UNIVERSITI PUTRA MALAYSIA

ASSESSMENT OF FELINE SPERMATOZOA QUALITY VIA MTT ASSAY FROM POST-ORCHIDECTOMY EPIDIDYMAL SALVAGE

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "ASSESSMENT OF FELINE SPERMATOZOA QUALITY VIA MTT ASSAY FROM POST-ORCHIDECTOMY EPIDIDYMAL SALVAGE" by Nadia Hussain

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DEDICATION

To Papa, Mumma & Sidra

To all my furrkids

To three year old me

- *Nadia Hussain -*

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LIST OF ABBREVIATIONS

AI	Artificial Insemination
MTT	(3-(4, 5-dimethylthiazol-2-y1)-2, 5-diphenyltetrazolium bromide)
OD	Optical Density
DMSO	Dimethyl sulfoxide

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 - Projek.

PENILAIAN KUALITI SPERMA KUCING MELALUI UJIAN MTT PASCA-PENYELAMATAN EPIDIDIMIS SELEPAS ORKIDEKTOMI

oleh

NADIA HUSSAIN

2023

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Ujian MTT (3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide) adalah kaedah kolorimetrik untuk mengukur aktiviti metabolik sel sperma yang aktif. Kajian ini bertujuan untuk menentukan korelasi kualiti sperma melalui penilaian sperma tradisional dan ujian MTT serta membandingkan dua teknik pasca-penyelamatan sperma epididimis pada kucing domestik. Dua puluh testis kiri dan kanan telah dikumpul dan sperma epididimis diperoleh melalui dua teknik berbeza iaitu cincangan dan hirisan. Penilaian sperma dijalankan untuk menilai corak gelombang, motiliti, peratusan hidup, peratusan sperma tidak normal, dan kepekatan sperma. Seterusnya, setiap sampel menjalani ujian MTT pada absorbans 570nm. Menggunakan analisis korelasi Spearman, terdapat korelasi

positif antara kadar penyerapan MTT dan motiliti (r=0.061) serta jumlah sperma tidak normal (r=0.296) bagi testis kiri tetapi keputusannya tidak signifikan ($P > 0.05$). Semua parameter lain bagi testis kiri seperti bilangan sperma hidup (r=-0.409), corak gelombang (r=-0.021), dan jumlah sperma keseluruhan (r=-0.201) menunjukkan korelasi negatif dengan kadar penyerapan MTT yang tidak signifikan ($P > 0.05$). Ujian yang sama diulangi bagi testis kanan yang menunjukkan korelasi positif antara kadar penyerapan MTT dan jumlah sperma tidak normal (r=0.396) yang tidak signifikan ($P > 0.05$). Manakala, semua parameter lain seperti motiliti (r=-0.313), bilangan sperma hidup (r=-0.396), corak gelombang (r=-0.305), dan jumlah sperma keseluruhan (r=-0.370) menunjukkan korelasi negatif dengan kadar penyerapan MTT yang tidak signifikan ($P > 0.05$). Ujian Wilcoxon dilakukan untuk membandingkan dua teknik pasca-penyelamatan sperma epididimis, iaitu cincangan dan hirisan. Keputusan menunjukkan bahawa tiada perbezaan yang signifikan ($P > 0.05$) antara teknik-teknik tersebut. Ketidaksignifikan korelasi antara ujian MTT dan parameter penilaian sperma tradisional menimbulkan kebimbangan terhadap penggunaan ujian MTT dalam mengukur kualiti sperma. Kajian lanjut diperlukan untuk menyempurnakan ujian MTT bagi penilaian yang lebih tepat terhadap kualiti sperma dalam kucing domestik. Walaupun tiada perbezaan yang signifikan antara teknik cincangan dan hirisan, teknik cincangan adalah lebih efisien untuk mendapatkan sperma.

Abstract

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4999 – Final Year Project.

ASSESSMENT OF FELINE SPERMATOZOA QUALITY VIA MTT ASSAY FROM POST-ORCHIDECTOMY EPIDIDYMAL SALVAGE

BY

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2023

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MTT (3-(4, 5-dimethylthiazol-2-y1)-2, 5-diphenyltetrazolium bromide) assay is a colorimetric method to measure cellular metabolic activity of active sperm cells. This study aims to determine the correlation of semen quality via traditional semen evaluation and MTT assay as well as to compare two epididymal semen salvage techniques in domestic cats. Twenty left and right testicles were collected and epididymal semen were extracted via two different techniques of mincing and slicing. Semen evaluation was performed to assess wave pattern, motility, live percentage, abnormal sperm percentage and concentration. Subsequently, each sample underwent MTT assay at absorbance of 570nm. Using Spearman's correlation coefficient analysis, there were positive correlation between MTT absorbance rates and motility ($r= 0.061$) and abnormal sperm count ($r = 0.296$) of the left testicles but the test was not significant ($P > 0.05$). All other parameters

for the left testicle such as live sperm count (r = - 0.409), wave pattern (r = - 0.021) and total sperm count (r = - 0.201) had a negative correlation with MTT absorbance rate that was not significant (P > 0.05). The same test was repeated for the right testicle which resulted in positive correlation between MTT absorbance rates and abnormal sperm count (r = 0.396) which were not significant (P > 0.05). All other parameters such as motility (r = - 0.313), live sperm count (r = - 0.396), wave pattern (r = - 0.305) and total sperm count (r = - 0.370) had a negative correlation with MTT absorbance rate that was not significant (P > 0.05). Wilcoxon signed ranked test was performed to compare the two epididymal semen salvage techniques of, mincing and slicing. The results showed no significant (P > 0.05) difference between the techniques. The lack of significant correlations between the MTT assay and traditional sperm evaluation parameters raises concerns regarding the MTT assay's utility for measuring sperm quality. Further research is necessary to refine the MTT assay for more accurate assessments of sperm quality in domestic cats. Although there was no significant difference between the mincing and slicing techniques, the mincing technique was more convenient for sperm salvage.

1.0 INTRODUCTION

Cats have been bred for companionship for many years now. There has been a growing trend in popularity of visually appealing cat breeds including Maine Coons, British Shorthairs, and Munchkins. Therefore, pedigrees and stud lines are important for the breeding program's genetic diversity and health. Alternative means to retain a high-producing stud tomcat's genetic material in the event of his death would be a useful asset in breeding.

In the male reproductive system, the epididymis is a crucial location where sperm develop and become motile. The testes produce spermatozoa, which are initially immotile and unable to fertilize. They pass via the epididymis, where they experience modifications that allow them to become completely functional and motile. Sperm cells contained within the epididymis are referred to as epididymal spermatozoa. These spermatozoa are in different phases of development. As they go through the epididymis, they develop motility and the capacity to fertilise an egg (Axnér et. al, 1999). In some circumstances, such as when a male cat has passed away and its genetic material needs to be preserved for future breeding, epididymal sperm can be extracted from the epididymis. The sperm obtained has the potential to be utilised in assisted reproductive procedures such as in vitro fertilisation or artificial insemination (AI). In reproductive research and veterinary medicine, it is crucial to comprehend the physiology and traits of epididymal spermatozoa, especially when natural breeding is impractical or when safeguarding priceless animal genetic material is involved (Hewwit et.al, 2001).

A colorimetric method for determining metabolic activity in living cells, including spermatozoa, is the MTT assay. The assay relies on the introduction of metabolically active cells, such as spermatozoa, to reduce the yellow-colored, water-soluble tetrazolium salt MTT to a purple formazan crystal that is non-water-soluble (Ghasemi, 2021). This conversion is carried out by the active mitochondria's succinate dehydrogenase system. For determining the proportion of viable spermatozoa in a variety of species, including humans, boars, horses, and cattle, the MTT test is an easy, quick, and accurate technique (Byun. J et.al, 2008) (Aziz, 2006).

The first objective in this study was to assess epididymal spermatozoa by employing two distinct methods namely conventional evaluation and via MTT assay. Known methods for evaluating sperm quality, such as motility, concentration, morphology, and viability, were used in conventional semen evaluation procedures. The MTT assay, on the other hand, gauges cellular metabolic activity as a proxy for sperm viability. The goal was to identify whether MTT assay and conventional evaluation techniques relate to each other while assessing the quantity and quality of epididymal sperm. The second objective compared two approaches to the preservation of epididymal sperm. There are several methods for obtaining epididymal sperm, including mincing and slicing. This goal was to assess and contrast the viability, effectiveness, and potentially even quality of sperm produced by these various techniques. A comparison may include things like sperm yield, simplicity of use, and possible effects on sperm quality.

1.1 Objectives

1. To determine the quality and quantity of epididymal spermatozoa using conventional semen evaluation and MTT assay.
2. To compare two methods for epididymal sperm salvage.

1.2 Hypothesis

- Null hypothesis 1 (H_0): There is no correlation with the quality of semen evaluated via conventional methods compared to MTT assay.
- Alternate hypothesis 1 (H_1): There is correlation with the quality of semen evaluated via conventional methods compared to MTT assay.
- Null hypothesis 2 (H_0): There is no significant difference in sperm quality via mincing or slicing technique.
- Alternate hypothesis 2 (H_1): There is significant difference in sperm quality via mincing or slicing technique.

1.4 Justification

Artificial insemination uses fresh semen that was collected by electroejaculation and artificial vagina in the tomcat. However, there is a possibility that viable epididymal spermatozoa can be extracted from castrated testes and be used in artificial insemination. This procedure would be invaluable to preserve genetic material.

2.0 LITERATURE REVIEW

2.1 Cats

Cats are enigmatic elegant animals that have long won people over with their special mix of independence and love. Cats are adored for their glossy coats, captivating gaze, and lively personalities (Parkhi et.al, 2012). Within the Felidae family, *Felis catus*, or the domestic cat, is an interesting species. They are obligatory carnivores with unique physiological and behavioural traits that have developed over millennia, allowing them to be successful hunters and human companions (Parkhi et.al, 2012). Because of decades of selective breeding, domestic cats, who are taxonomically categorized within the Felidae family and Felis genus, exhibit a broad range in coat colour, pattern, and size. Considering that they are descended from their wild predecessors through evolution, they have many characteristics in common with felines, like their sharp senses, retractable claws, and carnivorous diet (John et.al, 1968).

2.2 Cats in Malaysia

In Malaysia, there are plenty of cats in urban and rural settings. In addition to being kept as pets, they can also be found roaming around in places like marketplaces, temples, and residential areas. In Malaysia, many people view cats with affection and they have cultural significance (Cat, 2023).

Similar to other regions globally, cats are valued in Malaysia for their ability to provide companionship, manage pests, and simply be a charming presence. They frequently live side by side with people; in fact, some communities actively feed and care for stray cats. But there are other problems associated with the stray population, including overcrowding, which is why spaying, neutering, and adoption programmes have been implemented in an attempt to control it (Kousi, n.d.).

Culturally, Malaysians have a particular place in their hearts for cats. They frequently appear in regional legends, folklore, and customs, signifying a variety of qualities like fortuity, discernment, and dexterity. In addition, cat cafes are becoming more and more common in Malaysian cities, offering areas where people can unwind or socialise with cats (Munir et al., 2023).

Different people and communities in Malaysia have different opinions about cats. While cats are cherished as pets in some homes, their cultural or personal beliefs may lead to a different perspective on them in others. All things considered, cats play a significant role in Malaysian society, bringing charm and company to a great number of the nation's citizens.

2.3 Spermatozoa

Spermatozoa, or sperm cells, are essential to the health of the reproductive system. These are the male reproductive cells that fertilise the egg of the female and induce pregnancy. Within the female reproductive system, spermatozoa are highly specialised cells that have successfully developed to carry out their activity outside of the male body (Garner et.al, 2000). They can overcome a variety of physiological obstacles by swimming towards the egg because to their special shape and motility. Seminal plasma, the fluid that carries and maintains spermatozoa, is made up of substances that nourish the sperm cells and provide information about the quality and condition of the ejaculate. Furthermore, the movement of spermatozoa within the female reproductive canal is significantly influenced by sperm motility (Chao et al., 2009). The evaluation of sperm motility is essential in assisted reproductive technology for predicting fertilisation success (Salian et al., 2018). Thus, research on reproductive biology, fertility management, and the development of assisted reproductive technologies requires an understanding of the biochemistry, anatomy, and function of spermatozoa (Patel et al., 1999). Additionally, spermatozoa are important in the domains of genetics, artificial insemination, cellular and molecular biology, and reproductive biology, according to current studies.

2.4 Epididymal Spermatozoa

The sperm mature and acquire motility, or the capacity to travel, to fertilise eggs in the epididymis, a coiled tube that lies behind each testicle. The testes produce immature spermatozoa that cannot be fertilised at first. As they pass through the epididymis, they undergo significant modifications and acquire the capabilities required for fertilization.

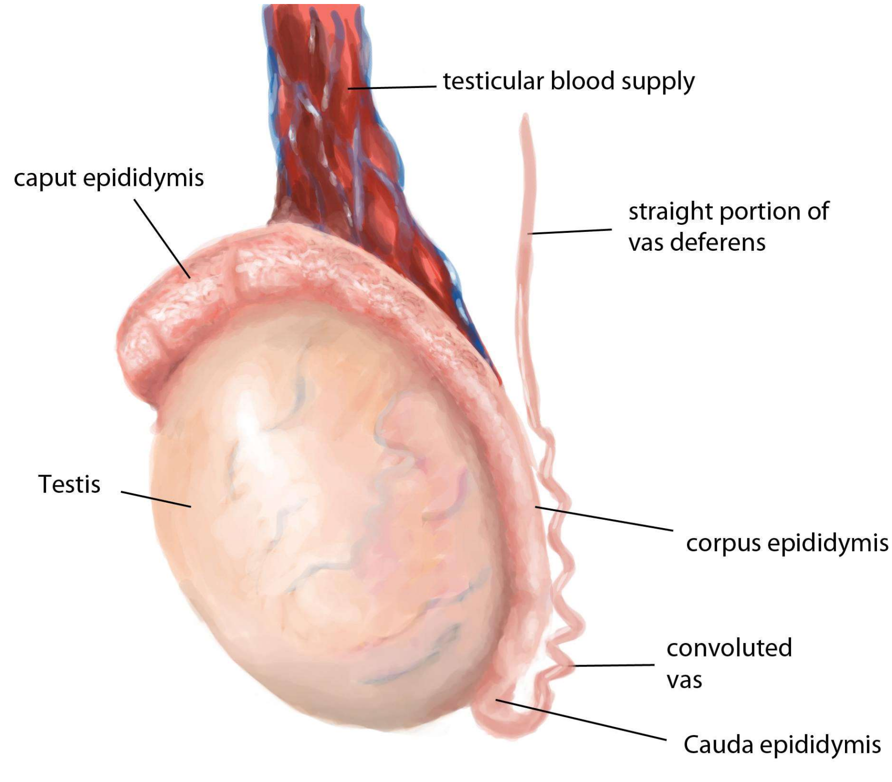

Fig. 1: Anatomy of the testes (Hassan, 2021)

A number of significant changes occur throughout the process of sperm maturation in the epididymis. Firstly, immature spermatozoa from the testes enter the epididymis. Here, they go through a maturation phase that, depending on the species, might take several days to weeks. They develop motility and the capacity to fertilise an egg during this phase. Mature spermatozoa are kept in storage in the epididymis (Angrimani, 2014). This structure holds sperm cells until a sexual activity occurs and they are released. Subsequently, spermatozoa undergo structural changes and gain vital components necessary for their activity as they pass through the epididymis. These modifications include enhancements in motility, modifications to the proteins on the surface of sperm, and modifications to the cell membrane (Roels, 2014).

Spermatozoa from the epididymides are essential for fertilisation to succeed. They must develop inside the epididymis in order to learn how to pass via the female reproductive system and fertilise an egg. Once discharged during ejaculation, these mature spermatozoa contain the genetic material required for fertilisation and the start of embryonic development (Hassan, 2021).

Barker & Gandier (1957) were able to artificially inseminate a mare successfully using epididymal sperm. Sperm cells are stored in the epididymis frequently characterised as immature, immotile, and infertile, in the epididymis they develop, acquire motility, and become fully fertile (Hassan, 2021). Feline reproductive technology can greatly benefit from the use of epididymal spermatozoa, particularly in situations where azoospermia occurs or when a donor male unintentionally passes away or has an orchiectomy (Luvoni, 2017). It is possible to gather and cryopreserve spermatozoa for later usage, which could open up breeding prospects. Artificial insemination and intracytoplasmic sperm injection (ICSI) are two assisted reproductive procedures that have made use of epididymal spermatozoa (Galarza et. al, 2021). In order to preserve the genetic diversity and survival of threatened or endangered species, epididymal spermatozoa can be gathered and kept in sperm banks (Joram, 2016). Epididymal spermatozoa collected from testicles stored under certain conditions can be utilised to preserve genetic material from domestic, endangered/threatened wild species (Strand, 2016).

2.5 Epididymal spermatozoa salvage techniques

Techniques for extracting and preserving sperm from the epididymis are known as epididymal spermatozoa salvage procedures, and they can be helpful in instances where important genetic material needs to be preserved, such as after an orchiectomy or accidental death. For this reason, many strategies have been created such as mincing, single incision epididymal sperm aspiration, slicing, retrograde flushing (Hassan, 2021). Rat, porcine and bovine epididymal sperm have been collected successfully by tissue mincing and incubation in various media (Tulsiani et al., 1993). Slicing technique proved to be effective in epididymal sperm salvage (Joram, 2016).

2.6 Conventional semen evaluation parameters

In veterinary medicine, the assessment of semen plays a crucial role in determining an animal's male fertility. Numerous factors, including semen volume, colour, pH, sperm concentration, motility, morphology, and sperm cell viability are analysed (Kustritz, 2007). The diagnosis of male infertility, the tracking of animals in a breeding programme, and the evaluation of the results of male fertility management and other interventions all depend on this examination. To maximise the precision and use of semen evaluation in veterinary practise, standardised procedures for the assessment of all semen parameters and quality control are crucial (Kustritz, 2007).

Male animals are semen sampled using a variety of techniques. This is frequently accomplished in cats by manual stimulation or the application of specific collection tools. The colour, consistency, volume, and anomalies of the collected semen are examined visually. If the sample seems normal, this can be ascertained through preliminary evaluation. The concentration of sperm per ml of semen can be precisely measured with a counting chamber or other specialised equipment. This number sheds light on the male's capability for conception (Christensen & Meyers, 2023). Sperm cells' motility and capacity to swim are evaluated under a microscope. Sperm need progressive motility in order to go to the female's eggs and fertilise them. Sperm cells are inspected for irregularities in size, shape, and structure. An irregular sperm morphology can hinder its ability to fertilise an egg. It is calculated what proportion of living sperm cells are in the sample via eosin-nigrosin staining. Sperm cells are the only ones that can fertilise (Zambelli & Cunto, 2006).

To effectively accomplish these tests, veterinarians and reproductive specialists use specialised tools such as counting chambers, staining procedures, and microscopes. The findings aid in the diagnosis of possible fertility problems, the making of breeding decisions, and the development of plans for enhancing the effectiveness of reproduction in animal breeding operations. Furthermore, in both domestic and endangered species conservation programmes, the examination of semen is essential for the selection of healthy and viable males for breeding reasons (Rijsselaere & Soom 2012).

2.7 MTT Assay

A colorimetric assay called the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is used to gauge the cytotoxicity, proliferation, and metabolic activity of cells. It is predicated on metabolically active cells reducing a yellow tetrazolium salt to purple formazan crystals (Reubel, 1989).

In many scientific researches, the MTT assay is frequently used to measure metabolic activity and determine the survival of cells. Although it is frequently applied to cell cultures, it can also be modified to evaluate the viability of sperm. Mitochondria in metabolically active cells are functioning. Succinate dehydrogenase enzymes found in these mitochondria have the ability to convert MTT into insoluble formazan crystals. This reduction process takes place inside of living cells, more precisely in the mitochondria. Viable cells with an active metabolism will transform MTT into formazan when it is introduced to the cells. These cells undergo this change gradually, leading to the development of purple formazan crystals. The number of cells that are metabolically active is directly correlated with the amount of formazan generated. Thus, the assay uses the intensity of the colour produced to indirectly quantify the vitality of the cells. A higher formazan production from more live cells will result in a stronger colour signal. Following the MTT incubation time, the cells are typically lysed in order to dissolve the formazan crystals that have grown inside of them. The extraction of the purple formazan solution is made possible by this procedure. A spectrophotometer is used to measure the formazan solution's absorbance or optical density. The quantity of live cells in the sample is directly correlated with the color's intensity. An estimate of cell viability or metabolic activity is given by this measurement (Jang, 2010).

One of the many cell types for which the assay is commonly used is sperm cells. Byun et al. (2008) developed an MTT assay for pig sperm and found that it is a simple and dependable method of verifying sperm vitality. A comparable MTT assay has been created to assess the viability of sperm from humans (Nasr-Esfahani et al., 2002), stallions (Aziz et al., 2005), bovines (Aziz, 2006), buffaloes (Iqbal et al., 2009), and poultry (Hazary et al.,2001). The mitochondrial enzyme succinate dehydrogenase has been shown by several authors to diminish MTT (Slater et al., 1963; Hazary et al., 2001; Aziz et al., 2005; Aziz, 2006). As a result, they believed that the formazan dye's production and precipitation occurred inside the mitochondria. The amount of formazan that is generated would reveal the quantity and activity of mitochondria. However, recent research (Berridge and Tan, 1993; Liu et al., 1997; Berridge et al., 2005; Diaz et al., 2007) has demonstrated that MTT is largely reduced in the cytoplasm. Therefore, the MTT test is a method that measures mitochondrial activity indirectly.

3.0 MATERIALS AND METHODS

3.1. Animals

Paired testes and epididymis were obtained from 20 tomcats, retrieved via orchidectomy (open technique) under general anesthesia by a licensed veterinarian from four small animal clinics. The anesthetic drugs used were ketamine (10mg/kg) and xylazine (0.5 - 1mg/kg) administered intramuscularly. The patients were placed on dorsal recumbency and the scrotal area was clipped, surgically scrubbed and prepared. To excise each testicle, an incision was made on each scrotal sac. Then the tunica vaginalis was incised and the testicle was exteriorised. The spermatic cord was tied off, cut and the testicle was removed.

3.2. Sample collection

Thereafter, either one of two techniques were used to harvest the sperm cells from each vas deferens and the cauda epididymis which were: slicing (float-up) and mincing. For slicing, a sterile scalpel blade #15 was used to was cut the epididymis in half longitudinally and then deposited into a conical tube with 5 ml of PBS. In order to allow the epididymal sperm to swim to the upper section of the solution, the solution was left to stand for 5–10 minutes. The PBS solution's upper two thirds were then pipetted out and transferred to a centrifugation tube for centrifugation at 10,000 rpm for 10 minutes (Kobuta model 2100). For the mincing technique, the epididymis was suspended in 5ml PBS on a petri dish and pairs of scissors was used to thoroughly mince the epididymis. It was then allowed to sit for 10 mins.

3.3. Semen evaluation

Prior to the process, plain glass tubes containing semen and PBS were submerged in a water bath at 37°C. A drop of nett semen was put on a glass slide and examined under a light microscope at 4X for wave pattern and 10X objective to observe general motility and individual motility. Scores for wave patterns ranged from 1 to 5 (David et.al, 2018). Through observation, the percentage of motile sperm was estimated and recorded as general motility (%). Individual motility was examined under the objective lens of 20X or 40X to check for variations of motility patterns of progressive, backward, rotating, and vibrating.

Verifying the live sperm percentage was another phase in the examination of the semen. Three to four drops of eosin-nigrosin stain and one drop of nett semen were applied to a glass slide and left to stand for three minutes. After that, a thin smear was made on a glass slide, air dried, and inspected at 40X objective. Under the microscope, the head of dead sperm were stained pinkish purple, whereas live ones were undyed. The live and dead percentages were counted using a cell counter.

Evaluating the semen concentration was the final step for semen evaluation. A dilution of 1:100 was obtained by diluting 10µl of fresh semen with 990µl of formal saline. A hemocytometer was used to do a concentration count on the diluted semen, which was then inspected at 40X objective magnification.

The Y1 and Y2 chambers, as well as five boxes with sixteen tiny boxes each that were selected and counted from each chamber, make up each hemocytometer. To reduce counting redundancy, the top-left grid method was employed to count the semen. After averaging the total semen from Y1 and Y2, the concentration of sperm sperm/ml is calculated using the formula: Y x Dilution Factor x Conversion Factor.

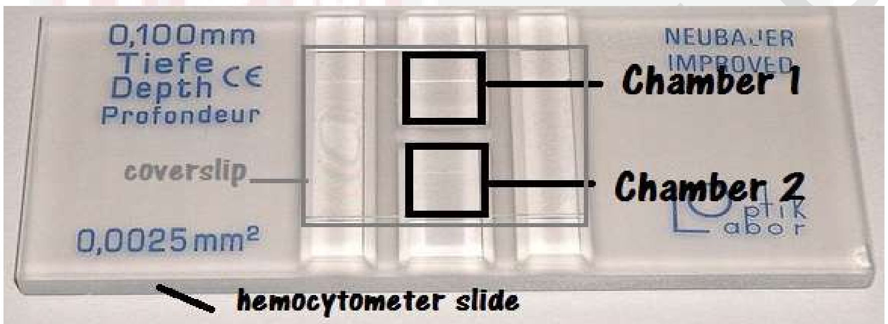

Fig. 2: Example of a hemocytometer and it's counting chambers (https://cellculture.altervista.org/).

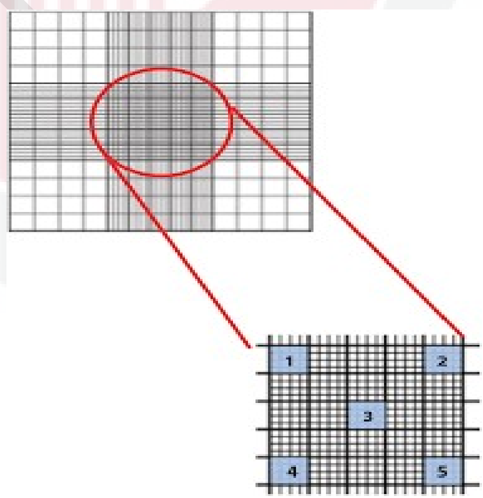

Fig. 3: Haemocytometer chamber containing 25 squares with each containing 16 smaller squares (abcam.com).

3.4. MTT assay

Each semen sample was immediately diluted in PBS to acquire sperm concentrations of 3 x 10^6 sperm/ml. In a 96 well plate, 100µl of diluted semen sample was added to each well plate with 10µl of MTT solution (10:1, v/v). The plate was then incubated for an hour at 37°C. Following incubation, the formazan crystals produced were dissolved in 80µl of dimethyl sulfoxide (solubilizing agent). At a wavelength of 570 nm, the OD of the samples was measured using an ELISA reader for microplates.

3.5. Statistical analysis

The data was tabulated using Google sheets and statistical analysis was run using IBM SPSS Statistics 27. The correlation between semen evaluation parameters and MTT assay absorbance results was determined via Spearman's correlation test wherein $P < 0.05$ was considered statistically significant. Wilcoxon signed ranks test was used to compare the two epididymal sperm salvage techniques run.

4.0 RESULTS

From the 20 tomcats sampled, the average age was 12.6 months old with a range of 7 to 38 months (Table 1). The motility of spermatozoa from the right testes was slightly lower than that of the left testes (67.5% vs. 64.7%). In the right testes, the wave pattern was marginally superior (2.7 vs. 2.9). In comparison to the left testes, the right testes had a higher percentage of viable sperm (79.5% vs. 82.1%). The percentage of abnormal sperm in both testes was comparable (17.9% vs. 18.15%). The total sperm count in the right testes was somewhat higher than in the left testes (13.87 vs. 14.57 million sperm per ml). The right testes exhibited a much greater MTT absorbance (0.0619 vs. 0.1047), suggesting that the right testes may have a higher metabolic activity. In terms of sperm parameters, these comparisons demonstrate some variations between the right and left testes, with the right testes exhibiting marginally better values in motility, live sperm percentage, total sperm count, and MTT absorbance. On the other hand, the aberrant sperm percentages in the two testes were likewise comparable.

Table 1

Averages of semen evaluation parameters collected from 20 post-orchidectomy tomcats.

PARAMETERS	LEFT TESTES	RIGHT TESTES
Age	12.6 months	
Motility (%)	67.5	64.7
Wave pattern	2.7	2.9
Live sperm (%)	79.5	82.1
Abnormal Sperm (%)	17.9	18.15
Total sperm count (10^6)	13.87	14.57
MTT Absorbance	0.0619 ± 0.0018	0.1047 ± 0.0939

Table 2

Spearman's correlation test between MTT assay parameters to conventional semen evaluation parameters of the left testes of 20 post-orchidectomy tomcats.

Correlations

			Motility_L	Wavepattern_L	Livesperm_L	Abnormal_sperm_L	concentration_L	MTT_L
Spearman's rho	MTT_L	Correlation Coefficient	.061	-.021	-.409	.296	-.201	1.000
		Sig. (2-tailed)	.799	.930	.074	.206	.396	.
		N	20	20	20	20	20	20

From table 3, all conventional semen evaluation parameters except for general motility and abnormal sperm percentage were negatively correlated to MTT absorbance values for the left testes. However, the correlation was significant with $P > 0.05$.

Table 3

Spearman's correlation test between MTT assay parameters to conventional semen evaluation parameters of the right testes of 20 post-orchidectomy tomcats.

Correlations

			Motility_R	Wavepattern_R	Livesperm_R	Abnormal_sperm_R	Concentration_R	MTT_R
Spearman's rho	MTT_R	Correlation Coefficient	-.313	-.305	-.398	.396	-.370	1.000
		Sig. (2-tailed)	.179	.192	.082	.084	.108	.
		N	20	20	20	20	20	20

From table 4, all conventional semen evaluation parameters except for abnormal sperm percentage are negatively correlated to MTT absorbance values for the right testes. However, the correlation was insignificant ($P > 0.05$).

Table 4

Comparing semen evaluation parameters of the left testes that underwent slicing and right testes that underwent mincing epididymal sperm salvage technique of 20 post-orchidectomy tomcats.

Test Statistics[a]

	Motility_R - Motility_L	Wavepattern_R - Wavepattern_L	Livesperm_R - Livesperm_L	Abnormal_sperm_R - Abnormal_sperm_L	Concentration_R - concentration_L	MTT_R - MTT_L
Z	-.595[b]	-.577[b]	-1.281[b]	-.593[c]	-1.499[b]	-1.682[b]
Asymp. Sig. (2-tailed)	.552	.564	.200	.553	.134	.093

All correlations between parameters are insignificant indicating that both methods are statistically effective.

Table 5

Comparing semen evaluation parameters of the right testes that underwent slicing and left testes that underwent mincing epididymal sperm salvage technique of 20 post-orchidectomy tomcats.

Test Statistics[a]

	Motility_R - Motility_L	Wavepattern_R - Wavepattern_L	Livesperm_R - Livesperm_L	Abnormal_sperm_R - Abnormal_sperm_L	Concentration_R - concentration_L	MTT_R - MTT_L
Z	-1.809[b]	-1.000[b]	-.561[c]	-.665[c]	-.358[b]	-1.070[b]
Asymp. Sig. (2-tailed)	.070	.317	.575	.506	.720	.285

All correlations between parameters are insignificant indicating that both methods are statistically effective.

5.0 DISCUSSION

Although the correlation shows a negative tendency, it was not statistically significant ($P > 0.05$), suggesting that the association between MTT absorbance and traditional semen evaluation criteria for the left testes is not significant. Like the left testes, the right testes showed a non-significant association between MTT absorbance and standard semen evaluation parameters, as indicated by the observed negative correlation that was not statistically significant ($P > 0.05$). Since there was no statistically significant link between the two ways for evaluating semen evaluation parameters, it was concluded that both the slicing and mincing methods are statistically effective and produce findings that are equivalent. For both the left and right testes, the MTT assay parameters and traditional semen evaluation parameters were not significantly correlated, according to the results of the Spearman's correlation tests.

The effectiveness of the MTT assay is called into question by the lack of significant connections with traditional semen evaluation parameters. The MTT assay's sensitivity may be affected by a few actors such as environmental factors, genetic variables, age and maturity (Buranaamnuay, 2021).

Temperature, length of storage, and chemical exposure are examples of environmental variables that can impact the outcome of the MTT experiment for semen viability. The motility, viability, DNA integrity, and apoptosis of spermatozoa can all be affected by temperature and storage duration (Kumar et al., 222). The MTT assay also assesses the mitochondrial metabolic rate, which in turn indicates the vitality of the cells.

As a result, variables that impact mitochondrial activity, such temperature and chemical exposure, can change the assay's outcomes. As a result, environmental elements like temperature and chemical exposure can significantly affect the MTT assay's results (Aziz, 2005).

Sperm viability might vary throughout individuals due to genetic variances. A number of variables can impact the metabolic activity measured by the MTT assay, including genetic mutations, polymorphisms, or differences in genes linked to sperm function, metabolism, or antioxidant defenses. Inherited genetic abnormalities or illnesses that alter sperm quality and function can lead to variations in viability (Golshan, 2020). Conditions such as genetic disorders affecting sperm development or structural problems can influence the metabolic activity evaluated by the MTT assay. Individual differences in how sperm cells react to environmental stressors or assay conditions can be caused by genetic variations (Reyes-San-Martin et al., 2023). Individual differences in reaction have the potential to impact the observed viability as determined by the MTT experiment.

When interpreting the results of the MTT experiment for semen viability, age and maturity levels must be taken into account. Due to more ideal sperm qualities, younger people or those in their reproductive prime may have better sperm viability, whereas aging-related changes in sperm quality and function may result in lower viability in older people (Aziz, 2005). These differences should be considered when assessing the prospective fertility and quality of the semen.

An important consideration is the caliber of sperm extracted from the epididymis. The capacity of sperm to convert MTT into formazan crystals is influenced by their metabolic activity, motility, and overall health (Aziz, 2005). The quality of the sperm sample can be greatly impacted by handling and processing epididymal tissue to extract sperm in an appropriate manner. Tissue dissociation, incubation, and other extraction techniques may have an impact on sperm viability, which in turn may have an impact on the assay's outcome (Buranaamnuay, 2021). Sperm viability and metabolic activity may be impacted by factors such as appropriate storage conditions (temperature, medium, etc.) and timing between sperm extraction from the epididymis and the MTT assay. The MTT decrease rates and the assay's dependability can be affected by the kind of medium—PBS, M16, F10, or BTS—that is used to dilute and incubate epididymal sperm (Nasr-Esfahani et. al, 2002).

The sperm count, motility, viability, and morphology of the semen sample itself can all have a big impact on the MTT assay's outcome. The assay's result will be impacted by samples that have greater sperm viability and metabolic activity because they will generate more formazan crystals (Aziz, 2005). The metabolic activity of sperm can be impacted by a variety of pre-assay procedures for handling and preparing semen, including centrifugation, washing, and dilution, which can then have an impact on the assay's outcomes (Matsuura, 2017). The amount of formazan production can vary depending on the MTT concentration employed and the length of time the sperm sample is incubated. Achieving correct results requires optimising these factors. It's crucial to keep the pH and temperature levels during the MTT test constant and suitable. Temperature or pH variations can change sperm metabolism and have an impact on the test result. The concentration of sperm and their metabolic activity can be impacted by the dilution of the semen sample prior to the experiment, which can therefore impact the

MTT assay results (Buranaamnuay, 2021). The MTT assay results can be affected by individual differences in semen samples, even if they come from the same donor or breed. To reduce variability, sample collection and handling must be consistent. Reliability and repeatability of the results can be greatly impacted by the quality and purity of MTT reagents, solvents, and other materials employed in the experiment (Buranaamnuay, 2021) (Matsuura, 2017). The species and the particulars of the experiment can also have an impact on the reliability of the MTT assay. For instance, research has demonstrated a robust association between sperm viability in equine semen and MTT decrease rates. However, sperm motility and MTT assay findings may not always correlate, depending on the species and circumstances of the experiment (Aziz, 2006).

It is crucial to standardise procedures, optimise settings depending on the unique properties of the sperm being assessed, and carefully control experimental variables in order to achieve accurate and dependable results when utilising the MTT assay for semen evaluation. To achieve meaningful results for sperm viability and metabolic activity assessment, it is essential to validate the assay under pertinent conditions and handle samples consistently.

A larger surface area is exposed during the mincing process, which involves coarsely chopping or mincing the tissue, enabling a greater volume of spermatozoa to be extracted. A greater amount of sperm is released into the buffer or collection medium as a result of the increased surface area, which improves access to the spermatozoa contained within the tissue (Joram, 2016). However, the slow release of spermatozoa into the buffer in the slicing approach—where the tissue is chopped into slices rather than coarsely minced—may result in a lower volume of sperm recovered.

The way the tissue is cut may have an impact on the accessibility of spermatozoa inside the tissue, which could explain this delayed release (Dong, 2008). These findings point to variations in the two techniques' sperm isolation effectiveness, which may have an effect on the total yield and applicability of each strategy for epididymal sperm salvaging.

For both the left and right testes, there were no significant connections between the parameters acquired using the two alternative approaches (slicing vs. mincing) for epididymal sperm salvaging strategies. Since, there were no statistically significant differences found when comparing the various methods for salvaging epididymal sperm, suggesting that all approaches are similarly efficient in assessing semen properties. It was discovered that both methods worked just as well for extracting sperm from the epididymis. However, because of its ease of use, quicker processing, and greater observed sperm concentration, mincing stood out as being more practical. Although effective, slicing was found to take longer and require more equipment such as a centrifugation machine, which may make it less feasible for regular use than mincing. The mincing technique was preferred because of its simplified procedure and possible increased sperm output, which makes it a more practical and effective option for epididymal sperm extraction.

6.0 LIMITATIONS AND RECOMMENDATIONS

We acknowledge the importance of repeating the study with a larger sample size considering the power analysis results, which show that robust statistical power requires at least 29 samples. To improve the study's reliability and generalizability and make sure that its findings are more reflective of the general public, it is essential to increase the sample size. The study's findings will be more credible and applicable to wider reproductive medicine contexts owing to the larger sample size's contribution to the statistical analyses' robustness.

To accomplish this, we will add semen extenders as soon as the epididymal sperm is salvaged, with the goal of greatly extending their viability. This tactical method is essential to maintaining the viability of sperm, which in turn allows for a careful and precise evaluation using the MTT assay in addition to conventional methods of evaluating semen. The ability of the semen extenders chosen for this purpose to preserve sperm integrity during the post-salvage period will be carefully considered. This method should yield insightful information about how well semen extenders maintain the viability of epididymal sperm and, in turn, whether they are suitable for use in assisted reproductive technologies in the future.

An essential part of determining whether cryopreserved sperm can be used for future artificial insemination is to analyze the quality of the epididymal sperm after cryopreservation. One popular method in reproductive technology is cryopreservation, which tries to maintain the viability and integrity of sperm over time. This study aims to determine the effectiveness of the preservation process by systematically examining sperm quality parameters, such as motility, morphology, and viability, after cryopreservation. Maintaining viability and functionality would indicate successful preservation, and these cryopreserved sperm samples may be suitable for artificial insemination. This not only covers the technical aspects of cryopreservation, but it also has consequences for the development of technologies related to assisted reproduction. Specifically, it offers insights that could improve the success rates of future operations utilizing epididymal sperm used in artificial insemination.

7.0 CONCLUSION

For both the left and right testes, the investigation did not show a statistically significant correlation between MTT absorbance and conventional parameters used to evaluate semen. Likewise, no noteworthy correlations were discovered between the two techniques (slicing versus mincing) for the rescue of epididymal sperm. These findings imply that the two approaches are equally useful for assessing the characteristics of semen. Furthermore, there was no strong correlation found between the MTT assay parameters and traditional semen evaluation measures, as evidenced by their lack of significant correlation. Numerous variables, including handling techniques, test settings, genetic diversity, age-related changes, and experimental variables, might complicate the MTT assay, which is used to assess semen viability. To improve the assay's accuracy in assessing sperm vitality, these components must be standardised.

In conclusion, more investigations and improvements are necessary to improve the MTT assay's accuracy and reliability in evaluating sperm quality in domestic cats, even though the study's results did not reveal any meaningful connections with conventional semen evaluation parameters. Convenience is another factor that could influence the adoption of specific salvaging techniques, such as mincing, when taking into account their practical characteristics considering that it is less time consuming and does not require a centrifuge machine compared to the slicing salvage technique.

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9.0 APPENDIX

9.1 Normality test of the parameters

Data obtained was not normally distributed as the *P* > 0.05 indicated that it was not significant.

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Motility_L	.163	20	.170	.892	20	.030
Motility_R	.157	20	.200*	.924	20	.121
Wavepattern_L	.309	20	<.001	.842	20	.004
Wavepattern_R	.309	20	<.001	.842	20	.004
Livesperm_L	.210	20	.021	.869	20	.011
Livesperm_R	.146	20	.200*	.930	20	.155
Abnormal_sperm_L	.120	20	.200*	.939	20	.227
Abnormal_sperm_R	.106	20	.200*	.967	20	.687
concentration_L	.170	20	.134	.951	20	.381
Concentration_R	.115	20	.200*	.983	20	.968
MTT_L	.177	20	.103	.845	20	.004
MTT_R	.122	20	.200*	.948	20	.334