UNIVERSITI PUTRA MALAYSIA

EFFECT OF VITRIFICATION ON SPERMATOZOA QUALITY IN BULL SEMEN

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EFFECT OF VITRIFICATION ON SPERMATOZOA QUALITY IN BULL SEMEN

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CERTIFICATION

It is hereby certified that we have read this project paper entitled “Effect of Vitrification on Spermatozoa Quality in Bull Semen”, by Lee Lian Yu and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999 – Project.

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DEDICATION

This project paper is dedicated

To my family,

Father

Mother

Brothers

And to all my teachers who have committed themselves towards the noble cause of education.

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It is with deepest appreciation and gratitude that I thank all those who have made this project paper a reality.

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Lastly, I wish to extend my sincere thanks to anyone that assisted me directly and indirectly in this project and my most heartfelt gratitude to my family; father, mother, aunties, brothers and my lab mate for their endless love and support throughout my studies.

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LIST OF ABBREVIATIONS

%	Percentage
<	Less than
>	More than
℃	Degree Celsius
kg	Kilogram
g	Gram
L	Liter
mL	Milliliter
M	Molar
hr	Hours
min	Minutes
s	Seconds
cm	Centimeter
x	Times
EE	Electro-ejaculation
TEYG	Tris-egg yolk glycerol extender
HS	Holding solution
VS	Vitrification solution
DMSO	Dimethyl sulfoxide
EG	Ethylene glycol
LN_2	Liquid nitrogen
CASA	Computer-assisted sperm analysis

ABSTRAK

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

KESAN VITRIFIKASI TERHADAP KUALITI SPERMA PADA SEMEN LEMBU

Oleh

Lee Lian Yu

2017

Penyelia: Prof. Dr. Abd Wahid Haron

Kriopreservasi sperma merupakan salah satu pembantuan bioteknologi reproduksi untuk meningkatkan kapasiti reproduksi dalam haiwan ternakan. Kriopreservasi sperma secara konvensional menggunakan teknik pembekuan berperingkat secara perlahan menyebabkan pembentukkan penghabluran ais dan kriokerosakan dengan menghasil kualiti sperma yang kurang memuaskan selepas dinyahbeku. Oleh itu, vitrifikasi diperkenalkan dengan memejal cecair kepada keadaan berkaca tanpa penghabluran dengan cepat dan murah. Kaedah penyejukan ultra-pantas ini memerlukan bahan kriopreservasi yang berkepekatan tinggi yang bakal tosik kepada sperma. Oleh itu, kajian ini dikendalikan untuk mengenal pasti kesan vitrifikasi kepada kualiti semen lembu. Sejumlah lapan (8) sampel semen lembu dikumpul menggunakan kaedah elektro-ejakulasi. Media berdasarkan Tris digunakan untuk membandingkan dengan media vitrifikasi yang berlainan konsentrasi krioprotectant pada kadar 10% (media vitrifikasi 1; VS-1) dan 20% (VS-2) mengandungi dimetilsulfoksida (DMSO) serta ethilen glikol. Keputusan memaparkan mortaliti yang tinggi serta parameter motiliti yang hampir sifar pada semua sperma vitrifikasi yang dinyahbeku. Akan tetapi, parameter motiliti keseluruhan dan progresif sperma bagi VS-1 pada penilaian awal adalah 22.45% dan 24.87% lebih baik dan ketara secara statistik berbanding dengan media berdasarkan Tris. Kesimpulannya, vitrifikasi berpotensi sebagai alternatif untuk kriopreservasi. Penyelidikan lanjut mengenai teknik vitrifikasi sperma dalam bidang penyejukan dan pemanasan haruslah dikaji.

Kata kunci: lembu, sperma, kriopresevasi, vitrifikasi, elektro-ejakulasi

ABSTRACT

Abstract of the project paper presented to the Faculty of Veterinary Medicine in partial requirement for the course VPD 4999 – Project.

EFFECT OF VITRIFICATION ON SPERMATOZOA QUALITY IN BULL SEMEN

by

Lee Lian Yu

2017

Supervisor: Prof. Dr. Abd Wahid Haron

Cryopreservation of spermatozoa is part of the assisted reproductive biotechnology to enhance reproductive capacity in livestock. Conventional cryopreservation applies slow-gradual freezing method permitted ice crystallization and causes cryodamage resulting in poor post-thawed semen quality. Hence, vitrification is introduced by solidifying the solution into glassy state without causing any crystallization in fast and inexpensive manner. This ultra-rapid cooling method requires high concentration of cryoprotectants that potentially toxic the spermatozoa. Therefore, this study was conducted to determine the effect of vitrification on the quality of bull semen. A total of eight (8) bull semen samples were collected using electro-ejaculation. Tris-based extender was compared with vitrification using solution of different concentration of cryoprotectants at 10% (Vitrification Solution 1;

VS-1) and 20% (VS-2) containing dimethyl sulfoxide (DMSO) and ethylene glycol respectively. The result revealed that high mortality and nearly zero motility in all post-warmed vitrified spermatozoa, but the general and progressive motilities parameters in VS-1 at initial evaluation was 22.45% and 24.87% respectively better than Tris-based extender and was statistically significant. In conclusion, vitrification has potential as an alternative for cryopreservation. Therefore, further research on spermatozoa vitrification technique on enhancement in cooling and warming should be conducted and investigated.

Key words: bull, spermatozoa, cryopreservation, vitrification, electro-ejaculation

CHAPTER I

GENERAL INTRODUCTION

Cryopreservation is the preservation of structurally intact living cells using very low temperatures (Wolkers and Oldenhof, 2015) which played an important role in livestock reproduction. Assisted reproductive biotechnology (ARB) has been applied in various species of animals to enhance reproductive capacity, to improve and preserve livestock genetics, as well as to develop new animal products. Artificial insemination is an example of ARB whereby various studies and development programmes have been conducted to improve the quality of cryopreserved semen. Bovine semen is the least sensitive of all species to freezing damage (Holt, 2000) but the biological effects of conventional cryopreservation is dominated by freezing of water resulting in high concentration of solutes, leading to osmotic shock as well as intracellular and extracellular damage. Hence, cryoprotective agents (CPA) were introduced to increase the total concentration of all solutes in the media and reduce the amount of ice formed at any given temperature (Pegg, 2015). The CPAs that administered must be biologically acceptable and able to penetrate into the cells with low toxicity, but the concentration that required varies according to species, cell types and procedures (Pegg, 2015).

The slow freezing technique applied in conventional cryopreservation causes intracellular freezing and extracellular ice formation that potentially damaged the spermatozoa. Thus, interventions have been conducted to avoid ice formation by vitrification. This technique allows production of a glassy state that is defined by the viscosity reaching a sufficiently high value of 10^{13} poise to act like a solid without causing any crystallization. However, toxicity due to high concentration of CPA that used in vitrification is the major obstacles (Pegg, 2015).

Conventional cryopreservation is rather expensive and time-consuming, even though lesser amount of CPA is required. Formation of ice crystal during slow freezing increases cyrodamage which produced poor to moderate result of viable spermatozoa after thawing. In contrast, vitrification is an inexpensive, ultra-rapid freezing method that preserve cells to sub-zero temperatures whereby fast cooling rate results in solidification of solution into glass-like structure rather than the ice crystals which reduces cryodamages. High CPA concentration is required and it is toxic to the cells, therefore exposure time to perform this technique must be conducted rapidly. However, complete understanding and development of vitrification in bovine spermatozoa is yet to be discovered.

Thus, the objectives of this study are to:

1. To determine the suitability of freezing bull semen by vitrification.
2. To evaluate the quality of vitrified bull semen.

Therefore, the hypothesis of this project is that the quality of vitrified bull semen is better than conventional cryopreservation.

CHAPTER II

LITERATURE REVIEW

2.1 Assisted Reproductive Biotechnology

The demand for high genetic merit bull semen leads to the development and refinement of storage technologies (Vishwanath and Shannon, 2000). Natural mating allows spread of genital diseases with decreased fertility and reduces effectivity of progeny testing schemes resulting in poor genetic gain. In addition, keeping herd bulls are expensive and possess potential danger to the farm personnel (Vishwanath, 2003). Commercialization of animal biotechnologies in the field of reproduction is known as ARB or assisted reproductive technologies (ART). It is intended to shorten the generational intervals as well as to propagate superior genetic materials among the breeding animal populations. The present biotechnological tools for reproduction in cattle including semen handling for artificial insemination (AI) remains the most important ARB in developing countries (Rodriguez-Martinez, 2011). Application of AI aid in diffusion of improved genotypes and the exchange of genotypes without transmitting diseases (De Pauw, 2003), and able to increase up to 50% genetic progress in bovine (Rodriguez-Martinez, 2011).

Spermatozoa can be cryopreserved via two methods, namely conventional cryopreservation or vitrification. Conventional cryopreservation is a slow-gradual freezing process that described as a slow process of dehydration to reduce intracellular ice crystallization. In contrast, vitrification applies ultra-rapid cooling method to solidify liquid into glassy state without ice crystal formation. Apart from that, these two methods has different cooling rates. According to Whittingham *et al.* (1972) and Willmut *et al.* (1972), the cooling rate for conventional cryopreservation or slow freezing of the oocytes is -0.3ºC/min, while vitrification able to achieve a faster cooling rate of -50ºC/min in 2 s (Rall and Fahy, 1985).

Besides that, both cryopreservation methods require CPA as agent to protect the cells from damage upon freezing and thawing. Cryoprotective agent aims to increase the total solutes concentrations and to reduce ice formation. The principle of administrating CPA ought to be biologically acceptable and able to penetrate into the cells with low toxicity (Pegg, 2015). Cryoprotective agents can be classify into permeable and non-permeable CPAs. Permeable CPAs comprised of small polyols and has hydrogen binding sites for intracellular water which able to stabilize the biomolecules. Meanwhile, non-permeable CPAs are made up of polymers and sugars that aim to perturb extracellular water or ice transition in order to achieve optimal cryogenic dehydration (Fuller, 2015). The balance between the concentration and the exposure time of CPAs to the cells determine the success of cryopreservation.

However, current advancement in preserving semen to produce good semen quality for AI on commercial basis do not allow full understanding on how spermatozoa lose their capacity to remain fertile upon freezing and thawing.

2.2 Conventional cryopreservation

Conventional cryopreservation developed along with AI allowed 100-times less spermatozoa required as compared to natural mating, as only 10 to 15 million frozen-thawed spermatozoa are needed to be deposited into the uterine body (Verberckmoes *et al.*, 2004). Back in 1940s and 1950s, AI was introduced by using fresh semen or semen stored at room temperature. Later, discovery of glycerol as CPA in diluents for freezing bovine semen and application of frozen-thawed semen procedure was wide spread in the 1960s. This discovery allowed fast distribution of highly valuable genes that enabled successful breeding programmes and encouraged development of highly specialization of AI establishments.

Bovine semen is least sensitive to freezing damage compared to other species. However, the freezing and thawing process has an irreversible impact on the spermatozoa including the recovery of motile, morphologically normal cells and the ensuing pregnancy rate (Holt, 2000). Even with the best preservation techniques, the optimal cell recovery is just over 50% (Vishwanath and Shannon, 2000).

According to Isachenko *et al.* (2003) and Saffa (2010), the freezing process of conventional cryopreservation can be divided into before and during freezing, as well as condition in the liquid nitrogen. As depicted in Figure V, fresh semen will be diluted with extender containing CPA initially. Low concentration of CPA in the extender and the slow-gradual freezing process causes occurrence of ice seeding whereby the dehydration process permitted crystallization resulting in both intracellular and / or extracellular cryodamage.

Conventional slow freezing protocol causes the spermatozoa undergo dramatic transformation in chemical and physical characteristics as the temperature drops from +37ºC to -196ºC gradually. Cells that underwent this process can lose up to 95% of their intracellular water resulting in osmotic shock due to increase in concentration of solutes. In addition, potential intracellular ice crystallization and mechanical deformation by the extracellular ice crystals might significantly injure the spermatozoa and leads to death (Moskovtsev *et al.*, 2011). Even though CPAs are added to reduce the freezing injury, but the concentration that used in conventional freezing is generally low.

The current slow freezing technique and storage in LN_2 has drawbacks, which results in loss of motility and vitality as well as increased damage to the membrane of spermatozoa resulting in non-physiological acrosome reaction and induction of apoptosis (Sanchez *et al.*, 2010).

2.3 Vitrification

Vitrification is the process of solidifying liquid into non-crystalline solid that known as glass, which act as an alternative approach for cryopreservation enabling hydrated living cells to be cooled to cryogenic temperatures without formation of ice crystals (Fahy and Wowk, 2015). It is induced by cooling where non-freezing aqueous solutions will elevate viscosity and reverted to glassy or vitreous state at 10^{13} poise (Wowk, 2010). The high viscosity level allows the solution to behave like a solid without crystallization and maintain an ice-free status. Initially, the semen was mixed with the carrier solutions and high concentration of CPA before plunging it directly into the LN_2. This method is fast and allow direct contact between cells with LN_2 to prevent ice formation (Lieberman *et al.*, 2003; Isachenko *et al.*, 2003), as depicted in Figure V. In addition, the ultra-rapid cooling rate allows the condition of the entire solution remain unchanged. So, water does not precipitate and no ice was formed (Safaa, 2010). This physical process of solidification resulted in absence of crystallization but it promotes viscous as it passes from liquid to solid state that similar to glass structure. Hence, this simple protocol minimizes osmotic injury and reduces procedural time which eliminates the cost of expensive programmable cooling equipment (Sanchez, 2010).

The conventional freezing of spermatozoa causes notorious chemical-physical damage to the extracellular and intracellular of spermatozoa membranes whereby alternative to preserve the spermatozoa without seminal plasma and permeable CPAs are required. In addition, conventional slow freezing only preserves 30% to 40% of spermatozoa viability but with vitrification it is closer to 80% (Sanchez, 2010). Apart from that, elimination of seminal plasma separate the spermatozoa from many contaminating agents that are potential sources of infection can be minimized.

Vitrification requires a vitrification solution that consists of CPAs which sufficiently concentrated to allow extracellular and intracellular vitrification of the system under the intended cooling conditions (Rall and Fahy, 1985). These CPAs are critical for cooling and warming rates as vitrification depends strongly on the total solute content of the system and the chemical nature of the solute which used to protect against freezing injury. According to Sanchez (2010), there are vast variables that influencing the effectiveness of spermatozoa vitrification. CPAs are the mediums that used to maintain the cells throughout the process. Since vitrification is a new-rising developing alternatives, the types, concentrations, volumes and the exposure time of these CPAs are yet to be discovered. Generally, CPAs are toxic to the cells and vitrification requires high concentration of these mediums for maintenance (Sanchez, 2010). Combination or sole usage of CPA must be biologically acceptable and possess low toxicity. Apart from that, experience of operator, quality of cells and contamination throughout the process influences the effectivity of vitrification. Ischanko *et al*. (2003, 2004, 2005, 2008) had proven vitrification has potential as an alternative to cryopreserve human spermatozoa. Combination of spermatozoa selection by swim-up technique, types and concentration of CPAs, exposure time, cooling and warming rates as well as elimination of the CPAs are the critical factors determining the success rate and effectiveness of vitrification.

CHAPTER III

MATERIALS AND METHODS

3.1 Animals

Four sexually matured Brangus bulls belonging to Beef Cattle Unit of University Agricultural Park, Universiti Putra Malaysia (UPM) were used for semen collection. Convenient sampling method was applied. The age of the bulls was within the range of 3 to 8 years old with body weight between 300 to 550 kg. All the bulls were kept under semi-intensive system, provided with a commercial feed. The bulls were also allowed to graze daily. Commercial mineral block and water were also given *ad libitum*.

3.2 Semen collection

A total of 8 semen samples were collected using convenient sampling method with automatic electro-ejaculator (Yimer *et al.*, 2011) for 2 consecutive weeks. The bulls were physically restrained with head crush. Anal probe of the electro-ejaculator was inserted after evacuation of feces manually and rectally. The ejaculated semen was collected directly into graduated collection centrifuge tube and kept in a 37°C insulated box temporarily. The average time for each collection cycle was 12 min. The bulls were allowed to rest for range of two days for the same week of collection, to four days for different week of collection.

3.3 Semen evaluation

The semen samples were evaluated macroscopically and microscopically. Macroscopic evaluation includes the volume and color of the semen, while microscopic evaluation includes the concentration, motility, morphology and livability of the spermatozoa. The component for motility included total motility and progressive motility. The semen sample was diluted with Tris-based extender of ratio 1:40 for initial evaluation and CASA machine was used to assess the first four parameters of microscopic evaluation. The livability and morphology of spermatozoa were evaluated using the Eosin-Nigrosine staining technique (Yimer *et al.*, 2014) that showed in Figure VI and VII.

3.4 Extenders preparation

Tris-egg yolk glycerol extender (TEYG) was prepared for conventional cryopreservation. Two extenders was prepared: Extender-A without glycerol and Extender-B with glycerol. To prepare this Tris-based extender, 2.42 g of Tris (hydroxymethyl), 1.48 g of citric acid, and 1.0 g of fructose (Mixture-A) were weighed and dissolved in 80mL of distilled water and mixed well manually. Then, 20 mL of freshly separated chicken egg yolk (20%) from the egg albumin (Yimer *et al.*, 2014) was added into dissolved Mixture-A. Extender-B was prepared with addition of 12.5 mL of glycerol (12.5%) into dissolved Mixture-A, followed by inserting 20 mL of egg yolk (20%) and distilled water to obtain a 100 mL extender.

3.5 Vitrification solution preparation

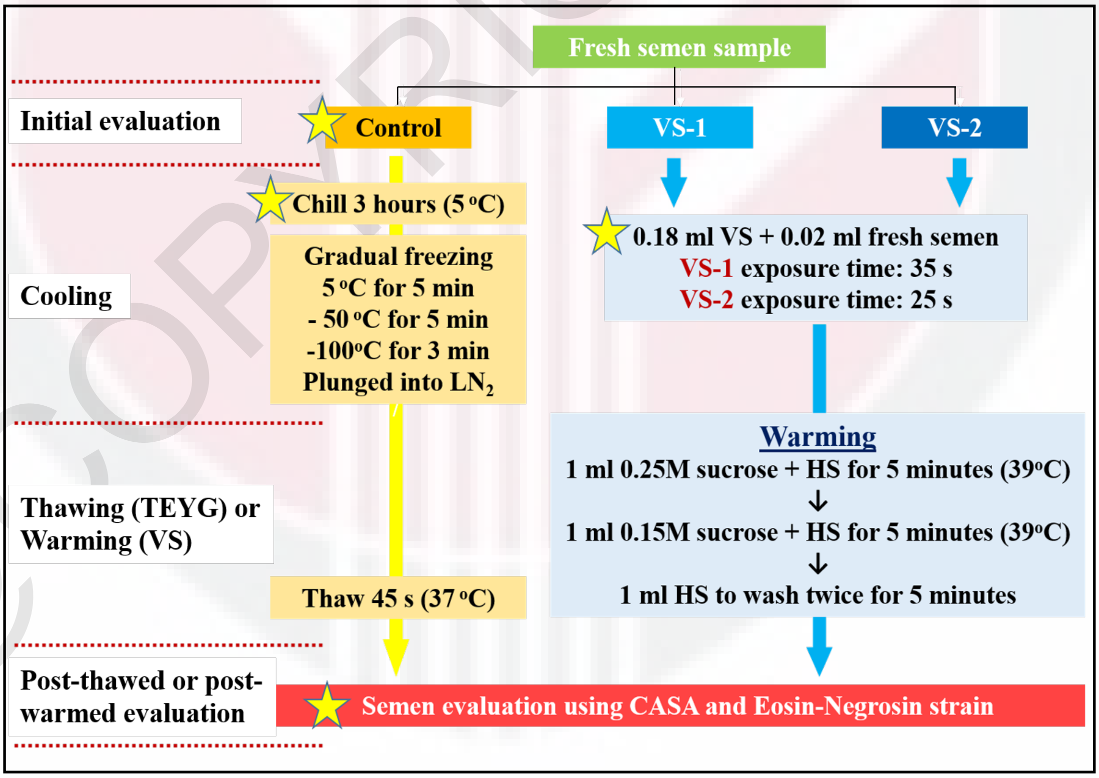
Two vitrification solution with different concentration were prepared. Holding solution (HS) is one of the major component in vitrification that prepared with Hepes-buffered medium 199 and 20% of calf bovine serum. VS-1 contained 10% of Dimethyl sulfoxide (DMSO) and 10% of Ethylene glycol (EG) in HS, while VS-2 contained 20% of DMSO and EG respectively with 0.5 M sucrose in HS. 0.5 M sucrose was calculated manually with formula of 0.5 mole/L x 342 grams/mole x 1 L whereby 171 g of sucrose was required to produce a 0.5 M sucrose in 1 liter of solution or 17.1 g of sucrose in 100 mL of solution or vice versa.

3.6 Experimental design

The semen collection and animal handling for this study had been approved by the Institutional Animal Care and Use Committee (IACUC). Each 0.25 mL straw contained targeted final concentration of 20 x 10^6 spermatozoa (Bearden *et al.*, 2004) for conventional cryopreservation. Initially, Extender-A was used for both initial evaluation and chilling at 5 ºC for 3 hr. Post-chilled suspension will be assessed after adding calculated volume of Extender-B. The extended semen was packed into straws and sealed. Straws were placed horizontally on a cold rack of 5 ºC for 5 min. Next, transferred them into LN_2 vapor of -50 ºC, about 3.5 cm above the surface of LN_2 for 5 min, followed by lowering the frozen straws were to -100 ºC for 3 min. Lastly, the straws were plunged into LN_2 and stored. Post-thawed evaluation will be done after 24 hr of storage by placing the straw in 37ºC water bath for 45 s (Lemma, 2011).

For vitrification, 0.02 mL of fresh semen will be added to pre-warmed 0.18 mL of vitrification solution with short exposure time of 35 s for VS-1 and 25 s for VS-2. Next, it will be packed and sealed in a 0.25 mL straw, and plunged directly into the LN_2. Straws were kept for 24 hr before evaluation. The straw will be thawed at 39 °C for 20 s and were cut. The content of the straw was immersed in 1 mL of pre-warmed 0.25 M sucrose with HS for 5 min at 39°C and transferred into 1 mL of pre-warmed 0.15 M sucrose with HS for 5 min. Lastly, it will be washed twice with HS for 5 min. 0.5 mL of HS will be added into the washed spermatozoa for post-warming evaluation. The methods above referred to Hadi *et al.* (2011) with modifications.

FIGURE I: Experimental design



☆ indicated stages where semen evaluations were performed.

3.7 Thawing and warming

All sealed straws were stored in LN_2 for 24 hr before evaluation. TEYG straws were removed immediately from LN_2 tank and thawed at 37°C water bath for 45 s. The straws were cut and the semen quality was evaluated again using CASA machine and stained with eosin-nigrosin stain. For vitrified straws, the methods for warming were described as above and post-warming semen quality evaluation was similar to conventional cryopreservation.

3.8 Statistical Analysis

Data collected including parameters of livability, normal morphology, general motility and progressive motility between the control group and new technique with two different concentrations were subjected to statistical analysis using IBM SPSS Statistics 20. Significance between the data were evaluated by Kruskal-Wallis H test followed by Mann Whitney U test between the groups. All differences with P value <0.05 were considered to be statistically significant.

CHAPTER IV

RESULTS

All data obtained were expressed into mean ± standard error mean (SEM) with TEYG as the control. The data obtained were not normally distributed ($P<0.05$) whereby non-parametric test of Kruskal-Wallis H (KWH) test and Mann Whitney U (MWU) test were applied. The result revealed both control and vitrification groups did not affect the morphology of spermatozoa with no significant difference among all the groups initially and post-thawed or post-warmed ($P>0.05$).

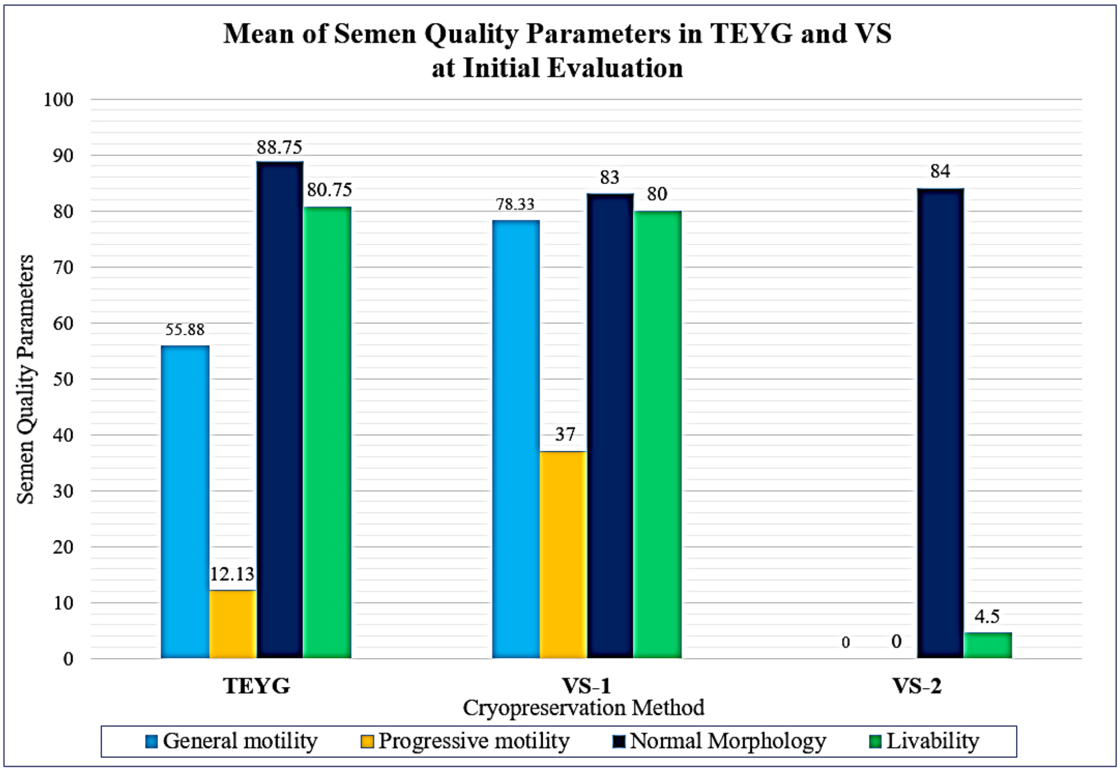
Table I showed the semen quality parameters of initial evaluation with VS-1 had the most superior motility parameters with 22.45% and 24.87% for general and progressive motilities respectively better than TEYG even though it was not statistically significant. VS-2 causes high mortality in spermatozoa and was statistically significant ($P<0.05$) for all parameters except for normal morphology when compared to the control. The progressive motility was statistically significant for both KWH and MWU tests ($P<0.05$) between all the groups. Livability parameter for initial evaluation was likely to be invalid due to the presence of missing data. The result showed no significant difference between groups for KWH test but was significantly different between control group and VS-2 only for MWU test, even though the livability parameter for both control and VS-1 were similar with 80.75% and 80% respectively. Figure II depicted the differences of each parameters during initial evaluation whereby VS-1 displayed the most superior semen quality, while VS-2 achieved the poorest parameters.

TABLE I: Mean ± SEM of semen quality parameters during initial semen evaluation

Parameters (%)	TEYG	VS-1	VS-2
General motility	55.88 ± 9.55[a]	78.33 ± 10.16[a]	0 ± 0.00[b]
Progressive motility	12.13 ± 4.98[a]	37 ± 11.03[b]	0 ± 0.00[c]
Normal morphology	88.75 ± 3.50	83 ± 13.00	84 ± 14.00
Livability	80.75 ± 4.74	80 ± 15.00	4.5 ± 0.50

[abc] with different superscript across the rows indicate significant differences (P<0.05). TEYG: Tris-based extender; VS: Vitrification solution.

FIGURE II: Mean of semen quality parameters during initial semen evaluation



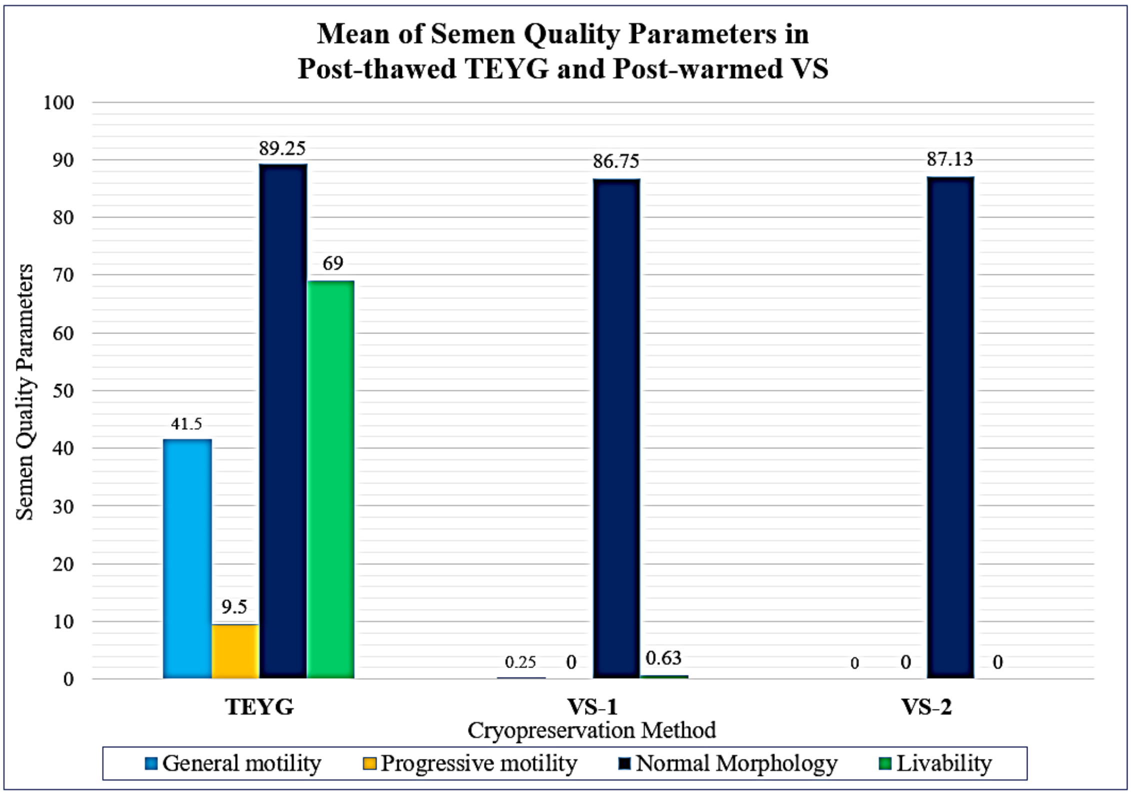
All semen quality parameters dropped after thawing and warming except for normal morphology. Table II and Figure III displayed the semen quality parameters after cryopreservation. The morphology of spermatozoa underwent conventional cryopreservation and vitrification was not statistically different (P>0.05) but vitrification drastically decreased and significantly affect both motility and livability parameters (P<0.05) and achieved nearly zero percent. For raw data, VS-1 had total of 2% general motility and 5% livability out of 8 samples with total of 4 live motile spermatozoa observed using CASA. Besides that, a few motile microorganisms were able to be observed in both VS-1 and VS-2. None of the spermatozoa underwent VS-2 survived with 0 ± 0.00 for both motility and livability parameters. There were no significance differences between VS-1 and VS-2 for all parameters.

TABLE II: Mean ± SEM of post-cryopreserve semen quality parameters

Parameters (%)	TEYG	VS-1	VS-2
General motility	41.5 ± 8.88^{a}	0.25 ± 0.16^{b}	0 ± 0.00^{b}
Progressive motility	9.5 ± 2.54^{a}	0 ± 0.00^{b}	0 ± 0.00^{b}
Normal morphology	89.25 ± 2.96	86.75 ± 3.85	87.13 ± 3.96
Livability	69 ± 7.04^{a}	0.63 ± 0.32^{a}	0 ± 0.00^{b}

[ab] with different superscript across the rows indicate significant differences (P<0.05). TEYG: Tris-based extender; VS: Vitrification solution.

FIGURE III: Mean of post-cryopreserve semen quality parameters



The semen quality parameters dropped as compared to the initial parameters of control group after cryopreservation. Table III and Figure IV depicted the effect of cryopreservation on the semen quality for this study. The result revealed that general motility and mortality were statistically significant (P<0.05) but had no significant difference between VS-1 and VS-2. For progressive motility, KHW test revealed no statistical significance but MWU test showed significant difference between control and VS-1 (P<0.05) with 2.63% and 12.13% respectively. Vitrified spermatozoa significantly dropped in general motility and has high mortality rate up to 55% and 80% respectively after warming. There were no significant difference between the spermatozoa underwent vitrification on all the parameters.

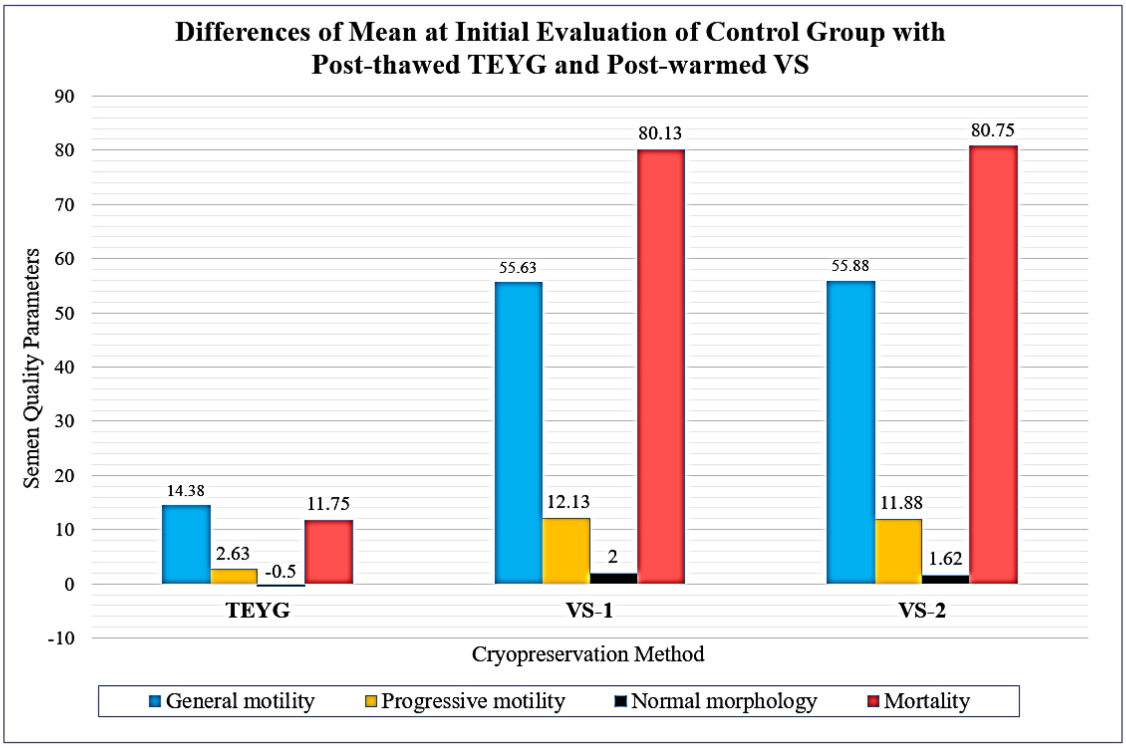
TABLE III: Mean ± SEM of differences between initial evaluation of control group and post-cryopreserve evaluation semen quality parameters

Parameters (%)	TEYG	VS-1	VS-2
General motility	14.38 ± 11.31[a]	55.63 ± 9.50[b]	55.88 ± 9.55[b]
Progressive motility	2.63 ± 6.51	12.13 ± 4.98	11.88 ± 4.96
Normal morphology	-0.50 ± 0.98	2 ± 1.98	1.62 ± 2.20
Mortality	11.75 ± 5.11[a]	80.13 ± 4.91[b]	80.75 ± 4.74[b]

[ab] with different superscript across the rows indicate significant differences ($P<0.05$).

TEYG: Tris-based extender; VS: Vitrification solution.

FIGURE IV: Differences of mean between initial evaluation of control group and post-cryopreserve evaluation semen quality parameters



CHAPTER V

DISCUSSION

During cryopreservation, spermatozoa are subjected to deleterious effect as they possess notorious chemical and physical stresses which inevitably reduce the quality of spermatozoa post-cryopreserve (Watson 2000; Andrabi 2007; Lemma 2011) which similar to this study. The main differences between gradual-slow freezing and ultra-rapid cooling are the concentration of CPAs, as well as the cooling and warming rates (Cuevas-Uribe *et al.*, 2015). The aim of cryopreservation is to prevent osmotic damage and intracellular ice formation that has negative effects on survival. In conventional method, these harmful effects are avoided by cellular dehydration, while in vitrification, ice formation is avoided by converting the solution into viscous glass state that correspond to a viscosity of 10^{13} poise (Mazur, Leibo and Seidel 2008; Fahy and Rall, 2007).

The post-thawed general motility and livability parameters of TEYG deteriorated 14.38% ± 11.31 and 11.75% ± 5.11 respectively as compared to the initial evaluation. Conventional cryopreservation applies slow equilibrium freezing method where straws were chilled and froze gradually from +37ºC to +5ºC, followed by -50ºC to -100ºC, and finally -196ºC in LN_2 had allowed freezing of water and resulted in high concentration of solutes leading to osmotic shock. At the same time, the spermatozoa potentially damaged by the ice crystals formed from freezing of water causes both internally and externally cryodamage. Hence, glycerol was added in the

Tris-based extender as CPA to increase the total concentration of solutes in the media and reduce crystallization at any given temperature (Pegg, 2015).

In contrast, vitrification requires higher concentration of CPAs and the exposure time is critical to ensure vitrification and maintenance of an ice-free state upon warming. The major problem of vitrification is the need of high concentration of CPAs but the spermatozoa are very sensitive to these agents (Isachenko *et al.*, 2003). Various researches had conducted over the year, but whether vitrification is a better alternative to cryopreserve spermatozoa is still uncertain (Agha-Rahimi *et al*., 2014).

In this study, VS-1 achieves the best motility parameters during initial evaluation. Hence, the concentration of CPAs in VS-1 was biologically acceptable and tolerable by the spermatozoa. For VS-2, the concentration of CPAs is doubled which cannot be tolerated by the spermatozoa and lead to high mortality even at initial stage although 0.5 M of sucrose was added as non-permeable CPA. Besides that, the experimental design for this project referred to vitrification of bovine oocytes which might be less compatible to spermatozoa as they are more sensitive with these agents. In addition, oocytes are bigger in size whereby its capacity to cope with stresses could be better. Succeeded vitrification of human spermatozoa by Isachenko *et al.* (2003, 2004, 2005, and 2008) applied spermatozoa selection by swim-up technique and used medium HTF-HSA 1% (Human Tubal Fluid – Human Serum Albumin) and 0.5 M sucrose at 37ºC, and maintain the suspension in the atmosphere of 5% carbon dioxide for 5 min before vitrification. Swim-up technique was not performed in this experiment where both good and poor quality spermatozoa were included which could affect the post-warmed semen quality. Next, supplementation of protein such as albumin and sucrose can be added as non-permeable CPA. Consideration of including antioxidants maybe be beneficial to reduce production of excessive free oxygen radicals (Ischanko *et al.*, 2005) that generated during mitochondrial electron transport which decreases the survival and fertility of spermatozoa by the reactive oxygen species formation and membrane lipid peroxidation.

The cooling and warming rates also affect the viability of spermatozoa. Since vitrification for bovine semen is new and yet to be discovered, there was no specific protocols or publications on this methodology. So, the cooling and warming rates for this experiment was referred and modified from previous work conducted by Hadi *et al.* (2011). The result for both post-warmed vitrified spermatozoa had zero motility parameters except for VS-1 with total 2% motility. For the cooling process of vitrification, a full-loaded sealed 0.25 mL straw causes breakage and even unsealed the straw. This phenomena could be due to sudden change in temperature from +37 ºC to -196ºC and the straw unable to exert the pressure. Hence, the straws were only filled with 0.2 mL and sealed to reduce the pressure. Apart from that, a 0.25 mL straw has thick-wall and contained large volume (Safaa, 2010) whereby directly plunging the straw into LN_2 might not cooled and vitrified the straws simultaneously. Successful example of human spermatozoa vitrification use cryoloop (Schuster *et al.*, 2003), vitrified solid spheres using pipettes and a double-layered straw contained 0.25 mL straw within a 0.5 mL sealed straw (Isachenko *et al.*, 2004, 2008, 2005) allowed generalize cooling. In addition, mishandling at any stages involving cooling can affect the post-warmed semen quality.

Warming is another critical factor determining the success of vitrification. Vitrification maintained at -130 ºC and below whereby any temperatures above than that may induce fracture of the glass and cleave the cells causing irreversible injury and additional ice nucleation (Fahy and Wowk, 2015). Once the straw was removed from LN_2 storage tank, the surrounding temperature begins the thawing process and allow crystallization. Hence, rapid warming is essential to avoid injury from devitrification and subsequent recrystallization which determines the success of vitrification. For this experiment, 39 ºC was used and it could be one of the factors contributing to the viability of spermatozoa. Warming from -196 ºC to 39 ºC in a thick-walled 0.25 mL straw might not fast enough to prevent crystallization from occurring. Therefore, increasing the warming rate can be considered. Apart from that, warming a straw would be harder as compared to warming a vitrified solid sphere or cryoloop. Vitrified spheres or cryoloop can be obtained directly from LN_2 which allowed direct rapid warming as compared to straws that stored in goblets in a LN_2 storage tank.

Even though the livability of vitrified spermatozoa achieved nearly zero in this experiment, but there are some motile microorganisms such as protozoa observed in this study that had survived the process of vitrification. In addition of superior semen quality of VS-1 during the initial stage indicated that there are potential and possibilities of vitrification for cryopreservation. Hence, improvising and enhancement of vitrification techniques potentially allow ultra-rapid cooling as an alternative to cryopreserve spermatozoa of bovine species in an ice-free manner.

CHAPTER VI

CONCLUSION

Based upon the findings of this study, the following conclusions could be made:

- Effect of conventional cryopreservation is better than vitrification of bull semen.
- Vitrification using VS-1 achieved highest semen quality parameters at initial evaluation with 10% of DMSO and ethylene glycol in HS.
- Vitrification has potential to cryopreserve bull spermatozoa.
- Further research on cooling and warming of vitrification technique in bull spermatozoa should be conducted and investigated.

CHAPTER VII

RECOMMENDATIONS

Concentration, types and exposure time of CPAs use in vitrification is critical but yet to be discovered. Supplementation of antioxidants can reduce the production of excessive free oxygen radicals too. All these supplementations are essential to ensure vitrification and maintenance of an ice-free status upon warming. Hence, further research on concentration and types of permeable and non-permeable CPAs should be conducted. Besides that, cooling and warming rates determine the success of vitrification and affects the post-warmed semen quality. Consideration of depositing the aliquots directly and rapidly into LN_2 forming solid spheres allowed complete simultaneous cooling. Besides that, these vitrified spheres allows uniform warming and able to determine the suitable warming temperature better. Hence, this can prevent devitrification and recrystallization that deteriorate the semen quality. In addition, warming temperature can be increase to allow rapid warming. However, the temperature should not be too high as it causes high mortality to spermatozoa.

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APPENDICES

FIGURE V: Differences of Cooling Process and Condition of Extracellular Cryopreservation between Conventional Cryopreservation and Vitrification

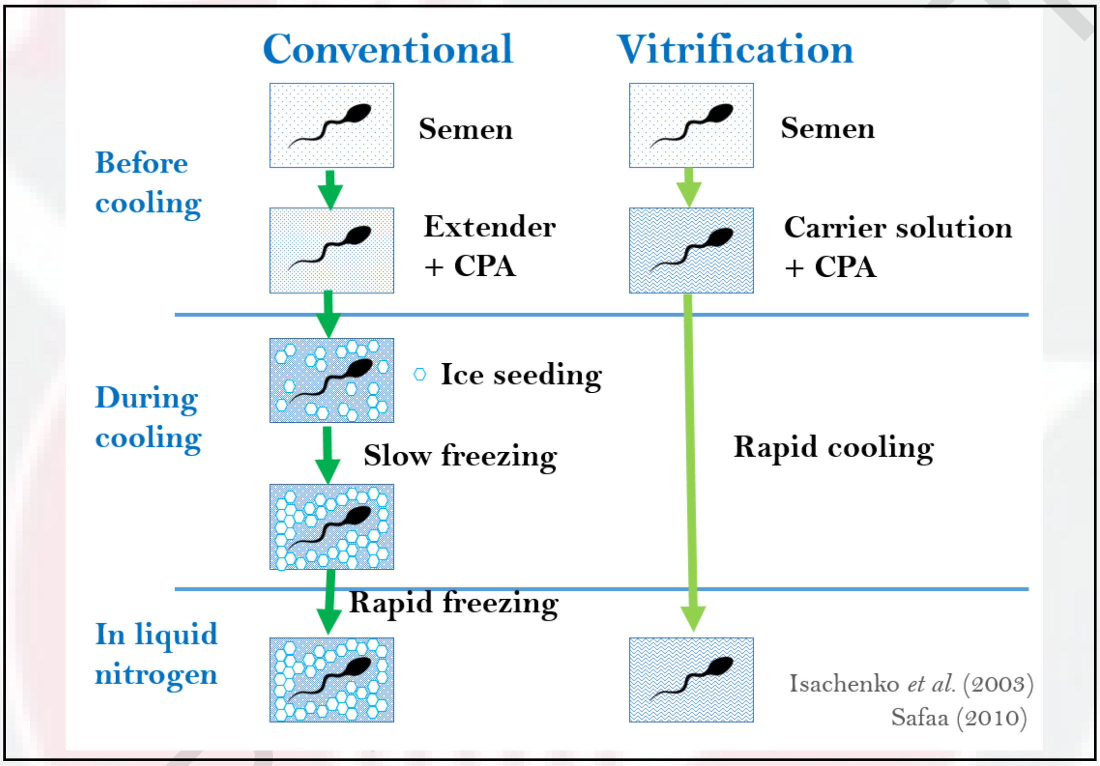
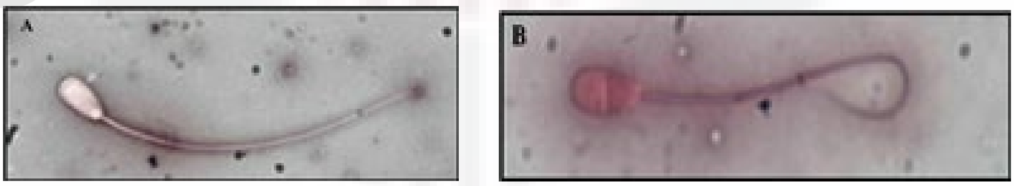


FIGURE VI: Differentiation of Live and Dead Spermatozoa Stained with Eosin-Nigrosin under Light Microscopy

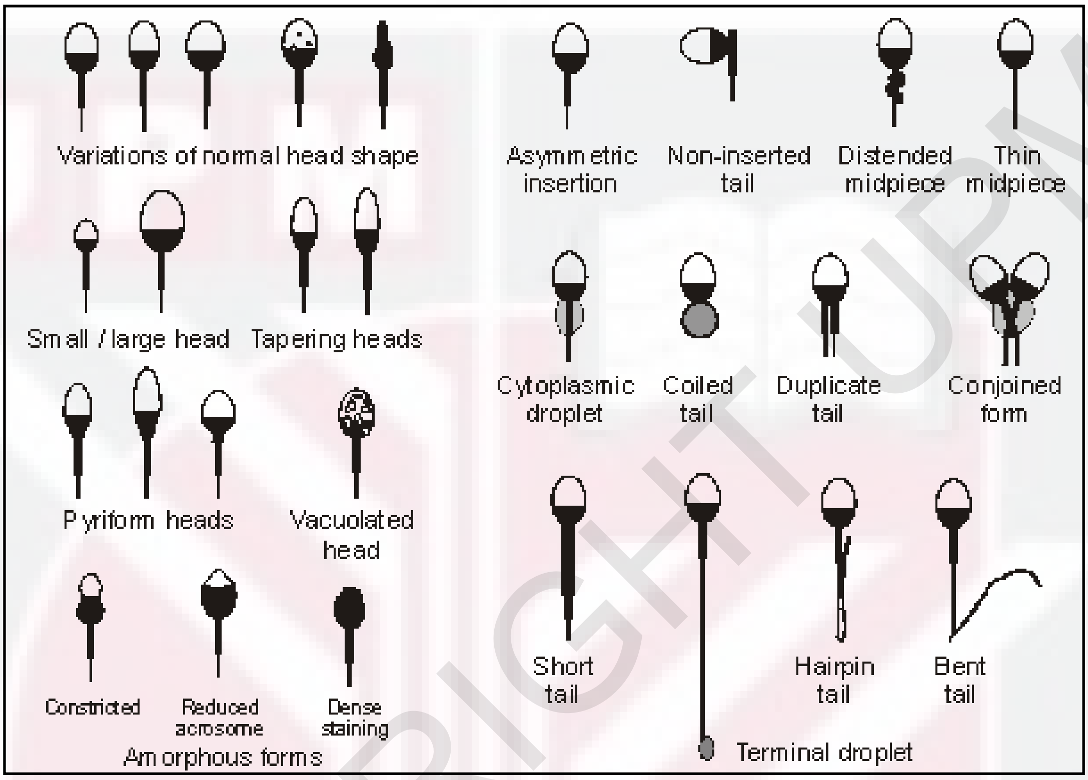


A: Live spermatozoa stained with Eosin-Negrosin stain

B: Dead spermatozoa stained with Eosin-Negrosin stain

Adapted from Faezah *et al.* (2012)

FIGURE VII: Morphology of Normal and Abnormal Spermatozoa



Adapted from Mortimer (2005)