QUALITY EVALUATION OF STALLION FROZEN SEMEN SUPPLEMENTED WITH CYSTEINE AND ASCORBIC ACID

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By

ALAMAARY, MOHAMMED SAAD M

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

July 2019

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DEDICATION

I would like to dedicate this thesis to my parents and my wife who gave me all the immense support and encouragement to continue my education.

To my brothers and sisters whom I grateful to their help and encouragement

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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July 2019

Chairman : Professor Abd Wahid Haron, PhD
Faculty : Veterinary Medicine

Semen cryopreservation offers numerous advantages for the livestock animal industry and new reproduction technologies. This technique increases the overall pregnancy rate and improves genetics. In horses, the conception rate using this technique remains lower, compared to using fresh semen. Stallion spermatozoa revealed high sensitivity to freezing and thawing procedures. During cryopreservation procedures, stallion spermatozoa are subjected to damage, primarily due to osmotic and oxidative stress that influence the sperm structure and functionality. Therefore, this study aimed to improve the cryopreserved semen quality and fertility prediction of cryopreserved semen in Arabian stallions. The primary objective of the current study was to determine the impact of adding antioxidants (cysteine and ascorbic acid) to the freezing extender, and investigating their capacity to counteract the reactive oxygen species ROS during freezing and thawing procedures.

Seven Arabian stallions were used for the semen collection, using either the Missouri model of artificial vagina, or the automated semen phantom collection (Equidame® phantom Haico-Finland). The gel was removed using a gauze filter from all samples, which were initially evaluated for volume, sperm concentration and motility. Only semen samples with at least 200×10^6 sperm/ml and motility $\geq$ 60 % were used in these experiments. The selected ejaculation was diluted (1:1) by a centrifuge media and divided into the number of samples required, then centrifuged at 800g for 10 minutes to remove the seminal plasma. The supernatant was discarded, and the pellet was re-suspended with the semen freezing extender. The extended samples were cooled to 4 °C for 90 minutes, before being packaged in 0.5 mL straws. The samples were then frozen using either the styrofoam box with liquid nitrogen vapor technology, or a programmable freezer (Automatic Freezer with Windows®-tablet, 230 V, Minitube, Germany) (60°C/min. to–140 °C). After one week, the straws were thawed in a water bath at 37ºC for 30 seconds, and evaluated for general motility, progressive motility,

VSL, VCL, VAP, LIN, STR, sperm concentration, normal and abnormal sperm morphology, sperm membrane integrity, viability, acrosome integrity and oxidative stress. The post-thawed semen was analyzed for glutamic oxaloacetic transaminase (GOT), glutamic-pyruvate (GPT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and γ-glutamyl transpeptidase (GGT) enzymes, to determine their efficiency in the fertility prediction for post-thawed semen.

A total of fifty mares were used for artificial insemination. The estrus mares with a follicle of ≥35 mm in diameter were injected with 3000 IU of human chorionic gonadotrophin (hCG), and inseminated using one dose after ovulation. Each dose contains 800×10^6 of total sperm. Flexible 75 cm pipettes (Minitube) were used to deposit the post-thawed semen dose in the uterine horn.

The effect of using four extenders in the quality of frozen semen in Arabian stallions was examined to determine the performance of frozen extenders prepared in the laboratory, compared to the commercial extenders. HF-20 and Tris-based extenders were prepared locally and cryopreserved in the same environment with the commercial extenders (INRA Freeze® IMV Technologies France, and EquiPlus Freeze® Minitube Germany). Cryopreserved samples from all extenders were evaluated in vitro, and were used for artificial insemination (AI). In the current study, the application of HF-20 extender revealed acceptable frozen semen quality, while Tris-based extender revealed poor post-thawed semen, compared to the commercial extenders.

The effect of adding cysteine and ascorbic acid at concentrations of 0, 0.25, 0.5, 1, 2 and 4 mg/ml on the quality of frozen semen in Arabian stallions were also evaluated through assessing the oxidative stress, motility pattern, sperm membrane integrity, viability and acrosome integrity. Subsequently, the best concentration of cysteine and ascorbic acid were used for AI to evaluate the effect of these antioxidants on the spermatozoa fertility. The supplementation of cysteine and ascorbic acid were shown to raise the oxidative stress (OS) on post-thawed semen samples, compared to the control. The increase of OS affected negatively the sperm motility, sperm membrane integrity and viability, especially with a high concentration of cysteine and ascorbic acid added. However, the addition of cysteine and ascorbic acid showed better sperm morphology and acrosome integrity. The ascorbic acid in this study exhibited poor post-thawed semen fertility, whereas cysteine exhibited a pregnancy rate that was in the same range with the control group.

The effect of cysteine and ascorbic acid on GOT, GPT, ALP, LDH, and GGT enzymes concentration on the cryopreserved semen samples were assessed to determine its efficiency as a marker of the post-thawed semen quality. The level of these enzymes was compared to the sperm motility pattern, viability, morphology and sperm membrane integrity. Using ALP, LDH and GGT, enzymes can act as a reliable marker of post-thawed semen quality. The GOT and GPT enzymes could not be used as reliable parameters for frozen semen in horses. Furthermore, the supplementation of cysteine and ascorbic acid to the semen freezing extender exposed a deleterious effect

on the ALP, LDH and GGT enzyme level and function on post-thawed stallion's semen.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KAJIAN KUALITI SEMEN BEKU KUDA JANTAN DITAMBAH DENGAN SISTEINA DAN ASID ASKORBIK

Oleh

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Pengkrioawetan semen menawarkan pelbagai manfaat bagi industri haiwan ternakan dan teknologi pembiakan baharu. Teknik ini meningkatkan keseluruhan kadar kebuntingan dan menambah baik genetik. Bagi kuda, kadar konsepsi menggunakan teknik ini kekal rendah, berbanding dengan penggunaan semen segar. Spermatozoa kuda jantan memperlihatkan sensitiviti yang tinggi terhadap prosedur penyejukbekuan dan pencairan. Semasa prosedur pengkrioawetan, spermatozoa kuda jantan tertakluk pada kerosakan, terutamanya disebabkan oleh tekanan osmotik dan oksidatif yang mempengaruhi struktur dan fungsi sperma. Oleh sebab itu, kajian ini bertujuan untuk meningkatkan kualiti semen pengkrioawetan dan ramalan kesuburan bagi semen yang dikrioawetkan pada kuda jantan Arab. Objektif utama kajian ini adalah menentukan impak penambahan antioksidan (sisteina dan asid askorbik) pada pengekal beku , dan mengkaji kapasiti mereka untuk bertindak balas dengan spesis oksigen reaktif ROS semasa prosedur penyejukbekuan dan pencairan.

Tujuh ekor kuda jantan Arab telah digunakan untuk pengumpulan sperma, menggunakan sama ada model vagina tiruan Missouri, atau pengumpulan fantom sperma automatik (Equidame® phantom Haico-Finland). Gel telah dibuang menggunakan sebuah turas kasa daripada semua sampel yang pada awalnya telah dinilai untuk isi padu, kepekatan dan motility sperma. Hanya sampel sperma dengan sekurang-kurangnya 200×10^6 sperma/ml dan motiliti $\geq$60% telah digunakan dalam eksperimen tersebut. Ejakulasi terpilih telah dilarutkan (1:1) dengan medium pengempar dan dibahagikan kepada bilangan sampel yang diperlukan, kemudian diemparkan pada 800g selama 10 minit bagi menyingkirkan plasma seminal. Supernatan telah dihapuskan, dan pelet telah diampai semula dengan pengekal penyejukbekuan sperma. Sampel yang diperluas telah disejukkan pada suhu 4°C selama 90 minit, sebelum dibungkus dalam straw 0.5 mL. Sampel tersebut kemudiannya telah dibekukan menggunakan sama ada teknik kotak polistrena dengan

wap nitrogen cecair, atau penyejuk beku automatik dengan Windows®-tablet, 230 V, (Minitiub, Germany) (60°C/min. hingga –140 °C). Seminggu kemudian, straw tersebut telah dicairkan dalam penangas air pada suhu 37ºC untuk 30 saat, dan dinilai bagi motiliti am, motiliti progresif, VSL, VCL, VAP, LIN, STR, kepekatan sperma, morfologi sperma normal dan tidak normal, integriti membran sperma, viabiliti, integriti akrosom dan stres oksidatif. Tambahan pula, sperma pascacair telah dianalisis untuk glutamik oksaloasetik transaminase (GOT), glutamik-piruvat (GPT), fosfatase alkalin (ALP), dehidrogenase laktat (LDH), dan enzim y-glutamiltranspeptidase(GGT), bagi menentukan efisiensi mereka dalam ramalan fertiliti bagi sperma pascacair.

Sejumlah lima puluh ekor kuda betina telah digunakan untuk permanian beradas. Kuda betina estrus dengan folikel ≥35 mm diameter telah disuntik dengan 3000 IU gonadotrofin korion manusia (hCG), dan diinseminasi menggunakan satu dos ovulasi. Setiap dos mengandungi 800×10^6 sperma. Pipet 75 sm yang fleksibel (Minitiub) telah digunakan untuk mendepositkan dos sperma pascacair dalam tanduk uterus.

Kesan menggunakan empat pengekal dalam kualiti sperma beku pada kuda jantan Arab telah diteliti bagi menentukan prestasi pengekal beku yang disediakan dalam makmal, berbanding dengan pengekal komersial. Pengekal HF-20 dan berdasarkan Tris telah disediakan secara lokal dan diawetkrio dalam persekitaran yang sama dengan pengekal komersial (INRA Freeze®IMV Technologies France,dan EquiPlus Freeze® Minitiub Germany). Sampel diawetkrio daripada semua pengekal telah dinilai secara in vitro, dan telah digunakan bagi permanian beradas. Dalam kajian ini, aplikasi pengekal HF-20 memperlihatkan kualiti sperma beku yang boleh terima, manakala pengekal berdasarkan Tris memperlihatkan sperma pascacair yang lemah, berbanding dengan pengekal komersial.

Kesan penambahan sisteina dan asid askorbik pada kepekatan 0, 0.25, 0.5, 1, 2 dan 4 mg/ml ke atas kualiti sperma beku pada kuda jantan Arab juga telah dinilai melalui penaksiran stres oksidatif, pola motiliti, integriti membran sperma, viabiliti dan integriti akrosom. Kemudian, kepekatan terbaik sisteina dan asid askorbik telah digunakan untuk permanian beradas bagi menilai kesan antioksidan tersebut ke atas fertiliti spermatozoa. Penambahan sisteina dan asid askorbik telah menunjukkan peningkatan stres oksidatif (OS) ke atas sampel sperma pascacair, berbanding dengan sampel kawalan. Peningkatan OS secara negatif mempengaruhi motiliti sperma, integriti membran sperma dan viabiliti, terutama dengan kepekatan sisteina dan asid askorbik tambahan yang tinggi. Walau bagaimanapun, penambahan sisteina dan asid askorbik menunjukkan morfologi sperma dan integriti akrosom yang lebih baik. Asid askorbik dalam kajian ini memperlihatkan fertiliti sperma pascacair yang lemah, manakala sisteina memperlihatkan kadar kebuntingan boleh terima dalam julat yang sama dengan kumpulan kawalan.

Kesan sisteina dan asid askorbik ke atas glutamik oksaloasetik transaminase (GOT), glutamik-piruvat (GPT), fosfatase alkalin (ALP), dehidrogenase laktat (LDH), dan kepekatan enzim y-glutamiltranspeptidase (GGT) ke atas sampel sperma diawetkrio

telah dinilai bagi menentukan keberkesanannya sebagai penanda kualiti sperma pascacair. Tahap enzim tersebut telah dibandingkan dengan pola motiliti sperma, viabiliti, integriti membran sperma dan morfologi. Menggunakan ALP, LDH dan GGT, enzim dapat bertindak sebagai penanda kualiti sperma pascacair yang boleh dipercayai. Enzim GOT dan GPT tidak dapat digunakan sebagai parameter boleh dipercayai bagi sperma beku pada kuda. Tambahan pula, suplementasi sisteina dan asid askorbik terhadap pengekal penyejuk beku sperma mendedahkan kesan yang memudaratkan ke atas tahap enzim ALP, LDH dan GGT dan fungsi ke atas sperma kuda jantan pascacair.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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AI	Artificial Insemination
IVF	In vitro fertilization
ICSI	Intracytoplasmic sperm injection
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
OS	Oxidative stress
WAHO	World Arabian Horse Organization
KAAH	King Abdulaziz Arabian Horses Center
HRU	Horse Research Unit
LDH	Lactate dehydrogenase
ALP	Alkaline phosphatase
GGT	Gamma glutamyl transferase
GOT	Glutamic oxaloacetic transaminase
ACP	Acid phosphatase
AST	Aspartate aminotransferase
CK	Creatine kinase
AV	Artificial vagina
CASA	Computerized assisted sperm analysis
MOT	General motility
PMOT	Progressive motility
VSL	Rectilinear speed
VCL	Curvilinear speed
STR	Straightness
LIN	Linearity index

LHD	Lateral head displacement
VAP	Average value
SMS	Slow motile sperm
MMS	Medium motile sperm
RMS	Rapid motile sperm
HOST	Hypo-osmotic swelling test
PI	Propidium iodide
AO	Acridine orange
RG	Reactive gel
LOS	Low oxidative stress
HOS	High oxidative stress
O_2	Oxygen electron
O_{2-}	Superoxide radical
H_2O_2	hydrogen peroxide
SOD	Superoxide dismutase
$OH^{\bullet}$	hydroxyl radical
CL	corpus luteum
FSH	follicle-stimulating hormone
GnRh	Gonadotropin releasing hormone
hCG	human chronic gonadotropin
EDHI	endoscopic deep horn insemination
RGDHI	rectally guided deep horn insemination
EED	Early embryonic

CHAPTER 1

INTRODUCTION

1.1 Background

The horse industry is still less successful than industries of other species due to the complicated estrus in the mares and challenges with stallion selection. The stallion used for breeding is not only selected because of his fertility but due to his athletic performance and pedigree (Šichtař et al., 2019). Recently, with improvements in semen preservation, the artificial insemination (AI) technique has become more commonly used in most farms and breeding centers. Using artificial insemination as an assisted reproductive technique in horses could decrease transmission of venereal diseases among horses and preserve the semen of superior stallions (Gomes et al., 2019). After the spermatogenesis stage, the sperm does not have the ability to conduct anabolism and repairs, hence induce excessive sperm metabolism which then minimizes the lifespan of spermatozoa. The metabolism in the sperm could be decreased by cooling or restriction by cryopreservation (Gibb and Aitken, 2016).

The long-term preservation of cells and tissues with minimal change in its structure and function is known as the cryopreservation technique. The cryopreservation method utilizes a low-temperature rate usually with liquid nitrogen around −196 °C (Hezavehei et al., 2018). This technique has been proven to be effective in protecting the cell organelles and ceasing the biochemical reactions and metabolism of a cell. Sperm cryopreservation plays a fundamental role in the domestic animal industry and breeding management. Employing frozen semen with artificial insemination techniques using selected semen and inseminating many females has expedited genetic improvement and maintained superior genes in various animal species (Masoudi et al., 2016). Moreover, Frozen semen is a fundamental technique in recent assisted reproductive technology such as intracytoplasmic sperm injection (ICSI) and in vitro fertilization (IVF) (Kopeika et al., 2015).

Over the past few years, there have been major improvements in semen cryopreservation and the methods used to achieve better post-thawed semen quality. In the beginning, glycerol with egg yolk extender was used, which is an acceptable protectant for spermatozoa against cold shock. Moreover, better sperm motility and viability were observed in both chilled and frozen semen after sugar supplementation with glucose. Later on, the addition of antibiotics such as streptomycin and penicillin to frozen semen extenders conferred the spermatozoa a high level of protection from contamination and venereal disease, thus enhancing overall frozen semen quality (Allai et al., 2018a). Significant improvement to the post-thawed semen was observed in various animals' semen, when antioxidants were supplemented to the freezing extender. The enzymatic antioxidants of catalase, glutathione peroxidase and superoxide dismutase were reported to improve sperm quality in rams and boars (Allai et al., 2018a; Silva et al., 2011; Zhang et al., 2012). Furthermore, beneficial effects of

non-enzymatic antioxidants such as glutamine, ergothioneine, taurine, Vitamin E, and trolox were reported in several studies on ram, boar, donkey and bull semen (Amini et al, 2013; Banday et al., 2017; Bottrel et al., 2018; Çoyan et al., 2011; Joaquín Gadea et al., 2005; Roostaei-Ali et al., 2013; Shah et al., 2017; Silva et al., 2013).

1.2 Problem Statement

Many changes occur in the sperm membrane behavior during cryopreservation procedures. Proteins and lipids in the sperm membrane are rearranged causing cold shock due to the transition temperatures during either cooling or thawing of the sperm. The liquid in the sperm membrane changes into a gel state due to the decrease in temperature. The metabolism decreases when the temperature reduces until it ceases at the freezing point. Furthermore, the unfrozen extracellular water could spread in the unfrozen channel causing cell shrinkage in the hyperosmotic condition (Loomis and Graham, 2008).

During semen cryopreservation, only 50% of the spermatozoa are able to resist the freezing and thawing procedures in order to maintain sperm fertility. The cryoprotectants in the freezing extenders vary in their ability to protect the spermatozoa from ice crystal formation that causes sperm damage. Cryopreservation technique revealed a high susceptibility to reactive oxygen species and lipid peroxidation in post-thawed semen samples. Moreover, the freezing technique showed a deleterious effect on the antioxidant levels in the frozen semen.

Various techniques have been conducted to improve sperm survival during semen cryopreservation including freezing-thawing protocols, packaging and extenders. The post-thawed semen quality is highly affected by the extender composition and the interaction between these components. The quality of the semen extender depends on its ability to protect the sperm from osmotic stress and cold shock as well as provide energy. Furthermore, a superior extender must be able to maintain the sperm's progressive motility, protect the sperm's plasma membrane and acrosome integrity, provide energy and preserve the enzymes needed to penetrate the zona pellucida (Layek et al, 2016b).

The physiologically reactive oxygen species (ROS) is fundamental in initiating reactions in the spermatozoa including the acrosome reaction, hyperactivity, capacitation and motility. Disorders in the level of antioxidants and its mechanisms or an excessive production of ROS are the main sources of oxidative stress (OS). Oxidative stress has adverse effects on male fertility due to the production of free radicals, which can cause disruption to most sperm functions. There are high levels of OS associated with DNA and mitochondria fragmentation, peroxidation of sperm plasma membranes, and decreases in sperm motility (Kumar and Singh, 2018). The spermatozoa are unable to repair the damage caused by OS unlike other cell types due to the absence of cytoplasmic-enzyme repair systems. Furthermore, spermatozoa membranes contain high concentrations of polyunsaturated fatty acids, which make

the spermatozoa more susceptible to oxidative stress than other cell types. Recently, the oxidative stress in the seminal plasma was revealed to be responsible for 30% to 40% of male infertility (Agarwal et al, 2014).

For many years, the subfertile male has been exposed to higher levels of ROS and lower levels of antioxidants compared with the fertile male. The disruption that caused by oxidative stress encountered by the reactive oxygen species scavengers which exist naturally in the spermatozoa and seminal plasma as enzymatic and non-enzymatic antioxidants. The cytoplasms of body cells have high concentrations of enzymatic antioxidants, but in the spermatozoa these enzymes are removed from the cytoplasm at the end of the spermatogenesis phase. Therefore, the antioxidant defense mechanisms in semen samples depend only on the antioxidants in the seminal plasma (Smits et al., 2018).

Many components were supplemented to improve the sperm function and fertilization ability. Most of these components have antioxidant action to counteract the excessive reactive oxygen species, by either breaking the chain reaction, or oxidative reduction (Amiri et al., 2018). Furthermore, numerous research works were conducted on the application of antioxidant supplementation to counteract the excessive ROS and improve the quality of frozen semen in various animal species (Banday et al., 2017; Bottrel et al., 2018; Ghallab et al., 2017). The consequence of adding either enzymatic or non-enzymatic antioxidants varies between different species and antioxidants types. The antioxidants have different mechanisms to counteract the ROS, which affects the antioxidant efficiency between the different species and extenders.

The low molecular weight and the thiol group in cysteine allows them to easily penetrate the sperm membrane and collaborate with glutathione, to improve the intracellular defense against the OS. Furthermore, cysteine can be metabolized in the sperm and produce taurine, which is able to combine with the fatty acid in the sperm membrane, and enhance the membrane osmolarity (Zhang et al., 2012). Ascorbic acid has the ability to penetrate the sperm membrane and prevent the peroxidation by breaking the chain reactions. Ascorbic acid produces monodehydroascorbate (MDHA) radicals, which inhibit the reactions with the oxygen and other molecules (Du et al., 2012).

However, there exists no studies that focus on the effect of cysteine and ascorbic acid, as ROS scavenges on the frozen semen extender and their capacity to protect the sperm from OS during cryopreservation. Furthermore, the frozen semen in stallion revealed poor pregnancy rates, compared to fresh semen (Squires et al., 2006; Lewis et al., 2015; Newcombe et al., 2011). In addition, no clear studies have investigated the effect of these antioxidants on the pregnancy rate using stallion frozen semen.

The semen enzymes such as GOT, GPT, ALP, LDH and GGT have been used as markers for fresh semen quality in horses and other livestock animals (Bucci et al., 2014; Pero et al., 2017; Stefanov et al., 2013; Žaja et al., 2016). There are no studies

that aim to determine the reliability of these enzymes as markers of frozen semen in horses.

1.3 Objectives

- To compare between prepared extenders in the laboratory (HF-20 and Tris-based) that contain different types of buffers with the commercial extenders EquiPlus Freeze® Minitube Germany, and INRA Freeze® IMV Technologies France on the fertility of cryopreserved semen from Arabian horses.
- To determine the effects of adding different concentrations of the antioxidants cysteine and ascorbic acid on the post-thawed semen motility, oxidative stress, viability, plasma membrane integrity, morphology and acrosome integrity in Arabian stallion semen.
- To evaluate and compare the pregnancy rates between cysteine and ascorbic acid cryopreserved Arabian stallion frozen semen extenders.
- To determine the effects of adding cysteine and ascorbic acid on sperm characteristics and levels of enzymes in post-thawed Arabian stallion semen.

1.4 Hypothesis

- There is significant difference between prepared extenders in the laboratory (HF-20 and Tris-based) with commercial extenders EquiPlus Freeze® Minitube Germany, and INRA Freeze® IMV Technologies France on the fertility of cryopreserved semen from Arabian horses.
- Supplementing of cysteine and ascorbic acid could significantly decrease the oxidative stress (OS) during semen cryopreservation procedures and improve the post-thawed semen parameters and fertility in the Arabian stallion.
- There is significant difference of pregnancy rates between cysteine and ascorbic acid cryopreserved Arabian stallion frozen semen extenders.
- Adding of cysteine and ascorbic acid could affect significantly on sperm characteristics and level of enzymes in post-thawed Arabian stallion semen.

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