (1.07±0.13, 0.93±0.09 and 0.89±0.09 ml, respectively). The result aligned with El-Hanoun *et al*. (2014), who found in comparison to the control, the 100 mg and 150 mg RJ groups' showed greater ejaculate volumes. It may be the result of enhanced seminal fluid flow from the sex accessory glands. However, in line with ideas of Elnagar (2010), who studied the exposure of male heat-stress rabbits to different doses of RJ, which were 200mg, 400mg, and 800mg, it can be concluded that the three RJ doses each significantly increased ejaculated volume by 36%, 31%, and 18% in comparison to the heat stressed control group.

The current study showed that there was an insignificant change in sperm count between groups. This finding is in line with Al-sanafi and Abdulla (2007), Ibrahim *et al.* (2017), and Waykar and Alqadhi (2020). Al-sanafi and Abdulla (2007) studied the effect of RJ on infertile men. The research found that in all groups receiving RJ treatment, the sperm count increased marginally but not significantly in infertile men treated with RJ. However, Ibrahim *et al.* (2017) finding showed that lithium carbonate with RJ treatment had no discernible effect on adult mice's total sperm count when compared to the control group. While Waykar and Alqadhi (2020) also found that sperm function indices such as sperm count, sperm viability, sperm motility, and weight of the epididymis and testis as compared to controls did not significantly change after honey and RJ were given orally to rats.

Nevertheless, the present results showed a disagreement with Ahmadnia *et al*. (2015), Asadi *et al*. (2019), and El-Hanoun *et al*. (2014). El-Hanoun *et al.* (2014) found sperm concentration was increased in RJ groups over the control group in rabbits under summer conditions. While Asadi *et al.* (2019) findings

showed that the sperm count increased significantly after royal jelly treatment in male rats. Nawar *et al*. (2015) stated that the development of the seminiferous epithelium as well as the start and upkeep of the mitotic stages of spermatogenesis required FSH. FSH affected the Sertoli cells by increasing the production of Androgen Binding Protein (ABP), which in turn stimulated spermatogenesis and induced proliferation. ABP was connected to the T hormone generated by the Leydig cells, which raised the levels of T hormone in the testes (Nawar *et al*., 2015).

As a result, raising FSH levels by RJ induced Sertoli cells to produce more ABP, which in turn raised T hormone levels in the testes. Male sexual development was impacted by genetic disorders and high FSH levels, which can harm the testicles. It frequently results in infertility. This may explain why the present result showed a decreased sperm count; it may be due to the excess FSH caused by the high concentration of RJ. FSH levels rise due to RJ, which encouraged the creation of ABP from Sertoli cells and then elevates T hormone levels in the testes, which negatively impact spermatogenesis (Nawar *et al*., 2015).

4.2 Effect of Royal Jelly on Sperm Quality in Rabbit

4.2.1 Sperm Viability

The results of Table 4.2 show the effect of RJ on viability of sperm in a control extender for 8 weeks. Based on the results, there was significant difference ($p<0.05$) between T1 and T3 (52.62±2.18 and 38.07±3.89, respectively). T1 showed the highest overall mean of sperm viability among

other groups while the lowest was T3. Control group and T1 showed the highest sperm viability at week 6 (58±2.08 and 61.33±2.73, respectively). However, T2 showed the highest sperm viability at week 8 (56±1.15) and T3 showed the highest sperm viability at week 2 (49±5.29). Next, Table 4.3 shows the effect of RJ on the viability of sperm in an RJ extender for 8 weeks. The results showed that T1 has a significant difference ($p<0.05$) with T2 and T3. The highest overall mean of sperm viability was T1 while the lowest was T3 (64.71±1.01 and 52.40±3.38, respectively). The highest sperm viability of control group was at week 2 while T1 showed the highest sperm viability at week 6 (67±3 and 68.33±0.33, respectively). However, the highest sperm viability of T2 was at week 4 and T3 showed the highest sperm viability at week 0 (64.33±0.88 and 59.66±8.45, respectively).

Table 4.2: Effect of royal jelly on sperm viability in control extender for 8 weeks

Sperm Quality	Week	Experimental group (Mean ± SEM)			
		C n = 5	T1 n = 5	T2 n = 5	T3 n = 5
Live Sperm (%)	0	11.67±10.2	36±3.51	38.33±5.36	26.33±14.5
	2	55±2.65	52±3.58	37.33±3.33	49±5.29
	4	54.33±1.2	53.5±3.69	45.33±3.93	25±2
	6	58±2.08	61.33±2.73	51.33±2.91	44.67±3.53
	8	57.33±4.1	60±2.89	56±1.15	45.33±3.71
Overall mean ± SEM		47.27±5.16^{ab}	52.62±2.18^{b}	45.67±2.37^{ab}	38.07±3.89^{a}

SEM, Standard error of Mean. ^{ab} Means with different superscripts in a same row differ significantly ($p<0.05$). C = Control Group, T1 = 100mg of Royal Jelly, T2= 200mg of Royal Jelly, T3 = 300mg of Royal Jelly

Table 4.3: Effect of royal jelly on viability sperm in RJ extender for 8 weeks

Sperm Quality	Week	Experimental group (Mean ± SEM)			
		C	T1	T2	T3
		n = 5	n = 5	n =5	n = 5
Live Sperm (%)	0	42.33±3.28	61.33±4.06	47.33±5.93	59.66±8.45
	2	67±3	63±2.58	36±5.29	59±2.9
	4	59±1.53	66.67±1.8	64.33±0.88	30.67±1.33
	6	61.67±1.2	68.33±0.33	59.67±4.84	57.67±2.96
	8	63.33±2.6	64±1.53	62.33±2.73	54.67±2.4
Overall mean ± SEM		58.67±2.47^{ab}	64.71±1.01^{b}	53.93±3.31^{a}	52.40±3.38^{a}

SEM, Standard error of Mean. ^{ab} Means with different superscripts in a same row differ significantly ($p<0.05$). C = Control Group, T1 = 100mg of Royal Jelly, T2= 200mg of Royal Jelly, T3 = 300mg of Royal Jelly

Based on the result, 100 mg of RJ had a significantly positive ($p<0.05$) effect on sperm viability, especially in both extenders. Present study has using 100 mg of RJ produces similar results to El-Hanoun *et al.* (2014), who found RJ at 50 mg, 100 mg, and 150 mg led to dramatically and gradually improved ($p<0.05$) live sperm and normal sperm counts in contrast to the control group. The effect of RJ was influenced by the doses consumed (Abdelnour *et al*., 2020; El-Hanoun *et al*., 2014).

T3 showed the lowest sperm viability in both tables 4.2 and table 4.3 (38.07±3.89 and 52.40±3.38, respectively). Present study showed that the higher the concentration of the RJ, the more negative impact on the sperm viability. Increased RJ components in the sperm extender may affect metabolic processes that help preservation of the sperm membrane and result in an imbalance in redox reactions (Abdelnour *et al*., 2020). Redox imbalance can cause lipid peroxidation, protein oxidation, DNA damage, and reactive oxygen species (ROS) interference with signal transduction pathways. These effects are made considerably more serious when genetic variants hinder the normal breakdown of altered proteins (Berg *et al*., 2004).

According to Asadi *et al*. (2019), RJ protect against the oxidative damage due to varicocele causes in rat testicles. This defense may result from increased production of antioxidant enzymes and the suppression of lipid peroxidation and free radical production. Vitamins E and C are well-known antioxidants that have been demonstrated to reduce lipid peroxidation in tissue and protect the delicate cell membranes of the testis from free radical damage. As a result, they significantly reduced MDA and raised glutathione levels. (Nawar *et al*., 2015).

Table 4.2 and 4.3 indicated that sperm viability in T3 of the control and RJ extenders (38.07±3.89 and 52.40±3.38, respectively) were lower than those in the control group of the same treatment (47.27±5.16 and 58.67±2.47, respectively). According to Elnagar (2010), who found in comparison to the heat-stressed control bucks with the three doses of RJ (200 mg, 400 mg, and 800 mg), dead sperm decreased by 27, 25, and 17%, respectively. Dead sperm concentrations showed a significant interaction between RJ treatments.

Hassan (2009) agree with the finding that the percentage of viable sperm significantly increased ($p<0.05$) following a treatment with just RJ and was then maintained at the control value. Abdelnour *et al.* (2020) explained that RJ increased the production of seminal fluid from secondary sexual organs, which were crucial for sperm viability and motility. Spermatozoa were suspended in seminal plasma, a fluid medium, to form the substance known as semen. Seminal plasma, the fluid component of semen, was released by the accessory glands and the epididymis both before and during ejaculation (Talluri *et al.*, 2017).

4.2.2 Sperm Motility

Table 4.4 shows the effect of RJ on motile sperm in a control extender for 8 weeks. Based on the findings, The results showed that there was significant difference ($p<0.05$) between T1 and Control group with T2 and T3. Other that, T2 showed a significant difference ($p<0.05$) with T3. The highest overall mean of sperm motility was T1 and the lowest T3 (73.1±2.43 and 34.07±5.02, respectively). The highest sperm motility of control group was at week 8 while T1 showed the highest sperm motility at week 6 (80.67±1.76 and 86.33±3.38, respectively). However, the highest sperm motility of T2 was at week 6 and T3 showed the highest sperm motility at week 0 (71.33±2.85 and 65.67±1.45, respectively). Table 4.5 shows the effect of RJ on motility sperm in an RJ extender for 8 weeks. There was significant difference ($p<0.05$) between T1 with T2 and T3. While control and T2 showed a significant difference ($p<0.05$) with T3. T1 showed the highest overall mean of sperm motility in RJ extender while T3 showed the lowest overall mean of sperm motility (79.43±3.00 and 35.93±5.68, respectively). The highest sperm motility of control group was at week 8 and the highest sperm motility for T1 was at week 6 (87±1 and 92.67±2.19, respectively). However, the highest sperm motility of T2 was at week 4 and the highest sperm motility of T3 was at week 0 (91.67±0.67 and 71.33±0.88, respectively).

Table 4.4: Effect of royal jelly on sperm motility in control extender for 8 weeks

Sperm Quality	Week	Experimental group			
		C n = 5	T1 n = 5	T2 n = 5	T3 n = 5
Sperm Motility (%)	0	71±0.33	64±0.58	66±0.58	65.67±1.45
	2	61±2.31	60±1.13	30.67±5.24	44.33±1.2
	4	77.33±1.2	79.67±1.43	63.67±1.2	15±1.15
	6	67±2.08	86.33±3.38	71.33±2.85	23.33±2.85
	8	80.67±1.76	82±0.58	19.33±6.57	22±2
Overall mean ± SEM		71.5±1.99^{c}	73.1±2.43^{c}	50.20±5.82^{b}	34.07±5.02^{a}

SEM, Standard error of Mean. ^{ab} Means with different superscripts in a same row differ significantly ($p<0.05$). C = Control Group, T1 = 100mg of Royal Jelly, T2= 200mg of Royal Jelly, T3 = 300mg of Royal Jelly

Table 4.5: Effect of royal jelly on sperm motility in RJ extender for 8 weeks

Sperm Quality	Week	Experimental group			
		C n = 5	T1 n = 5	T2 n = 5	T3 n = 5
Sperm Motility (%)	0	75±1.53	76±1.53	77±1.15	71.33±0.88
	2	65.67±3.53	63±5.7	37.67±4.33	46.33±3.18
	4	82.33±2.33	85.83±0.79	91.67±0.67	19.67±5.17
	6	81.33±2.33	92.67±2.19	78.33±2.03	28±2.89
	8	87±1	89.67±1.2	25.33±2.91	14.33±2.33
Overall mean ± SEM		78.27±2.16^{bc}	79.43±3.00^{c}	62.00±6.94^{b}	35.93±5.68^{a}

SEM, Standard error of Mean. ^{ab} Means with different superscripts in a same row differ significantly ($p<0.05$). C = Control Group, T1 = 100mg of Royal Jelly, T2= 200mg of Royal Jelly, T3 = 300mg of Royal Jelly

Table 4.4 and Table 4.5 showed that there was a significant difference ($p<0.05$) in T2 and T3 with T1 ($p<0.05$) while there was a non-significant difference ($p>0.05$) between control and T1 ($p>0.05$). T1 showed significantly higher sperm motility than T2 and T3 in both extenders ($p<0.05$). The result showed that T1 in both control and RJ extenders has the highest sperm motility compared to other groups (73.1±2.43 and 79.43±3.00, respectively). This result in line with El-Hanoun *et al.* (2014), who stated that RJ substantially ($p<0.05$) enhanced sperm motility in groups by 9.7, 23.6, and 25.9% compared to the control group with a 50 mg, 100 mg, and 150mg dose of RJ, respectively.

The ability of antioxidant protein components and the presence of micronutrients with beneficial impacts on the health and metabolism of bucks may be a significant factor in the enhancement of sperm motility in bucks consuming RJ (Abdelnour *et al*., 2020; Asadi *et al*., 2019; Park *et al.*, 2019). The antioxidative activity of RJ has been proven by Nagai and Inoue (2004), who studied the antioxidant properties of RJ in enzymatic hydrolysates, water, and alkaline extracts. Kanbur *et al*. (2009) and Silici *et al.* (2009) have also proven that RJ antioxidative activity can protect against oxidative stress in animals. Pizzino *et al*. (2017) stated that a biological system's ability to detoxify these reactive products and the production and accumulation of oxygen reactive species (ROS) in cells and tissues create an imbalance that leads to the phenomenon known as oxidative stress. Glutathione (GSH), glutathione S-transferase (GST), glutathione S-transferase theta 1 (GSTT1), Bax, and Bcl-2, which are present in RJ, regulate oxidative stress. Direct ROS scavengers like GSH, GST, and GSTT1 are essential for eliminating oxidative stress from cells (Pasupuleti *et al*., 2017).

However, T3 has the lowest sperm motility in both extenders (34.07±5.02 and 35.93±5.68, respectively). According to Xu *et al*. (2011), rabbits in the low RJ dose group had considerably higher semen motility than those in the control group ($p<0.01$), while those in the control group had significantly higher semen motility than rabbits in the high RJ dosage ($p<0.01$). This appeared to be a case of excessive antioxidant activity in RJ. A redox imbalance results from the loss of equilibrium between oxidants and antioxidants. The most common oxidants were ROS, which influenced numerous cellular processes by either healthily transmitting signals as second

messengers or pathologically oxidizing proteins, lipids, and DNA (Liu *et al*., 2017).

ROS, such as hydroxyl radicals, directly damage cells, tissues, and blood vessels. The tissue response to stomatotoxic injury appeared to be characterized by ROS activation and its subsequent capacity to trigger several transcription factors. Numerous genes, including those responsible for the production of pro-inflammatory cytokines, are up-regulated as a result of transcription factors such as nuclear factor-B (Watanabe *et al*., 2013). Pro-inflammatory cytokines spread inflammation by concentrating on cells. The research makes it abundantly evident that the somatic cells of the male reproductive tract were responsible for the initial release of the pro-inflammatory cytokine. Inflammation-related changes in immunological homeostasis can result in male infertility (Sadia *et al*., 2023).

A finding by Elnagar (2010) showed RJ treatments (200 mg, 400 mg, and 800 mg) considerably ($p<0.001$) enhanced sperm motility; the improvements were by 15, 18, and 5% when compared to the heat-stressed control in rabbits. Furthermore, Nawar *et al*. (2019) stated that the RJ group and the RJ + PG group showed a significant increase in sperm motility in male albino rats. Nawar *et al*. (2019) explained that RJ contains sperm motility-enhancing substances such as adenosine and adenosine monophosphate. Al-sanafi and Abdulla (2007) and Asadi *et al*. (2019) stated that the RJ boosts the secondary sex organs' ability to produce seminal fluid, which is crucial to the viability and movement of sperm.

4.2.3 Sperm Progressive Motility

Table 4.6 shows the effect of RJ on progressive sperm in a control extender for 8 weeks. Based on the result, T1 has a significantly higher progressive sperm count than T2 and T3 ($p<0.05$). However, there was no significant difference between control, T2 and T3. The highest overall mean of sperm progressive motility was T1, and the lowest sperm progressive motility was T3 (11.21±2.45 and 1.47±0.42, respectively). The control group showed the highest sperm progressive motility at week 8 and T1 showed the highest sperm progressive motility at week 6 (14.67±2.91 and 28.33±5.6, respectively). Both T2 and T3 showed the highest sperm progressive motility at week 2 (4.33±2.85 and 3±1.15, respectively). Table 4.7 shows the effect of RJ on progressive sperm in an RJ extender for 8 weeks. Based on the result, T1 showed a significant positive ($p<0.05$) effect on sperm progressive motility between T2 and T3. But there was an insignificant difference ($p>0.05$) between control group and T1. The result showed that the highest overall mean of sperm progressive motility was T1 while the lowest was T3 (15.05±3.22 and 3.00±0.54, respectively). Next, the control group showed the highest sperm progressive motility at week 8 while T1 showed the highest sperm progressive motility at week 6 (17±1.53 and 43±3.06, respectively). Other than that, T2 showed the highest sperm progressive motility at week 4 and T3 showed the highest sperm progressive motility at week 2 (6±2.31 and 5.33±1.33, respectively).

Table 4.6: Effect of royal jelly on sperm progressive in control extender for 8 weeks

Sperm Quality	Week	Experimental Group			
		C n = 5	T1 n = 5	T2 n = 5	T3 n = 5
Progressive Sperm (%)	0	2.67±0.33	3.67±0.33	2±0.58	3±0.58
	2	7.67±2.91	4.17±2.09	4.33±2.85	3±1.15
	4	4.33±1.45	11.67±4.8	3.33±1.76	0.33±0.33
	6	5±2.52	28.33±5.61	2±0	1±0.58
	8	14.67±2.91	15.67±3.93	0.33±0.33	0±0
Overall mean ± SEM		6.87±1.41^{ab}	11.21±2.45^{b}	2.40±0.68^{a}	1.47±0.42^{a}

SEM, Standard error of Mean. ^{ab} Means with different superscripts in a same row differ significantly ($p<0.05$). C = Control Group, T1 = 100mg of Royal Jelly, T2= 200mg of Royal Jelly, T3 = 300mg of Royal Jelly

Table 4.7: Effect of royal jelly on sperm progressive in RJ extender for 8 weeks

Sperm Quality	Week	Experimental group			
		C n = 5	T1 n = 5	T2 n = 5	T3 n = 5
Progressive Sperm (%)	0	3.67±0.88	5±0.58	3.67±0.88	4.67±0.33
	2	4.67±1.76	5.17±2.14	5±0.58	5.33±1.33
	4	10.33±2.91	10.67±3.79	6±2.31	2.33±0.88
	6	7±1.15	43±3.06	2.67±0.88	1±0.58
	8	17±1.53	25.67±2.33	1.33±0.67	1.67±0.33
Overall mean ± SEM		8.53±1.45^{ab}	15.05±3.22^{b}	3.73±0.64^{a}	3.00±0.54^{a}

SEM, Standard error of Mean. ^{ab} Means with different superscripts in a same row differ significantly ($p<0.05$). C = Control Group, T1 = 100mg of Royal Jelly, T2= 200mg of Royal Jelly, T3 = 300mg of Royal Jelly

The current result showed that there were insignificant changes throughout the week, but there was a significant difference between T1 and other RJ groups. A similar pattern of results was obtained by Al-sanafi and Abdulla (2007) and Khadr *et al*. (2015). Al-sanafi and Abdulla (2007) findings showed that patients receiving RJ for infertile males at doses of 25, 50, and 100 mg/day experienced a substantial ($p<0.01$) increased in active sperm motility. According to other researchers findings, the ejaculate volume, sperm concentration, sperm

progressive motility percentage, and seminal fructose content were all significantly ($p<0.05$) increased by RJ or honey treatments (Khadr *et al*., 2015).

This may be attributed to the impact of RJ, which was known to enhance sperm motility by reducing phosphodiesterase activity and contained motility stimulants including adenosine and adenosine monophosphate((AMP) N (1)-oxide). This enhanced the phosphorylation of both cAMP/calcium-responsive element-binding protein and mitogen-activated protein kinase and elevated cAMP at the level of the sperm tail (Nawar *et al*., 2015). Due to the high concentration of calcium ions in RJ, it may entirely yield sperm cells to increase motility (Nawar *et al*., 2015). Consequently, a rise in cAMP causes a steady increase in sperm motility. Through its impact on glycolysis, cAMP contributes significantly to the sperm's glycolytic pathway. It may affect the energy needed to generate motility in the sperm (Nawar *et al*., 2015)

Maghsoudlou *et al*. (2019) also explained that fructose, glucose, and sucrose make up the majority of carbohydrates. Similar to how honey has a roughly consistent ratio of fructose to glucose. During the incubation stage, fructose uptake fluctuates. Within 0.5–1 hour of incubation, fructose absorption may rise. Then, to make up for the high intake during the first 0.5–1 hour, the fructose uptake may be delayed between 1.5–2 hours. The rate of sugar utilization in the seminal plasma was correlated with the motility of spermatozoa (Abd-Allah, 2010). This may explain why the percentage of progressive sperm was less than 50% because the evaluation was conducted 2 hours after the sample was taken.

4.2.4 Sperm Morphological Abnormalities

Table 4.8 shows the effect of RJ on sperm morphological abnormalities in the control extender for 8 weeks. The result showed that there was no significant difference ($p>0.05$) between all groups. The highest overall mean of sperm abnormal was T2 while the lowest sperm abnormal was control group (46.73±4.86 and 41.47±2.86, respectively). Next, the lowest sperm abnormal in control group throughout the week was at week 8 and the lowest sperm abnormal for T1, T2 and T3 was at week 0 (34.33±4.1, 28.33±10.84, 21.33±8.01 and 17±4.36, respectively). Table 4.9 shows the effect of RJ on morphological abnormal sperm in an RJ extender for 8 weeks. The result showed that the was insignificant difference ($p>0.05$) for all treatments. The lowest overall mean of sperm abnormal was control group while the highest was T3 (37.00±2.62 and 45.80±5.05, respectively). The lowest sperm abnormal for control group was at week 8 while T1 and T2 has the lowest sperm abnormal at week 0 (29.33±2.6, 26.67±6.94 and 20.33±4.37, respectively). However, the lowest sperm abnormal for T3 was at week 4 (21.67±4.67).

Table 4.8: Effect of royal jelly on sperm abnormal in control extender for 8 weeks

Sperm Quality	Week	Experimental group			
		C n = 5	T1 n = 5	T2 n = 5	T3 n = 5
Abnormal Sperm (%)	0	43±9.50	28.33±10.84	21.33±8.01	17±4.36
	2	37.67±6.98	44.17±2.36	36±1.15	59.33±8.41
	4	53.33±2.4	46.5±3.5	47.33±6.49	30±2.08
	6	39±4.04	49.67±4.7	68±3	66.67±4.37
	8	34.33±4.1	47.67±2.03	61±0.58	38±5.03
Overall mean ± SEM		41.47±2.86[a]	43.86±2.34[a]	46.73±4.86[a]	42.20±0.31[a]

SEM, Standard error of Mean. [ab] Means with different superscripts in a same row differ significantly ($p<0.05$). C = Control Group, T1 = 100mg of Royal Jelly, T2= 200mg of Royal Jelly, T3 = 300mg of Royal Jelly

Table 4.9: Effect of royal jelly on sperm abnormal in RJ extender for 8 weeks

Sperm Quality	Week	Experimental group			
		C n = 5	T1 n = 5	T2 n = 5	T3 n = 5
Abnormal Sperm (%)	0	38.67±7.54	26.67±6.94	20.33±4.37	36±8.39
	2	32±7.21	40±4.34	26±5.29	55.33±5.21
	4	48.67±2.33	42±3.06	50.33±4.7	21.67±4.67
	6	36.33±2.91	49±2	65.33±5.21	72.67±2.67
	8	29.33±2.6	27.67±1.33	48.67±1.2	43.33±4.37
Overall mean ± SEM		37.00±2.62[a]	38.19±2.37[a]	42.13±4.75[a]	45.80±5.05[a]

SEM, Standard error of Mean. [ab] Means with different superscripts in a same row differ significantly ($p<0.05$). C = Control Group, T1 = 100mg of Royal Jelly, T2= 200mg of Royal Jelly, T3 = 300mg of Royal Jelly

Based on the result, there was no significant difference ($p>0.05$) between the groups in both extenders, which agreed with Ibrahim *et al*. (2017). Ibrahim *et al*. (2017) studied the RJ effect on sperm quality in adult mice treated with lithium carbonate. It was stated that when compared to the control group, sperm abnormalities in the lithium carbonate group somewhat increased, but there was no statistically significant difference in the lithium carbonate + RJ group ($p\leq0.05$) (Ibrahim *et al*., 2017). The researcher stated it may be due to the RJ substantially raising LH levels, which is used to treat impotence and infertility.

When comparing our results to those of older studies, it was pointed out that El-Hanoun *et al*. (2014) studied the impact of RJ on reproductive performance of male rabbits under hot summer conditions. It was stated that the aberrant and dead sperm displayed the opposite pattern, showing a significant drop that appeared dose-dependent (El-Hanoun *et al*., 2014). Elnagar (2010) also stated that the effects of RJ treatments on abnormal and dead sperm concentrations were significant ($p<0.001$).

The current results did not aligned with Elnagar (2010) and Ghanbari *et al.* (2015). In addition to the earlier benefits of RJ on the reproductive health of heat-stressed males, RJ treatments were able to drastically minimize the

concentrations of abnormal and dead sperm (Elnagar, 2010). According to Elnagar (2010), the antioxidant protective effects of feeding rabbit diets with various vitamin C concentrations enhanced spermatic concentration and decreased abnormal sperm count. The fact that RJ was found to contain testosterone and to have steroid hormone-like properties could be another reason for its favorable effects on rabbits' reproductive health. (Elnagar, 2010). Ghanbari *et al.* (2015) demonstrated that oral administration of 100 mg/kg BW of RJ to diabetic male rats for 42 days increased the weight of the testes, the percentage of viable sperm, the level of serum testosterone, and the motility of the sperm while lowering the quantity of abnormal sperm. These outcomes were related to RJ's antioxidant and estrogenic effects, which were attributed to its improvement of reproductive indices in diabetic male rats (Ghanbari *et al*., 2015).

Under certain assumptions, this can be construed as mishandling. Castellini (2008) stated that, in general, semen characteristics were negatively impacted by all semen handling. Generally, any shock to the sample during semen processing (temperature, chemical, oxygen) may decrease fertility. The sperm properties of some genetic strains were exposed to stringent rearing procedures (light, temperature, nutrition, and collection frequencies); however, they have demonstrated less variability within and between bucks. (Castellini, 2008). Castellini (2008) observed that males from various genetic lines, as well as crossbred and purebred males, differ in their semen qualities. Although semen from these bucks was utilized for artificial insemination, a negative heterotic effect was seen. Crossbred sires often expressed a moderate advantage for several semen features.

4.3 Effect of Sperm Quality between Control and Royal Jelly Extender

Table 4.10 shows the difference in sperm quality between the control and RJ extender. Based on the result, the RJ extender has significantly higher ($p<0.01$) sperm viability than the control extender (58.10±1.37 and 46.52±1.79, respectively). The percentage of motility and progressive motility sperm in RJ (65.32±3.11 and 8.26±1.24, respectively) was higher than in control (58.65±2.76 and 6.05±0.99, respectively) extenders, while the percentage of abnormal sperm in control (43.59±1.88) was higher than in RJ (40.55±1.85) extenders.

Table 4.10: Differences in sperm quality between control extender and RJ extender

Parameter	Experimental Group (Mean ± SEM)		P = Value
	Control	Royal Jelly	
Viability	46.52±1.79	58.10±1.37	**
Motility	58.65±2.76	65.32±3.11	0.11
Progressive Motility	6.05±0.99	8.26±1.24	0.17
Abnormalities	43.59±1.88	40.55±1.85	0.25

SEM, Standard error of Mean. ** indicate ($p<0.01$).

The concentration of RJ was determined based on previous research; 10mg of RJ/6mL of TCY was selected as the RJ extender. El-Sherbiny (2014) stated that after freezing and thawing, the greatest outcome for diluted rabbit semen quality and fertilizing potential when RJ at 10 mg/6 mL TCFY extender was added. The optimum post-thaw sperm quality was achieved with a dose of 0.1% RJ, which can be included in freezing extenders to improve the structural and functional properties of sperm during freezing and thawing (Kaleem *et al.*, 2017).

The presence of RJ in extender significantly improved sperm viability compared to control extender ($p<0.05$). The findings were directly in line with previous findings by Kaleem *et al*. (2017) and Shahzad *et al*. (2016). *Shahzad et al*. (2016) found that, in comparison to other treatment groups, the 0.1% RJ group's sperm vitality was significantly greater in buffalo bull semen. *Kaleem et al*. (2017) also found that comparing the 1% RJ group to other treatment groups, sperm viability was significantly higher in the 1% RJ group of Beetle Buck. According to Yaman *et al*. (2022), the length of the storage period had an impact on the viability percentage of spermatozoa. As storage time increased, the percentage of spermatozoa that were still viable decreased because the sperm's access to nutrient and energy sources declined. Treatment with propolis and RJ contained carbohydrates that were inferior to the fructose in honey *(*Yaman *et al*., 2022). According to El-Sherbiny (2014), progressive motility was reduced based on the storage period as honey and RJ extender were stored for longer periods of time.

The present results agree with Atalla *et al*. (2019), Coskun Cetin *et al*. (2020) and Kaleem *et al*. (2017) that RJ presence in the semen extender improved semen motility and progressive motility. Atalla *et al*. (2019) found that after the second hour of incubation, post-thaw data showed that 0.5% RJ supplementation significantly ($P<0.05$) enhanced total and progressive motility compared to the 0%, 1%, or 2% RJ groups in ram semen. The control group and 0.5% RJ-supplemented group didn't differ significantly in these parameters throughout the first hour of incubation (Atalla *et al*., 2019). According to earlier findings, adding RJ to media that had been frozen at a low concentration (0.5%) significantly ($p<0.5$) improved the sperm's progressive motility. When compared to an incubation medium without RJ, there may be a considerable improvement

in post-thaw sperm motility and membrane integrity that was due to the antioxidant efficacy of RJ. In comparison to the control and low RJ concentration groups, spermatozoa incubated at higher RJ concentrations (1.0 and 2.0%) displayed noticeably reduced motility rates (Atalla *et al*., 2019).

Other researchers also found that in Damascus buck, at 0 h, the Tris egg yolk control (TEY control) group demonstrated noticeably higher motility than the TEY with 1% RJ (TEY-1) group while being comparable to the other royal jelly-supplemented groups which is TEY with 0.25%, 0.5% and 0.75%. The control and TEY-0.25 groups had the lowest motility at 24 and 36 hours. The TEY-1 groups showed the maximum motility at 48 and 60 hours ($p<0.05$) (Cetin *et al*., 2020). The study of El-Sherbiny (2014) in rabbit semen found that when kept at room temperature, the addition of RJ improved progressive motility at 0 hours, 24 hours, and 48 hours. Better progressive motility was seen for all concentrations examined for RJ addition compared to controls and bee honey extenders. Depending on conservation temperatures, the effective dosages of RJ change (El-Sherbiny, 2013, 2014). Shahzad *et al*. (2016) also discovered the post-thaw in buffalo bull semen results showed that 0.1% RJ supplemented considerably enhanced ($p<0.05$) progressive motility when compared to 0 or 0.4% RJ group; however, a non-significant difference in progressive motility was detected among 0.05, 0.1, 0.2, and 0.3% RJ supplemented groups. According to earlier findings, supplementing freezing extender with a mild dose of RJ (0.1–0.3%) significantly increased sperm motility (Shahzad *et al*., 2016).

The current result was similar to Yaman *et al*. (2022), who found no significant difference between treatments ($p>0.05$) for abnormalities in the hybrid chicken sperm. However, RJ extender proved to reduce the percentage of abnormal sperm. Coskun Cetin *et al*. (2020) found that at 0 hours, the TEY-1

group showed a larger percentage of unacceptable spermatozoa than the TEY-0.50 and TEY-0.25 in tris egg-yolk groups. At 24 hours, the TEY-0.25 group had the largest percentage of aberrant spermatozoa, while the highest RJ supplemented group with tris soybean lecithin extenders had the highest percentage of abnormalities. This does seem to depend on genetics and the storage environment. It has an impact on spermatozoa abnormalities. Anomalies could arise as an outcome of storage duration. The percentage of anomalies brought on by osmotic imbalance and cold stress caused by continuous metabolic processes during storage increases with storage time (Yaman *et al*., 2022). Yaman *et al*. (2022) also stated that the metabolism of spermatozoa will convert fructose and carbohydrates into energy, but this process takes time and requires adaptation. Therefore, the process by which spermatozoa adjust to concentration diluents may affect membrane permeability, lessen metabolic activity in cells, harm cells, and reduce the motility of individual spermatozoa (Yaman *et al*., 2022).

4.4 Effect of Royal Jelly on Testis of Rabbit

Based on the present results, there were non-significant differences ($p>0.05$) in testis weight between groups. However, T1 showed a significantly higher testis weight than other groups (4.4±0.8g). Nonetheless, the average seminiferous tubule diameter in T1 was significantly higher ($p<0.05$) than other treatment groups. T1 showed the highest seminiferous tubule diameter (246.03±1.92µm) and control group showed the lowest seminiferous tubule diameter (194.72±1.66µm).

Table 4.11: Effect of royal jelly on testis of rabbit

Experimental group	Parameters	
	Testis Weight (g)	Seminiferous tubule diameter (µm)
Control	2.6±0.1[a]	194.72±1.66[a]
T1	4.4±0.8[a]	246.03±1.92[c]
T2	3.3±0.6[a]	212.33±1.96[b]
T3	3.8±0.1[a]	196.42±1.45[a]

SEM, Standard error of Mean. [ab] Means with different superscripts in a same column differ significantly ($p<0.05$). C = Control Group, T1 = 100mg of Royal Jelly, T2= 200mg of Royal Jelly, T3 = 300mg of Royal Jelly

Figure 4.2 shows photomicrograph of transverse section of rabbit's testis while Figure 4.3 showed the difference in seminiferous tubule of rabbit's testis. A indicates control group, B indicates T1, C indicates T2, and D indicates T3. Figure 4.3 showed that B has the biggest seminiferous tubule compared to other treatment groups.

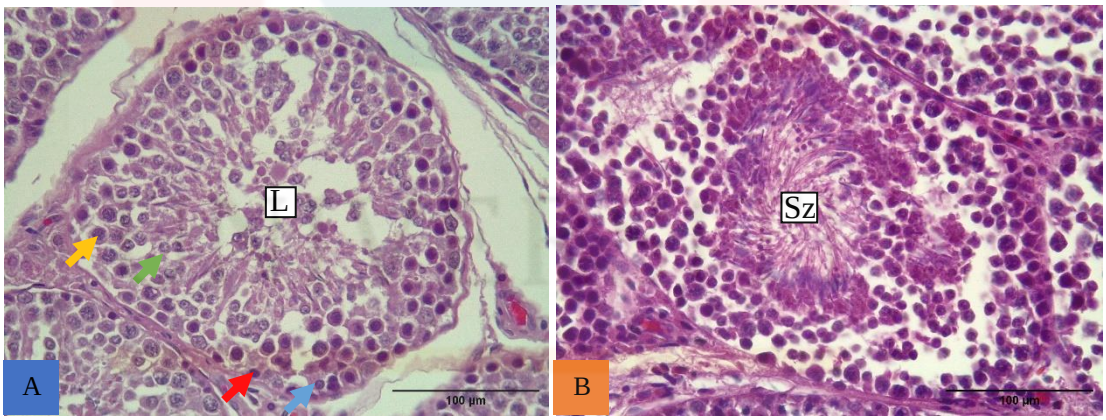

Figure 4.2: Photomicrograph of transverse section of rabbit's testis. A normal seminiferous tubule has numerous Sertoli cells (blue arrow), spermatogonium (red arrow), spermatocytes (yellow arrow), spermatids (green arrow), and spermatozoa (Sz) in the lumen of tubule (L). (A) Control group of seminiferous tubules and (B) T1 group of seminiferous tubules.

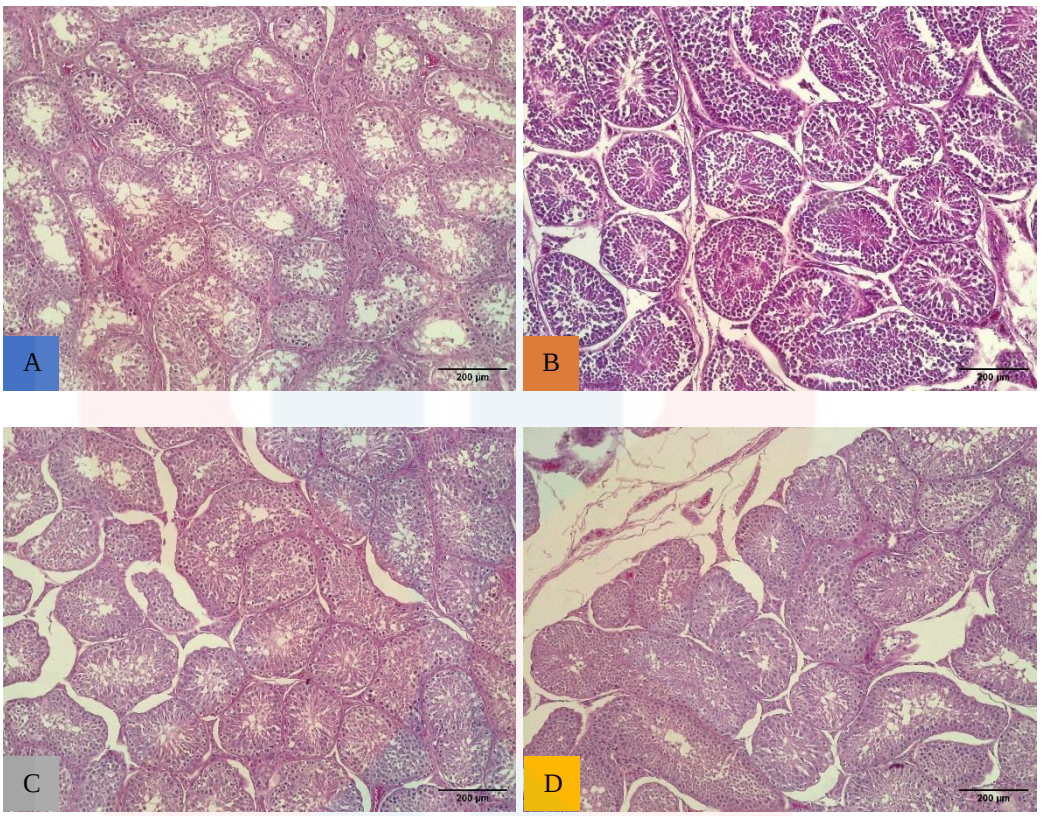

Figure 4.3: Seminiferous tubule of rabbit's testis. Spermatozoa in the lumen of tubule (L). (A) control group showed less spermatozoa in the lumen. (B) T1 group and (C) T2 group showed a normal seminiferous tubule. (D) T3 group showed less spermatozoa in seminiferous tubule.

These basic findings are consistent with research showing that there was a non-significant difference ($p<0.05$) in testis weight between the four groups. However, RJ oral administration does seem to improve the testis weight of the rabbit. The present result was in agreement with Kohguchi *et al*. (2004) and Waykar & Alqadhi (2020). Kohguchi *et al*. (2004) findings showed that histological observations between the three groups (0, 50 and 500 µg of RJ/g diet) showed no change in the testicular weight of the hamster. According to Waykar and Alqadhi (2020), when honey and royal jelly were given orally to rats (honey (500 mg/kg/day) + Royal jelly (100 mg/kg/day)), it was found that these rats' sperm function parameters—such as sperm count, sperm viability, sperm motility, and weight of the epididymis and testis—showed a non-significant rise in comparison to the controls.

Even though there was no significant difference ($p>0.05$) in the testis weight of the rabbit, the addition of RJ increased the testis weight of the rabbit. This was in line with Ghanbari *et al*. (2015), who found that male rats treated with RJ (100mg RJ/kg orally) significantly increased the testicular weight. Other researchers also found that, regarding the means for body weight and testicular weights, there was a significant difference between the diabetic group receiving RJ (400 mg/kg/day BW) treatment and the untreated diabetic group ($p<0.05$) (Karaca *et al*., 2015). Shi *et al*. (2019) also found that at postnatal day 14, oral administration of a moderate dose of RJ (250 mg/kg/day) significantly ($p<0.05$) enhanced the testis weight, the diameter of the seminiferous tubule, and the height of the seminiferous epithelium in the offspring mice. However, seminiferous tubule width was lowered by high-dose RJ (500 mg/kg/day). High doses of RJ decreased testicular weight and size (seminiferous tubule diameter and seminiferous epithelium height). This was included to verify that a higher dosage of RJ can have a negative effect on the male reproductive organ.

A similar conclusion was reached by Karaca *et al*. (2015) and Raafat & Hamam (2012). Raafat and Hamam (2012) finding showed that the mean diameter of the seminiferous tubules increased significantly ($p<0.05$) in the RJ-treated group when compared to the cisplatin group, but not significantly ($p>0.05$) when compared to the control group and the RJ group. According to earlier findings, the diabetic group receiving RJ treatment had larger seminiferous tubules and a higher Johnsen score than the diabetic group not receiving treatment. When compared to the control group, these values were significantly lower in the untreated diabetes group (Karaca *et al.*, 2015). Najafi *et al.* (2014) stated that seminiferous tubules were present and actively involved in spermatogenesis in the testes of mice treated with RJ, which showed a

significant difference when compared to the oxymetholone group at $p<0.05$ and left untreated, which showed a significant difference when compared to the control group at $p<0.05$.

El-Hanoun *et al.* (2014) explained that RJ had positive impacts on male reproductive capabilities because it boosted semen production from seminiferous tubules, which in turn produced complete, highly mobile sperm. Furthermore, FSH is necessary for the growth of the seminiferous tubule epithelium, maintenance of the mitotic stages of spermatogenesis, and increased production of androgen binding protein, which promotes proliferation in the spermatogenesis process (Inoue *et al.*, 2003), although RJ contains L-arginine and carnitine amino acids, which are essential for spermatogenesis (Nagai & Inoue, 2004), the blood testes, barrier (BTB) will evolve as a result if specific protein expressions and/or assembly are changed because this allows toxins to more easily penetrate the seminiferous epithelium (Abdel-Kawi, 2021).

4.5 Correlation between Histology of testis and Sperm Quality

Table 4.12 shows the correlation between testis weight (TW), seminiferous tubule diameter (STD), and sperm quality in the control extender. sperm count (SC) has a negative effect on viability sperm (VS), motility sperm (MS), progressive motility (PM), TW, and STD (-0.129, -0.208, -0.163, -0.108, and -0.075, respectively) but the relationship was not significant ($p>0.05$), while SC has a positive effect on AB (0.074). However, VS has a significantly positive effect ($p<0.05$) on MS and PM (0.398 and 0.255, respectively). Other than that, MS has a significantly positive effect ($P<0.01$) on PM (0.644). Meanwhile, PM has a negative effect on abnormal sperm (AB), TW, and STD (-0.003, -0.267, and -0.049, respectively). Sperm abnormality has a negative effect on TW and STD (-0.108 and -0.228, respectively), while TW has a significantly positive effect ($p<0.01$) on STD (0.970).

Table 4.12: Correlation between histology of testis and sperm quality for control extender

	SC	VS	MS	PM	AB	TW	STD
SC	1						
VS	-0.129	1					
MS	-0.208	0.398**	1				
PM	-0.163	0.255*	0.644**	1			
AB	0.074	0.242	0.016	-0.003	1		
TW	-0.108	-0.758*	-0.614	-0.267	-0.108	1	
STD	-0.075	-0.197	0.102	-0.049	-0.228	0.970**	1

** Correlation is significant at the 0.01 level.

* Correlation is significant at the 0.05 level.

Sperm Count (SC), Viability Sperm (VS), Motility Sperm (MS), Progressive Motility (PM), Abnormal Sperm (AB), Testis Weight (TW), Seminiferous Tubule Diameter (STD)

Based on table 4.13, there was a significantly positive effect ($p<0.01$) on VS with MS (0.483), while there was a negative correlation ($p<0.01$) on VS with TW and STD (-0.564 and -0.110, respectively). Other than that, MS has a significant positive effect ($p<0.01$) on PM (0.645). SC in RJ extenders showed a negative effect on VS, MS, TW, and STD (-0.073, -0.137, -0.108, and -0.075, respectively) but the relationship was insignificant ($p>0.05$). However, PM showed a negative effect with AB and STD (-0.129 and -0.065, respectively). AB showed a positive effect on SC, VS and MS (0.100, 0.107 and 0.019, respectively) but there was negative effect on TW and STD (-0.145 and -0.135, respectively).

Table 4.13: Correlation between histology of testis and sperm quality for RJ extender

	SC	VS	MS	PM	AB	TW	STD
SC	1						
VS	-0.073	1					
MS	-0.137	0.483**	1				
PM	0.007	0.214	0.645**	1			
AB	0.100	0.107	0.019	-0.129	1		
TW	-0.108	-0.564	-0.096	0.265	-0.145	1	
STD	-0.075	-0.110	0.016	-0.065	-0.135	0.970**	1

** Correlation is significant at the 0.01 level.

Sperm Count (SC), Viability Sperm (VS), Motility Sperm (MS), Progressive Motility (PM), Abnormal Sperm (AB), Testis Weight (TW), Seminiferous Tubule Diameter (STD)

Table 4.14 shows the correlation between histology of testis and sperm quality for both control and RJ extenders. The present result showed that SC has a positive effect on PM and AB (0.151 and 0.029, respectively). However, VS has a significantly positive effect ($p<0.01$) on MS and PM (0.512 and 0.316, respectively) but it has a significantly negative effect ($p<0.01$) on STD (-0.338). PM has a significantly positive effect ($p<0.01$) on VS and MS (0.316 and 0.651, respectively). The correlation coefficient in TW shows a negative effect on sperm quality such as SC, VS, MS, and AB (-0.108, -0.564, -0.096, and -0.145, respectively), while STD shows a significantly negative effect on VS and MS (-0.338 and -0.188, respectively). Lastly, the correlation coefficient of STD shows a significantly positive ($p<0.01$) with testis weight (0.970).

Table 4.14: Correlation between histology of testis and sperm quality for control extender and RJ extender

	SC	VS	MS	PM	AB	TW	STD
SC	1						
VS	-0.070	1					
MS	-0.032	0.512**	1				
PM	0.151	0.316**	0.651**	1			
AB	0.029	0.115	-0.009	-0.080	1		
TW	-0.108	-0.564	-0.096	0.265	-0.145	1	
STD	-0.075	-0.338**	-0.188*	-0.124	0.075	0.970**	1

** Correlation is significant at the 0.01 level.
* Correlation is significant at the 0.05 level.
Sperm Count (SC), Viability Sperm (VS), Motility Sperm (MS), Progressive Motility (PM), Abnormal Sperm (AB), Testis Weight (TW), Seminiferous Tubule Diameter (STD)

Based on the present result, there was a significant correlation between VS, MS, and PM ($p<0.001$), while TW has a significant correlation with STD. However, there were no references to the correlation coefficient between TW, STD, and sperm quality in rabbits supplemented with RJ. However, the result shows a negative correlation between SC and VS in Tables 4.12, 4.13, and 4.14,

similar to Mahmood *et al*. (2014). That stated, there was a significantly negative correlation between sperm concentration and sperm viability in Cholistani bulls ($r = -0.839$, $p<0.05$) (Mahmood *et al*., 2014). Based on the correlation between STD and VS, MS, and PM in both extenders, it can be concluded that changes in STD can have a negative effect on sperm quality. It also shows STD has a strong relationship with TW. Silber and Rodriguez-Rigau (1981) found a strong correlation between the number of mature spermatids per tubule and the quantity of sperm per milliliter in male patients without ductal blockage.

Tables 4.12 and 4.13 showed a change in the correlation between SC and PM (-0.163 and 0.007, respectively). The sperm count and FSH values also exhibited a strong positive correlation, while the correlation coefficient for the latter was lower (0.2594) than it was for the former for LH and prolactin (0.674 and 0.5318, respectively) (Biswas *et al*., 1978). FSH only enhances sperm motility at low levels. FSH improved sperm morphology and concentration at intermediate doses. Progressive motility and overall sperm count both indicated an upward trend. Sperm concentration, total sperm count, and progressive motility were all boosted by large doses of FSH (Cannarella *et al*., 2020). According to Abdelnour *et al*. (2020), RJ contributes to the production of hormones such as testosterone, LH, and FSH. Other than that, the correlation of progressive sperm with testis weight also changes between control extenders and RJ extenders (-0.267 and 0.265, respectively).

CHAPTER 5

CONCLUSION

5.1 Conclusion

Current research confirmed that RJ enhanced the sperm viability in rabbits when administered orally and as an extender. However, excessive consumption of RJ has the reverse effect. Based on our findings, RJ extender showed a significant positive compared with other groups in term of sperm viability. The weight of the rabbit's testicles also remained unchanged when RJ was used. However, there was a significant difference between the groups in terms of the diameter of the seminiferous tubule in 100 mg RJ. Oral administration of 100 mg of RJ was proved to significantly increase the seminiferous tubule diameter compared with control groups.

In conclusion, it has been demonstrated that RJ has a favorable impact on the sperm quality and testis of rabbits. RJ improved the sperm quality, however, varied according to the amount administered to the rabbit. Rabbits' reproductive traits can have negative effect by high RJ concentrations. As a result, oral treatment with 100 mg of RJ along with RJ extender was effective in improving rabbit sperm quality. RJ has been shown to be useful as an extender and oral supplement in rabbit feed.

5.2 Recommendation

Future research should be done based on the climate changes in Malaysia. Royal jelly can be studied more carefully in rabbits based on the suitability of the weather in Malaysia. It can help to determine the difference in the effect of royal jelly on the reproductive traits of rabbits in the monsoon season and the dry season. The temperature changes in the surrounding area can affect the quality of the semen. It can help to determine which season is better for breeding and has good sperm quality.

In addition, studies on AI can be improved by adding certain hormones, such as PMSG, to increase the effectiveness of AI and study the difference in the effect of RJ on rabbits. Due to the lack of a female sample, the doe used had not given birth for a long period of time. That is why using PMSG can stimulate lactation receptivity. Other than that, research on AI can also be done according to the different seasons in Malaysia. It can help breeders facilitate rabbit breeding planning by using AI. The effect of extender control and RJ can also be studied further by using AI to obtain the fertility rate of the rabbit.

Based on the current research, Redox imbalance seems to be the main cause of the decreasing of the sperm quality. It was the effect of high consumption of RJ in the rabbits. In future research, ROS can be measured based on the different concentration of RJ supplementation in rabbits. It can help to determine the antioxidant activity of RJ that is consumed by the rabbits.

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APPENDICES

Extender composition

Ingredient	Quantity
Tris	3.028 g
Citric Acid	1.675 g
Fructose	1.250 g
Egg Yolk	20 ml
Penicillin	0.1g

List of publication

1. Effect of Royal Jelly as Supplementation in Feed on Sperm Progressive Motility and Histology of Testis for Rabbits.