UNIVERSITI PUTRA MALAYSIA

EFFECTS OF Panax ginseng AND Eurycoma longifolia JACK EXTRACTS ON CHILLED AND FROZEN-THAWED CROSSBRED BULL SEMEN COLLECTED USING MODIFIED ELECTRO-EJACULATION METHOD

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By

FALAH HASAN ALI BAIEE

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

August 2017

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DEDICATION

I dedicate my dissertation work to my family.

In appreciation of their love, sacrifices, faith, and eternal goodness, I would like to dedicate my dissertation to my dear loving parents, Hasan and Salma.

I will always appreciate all they have done, especially my wife Ruaa' for helping me all the time throughout the entire doctoral program.

I dedicate this work and give special thanks to my best friends I mean my wonderful sons Ahmed and Mohammed Hasan for being there for me throughout the entire doctorate program.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Doctor of Philosophy

EFFECTS OF *Panax ginseng* AND *Eurycoma longifolia* JACK EXTRACTS ON CHILLED AND FROZEN-THAWED CROSSBRED BULL SEMEN COLLECTED USING MODIFIED ELECTRO-EJACULATION METHOD

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August 2017

Chairman : Professor Abd Wahid b Haron, PhD
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Collection of semen from bulls can be done using different methods. Nowadays, electro-ejaculator technique is known as the most popular method to obtain semen samples from wild and domestic males. Chilled semen does not submit to the freeze-thaw procedure and suffers fewer damages, leading to greater capability and an improved capacity to fertilization. Cryopreservation of sperm plays a considerable role in economization of breeding programs in the cattle herd industry, genetic improvement of domestic animals, preservation of endangered species, and is clinically worthy in the controlling of infertility. Hence, the objectives of the present thesis were: to minimize the discomfort signs during semen collection using electro-ejaculator. Secondly, to evaluate the effect of *Panax ginseng* aqueous extract on the quality of chilled and frozen-thawed bull semen. Thirdly, to evaluate the effect of Tongkat Ali aqueous extract on the quality of chilled and frozen-thawed bull semen. Finally, to assess the synergistic effect of Tongkat Ali and Panax ginseng extracts on the quality of frozen-thawed bull semen. For all experiments, a total of 84 ejaculates were obtained from six crossbred bulls. The normal automatic electro-ejaculation method of semen collection (Method I) was compared to a modified method involving three stages (stage one, two and three) of gradation electrical stimulation (Method II). Discomfort signs, bulls' response to electro-ejaculator and fresh semen samples were assessed. Tris-egg yolk extender was used to dilute the semen sample. The extender was prepared into two parts, part one (P1) which did not contain glycerol while part two (P2) contained double amount of glycerol (12.8%). Each semen extender was divided into seven groups containing different concentrations either Panax ginseng aqueous extract, Tongkat Ali aqueous extract or combination between them. Chilled and frozen-thawed semen were carried out. Chilled semen groups were placed in test tube and kept in refrigerator at 5 ºC for 6 days. The frozen semen were packaged in (0.25 mL French straws and cryopreserved in liquid nitrogen (-196 ºC). Sperm motility, morphology, viability, membranes integrity,

DNA integrity, and lipid peroxidation were carried out to assess the extracts on chilled and frozen-thawed crossbred bulls. The data were analysed as mean ± standard error of the mean and checked for normal distribution. Descriptive statistical analysis of data, independent samples *t*-test, one or two-way analysis of variance (ANOVA), Fisher's exact test, and chi square test were used to analyse the data. The results showed that the discomfortness signs were reduced ($P < 0.05$), as well as using Method II than Method I. The total time taken for semen collection was similar in both methods. Also, there was no significant difference in fresh semen parameters. *Panax ginseng* aqueous extract did not improve the functional parameters of chilled and frozen-thawed bull semen. Moreover, sperm DNA integrity of frozen-thawed semen was not improved either. The low dosages (0.25 mg/mL) and (0.5 mg/mL) were not significant compared to control group. However, dosages more than (0.5 mg/mL) showed marked decrease in sperm characteristics ($P < 0.01$). Tongkat Ali aqueous extract significantly improved the chilled and frozen-thawed semen compared to control. The ideal dose of Tongkat Ali in chilled semen was (1 mg/mL) and (5 mg/mL) in frozen-thawed semen. DNA integrity and lipid peroxidation significantly improved in (5 mg/mL) compared to control group of frozen-thawed semen. In addition, different concentrations of combination between PGe and TAe into Tris-egg yolk extender did not improve the frozen-thawed bull semen quality. In conclusion, the discomfort signs prior and during semen collection were reduced by modification of the automatic mode of the electro-ejaculator device. *Panax ginseng* aqueous extract did not improve the quality of preserved semen. The quality of chilled and frozen-thawed crossbred bull semen was improved by adding Tongkat Ali aqueous extract to the semen diluent. The combination between *Panax ginseng* and Tongkat Ali extract also did not improve the quality of frozen-thawed bull semen.

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

KESAN EKSTRAK *Panax ginseng* DAN *Eurycoma Longifolia* KE ATAS SEMEN LEMBU JANTAN KACUKAN YANG DISEJUKKAN DAN DIBEKU-CAIRKAN YANG DIKUMPULKAN SECARA PENGUBAHSUAIAN KAEDAH ELEKTROEJAKULATOR

Oleh

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Pengumpulan semen dari lembu jantan boleh dilakukan menggunakan kaedah yang berbeza. Pada masa kini, teknik elektro-ejakulator dikenali sebagai kaedah yang paling popular untuk mendapatkan sampel semen dari haiwan jantan liar dan belajinak. Semen yang disejukkan tidak menlalui prosedur beku-cair dan mengalami kurang kerosakan, yang membawa kepada keupayaan yang lebih besar dan kapasiti yang lebih baik untuk persenyawaan. Kriopengawetan sperma memainkan peranan penting dalam mengekonomikan program pembiakan dalam industri kelompok lembu, pembaikan genetik haiwan domestik, pengawetan spesis terancam, dan secara klinikal adalah berguna untuk mengawal kemandulan. Oleh itu, objektif tesis ini pertamanya ialah untuk mengurangkan tanda ketidakselesaan semasa pengumpulan semen menggunakan elektro-ejaculator dan kedua, untuk menilai kesan ekstrak *Panax ginseng* (PGe) ke atas kualiti semen lembu jantan yang disejukkan dan dibeku-cairkan. Ketiga, ia adalah untuk menilai kesan ekstrak Tongkat Ali (TAe) ke atas kualiti semen lembu jantan yang disejukkan dan dibeku-cairkan. Akhirnya, ia adalah untuk menilai kesan sinergi ekstrak Tongkat Ali dan Panax ginseng ke atas kualiti semen lembu jantan yang dibeku-cairkan. Untuk semua eksperimen, sejumlah 84 hasil ejakulasi diperoleh daripada enam ekor lembu jantan kacukan. Kaedah automatik elektro-ejakulasi biasa untuk pengumpulan semen (Kaedah I) dibandingkan dengan suatu kaedah yang diubahsuai yang melibatkan tiga peringkat (peringkat satu, dua dan tiga) penggredan rangsangan elektrik (Kaedah II). Tanda-tanda ketidakselesaan, respons lembu jantan terhadap elektro-ejalulator dan sampel semen segar dinilai. Pengekal Tris-kuning telur digunakan untuk mencairkan sampel semen. Pengekal ini disediakan dalam dua bahagian, bahagian satu (P1) yang tidak mengandungi gliserol manakala bahagian dua (P2) mengandungi dua kali ganda jumlah gliserol (12.8%). Setiap pengekal semen dibahagikan kepada tujuh kumpulan yang mengandungi kepekatan yang berlainan sama ada ekstrak akueus

Panax ginseng, ekstrak akueus Tongkat Ali atau gabungan di antara kedua-duanya. Ujian ke atas semen yang disejukkan dan yang dibeku-cairkan telah dijalankan. Kumpulan semen sejuk diletakkan di dalam tabung uji dan disimpan di dalam peti sejuk pada 5 ºC selama 6 hari. Semen beku disimpankan di dalam straw Perancis 0.25 mL dan dikrioawetkan di dalam nitrogen cair (-196ºC). Motiliti, morfologi, daya maju, integrity membran, dan integriti DNA sperma, serta ujian peroksidaan lipid telah dijalankan untuk menilai kesan ekstrak pada semen lembu jantan kacukan yang disejukkan dan yang dibeku-cairkan. Data dianalisis sebagai min ± ralat piawai min dan diperiksa untuk taburan normal. Analisis statistik deskriptif data, ujian-*t* sampel bebas, analisis varians satu atau dua-hala (ANOVA), ujian tepat Fisher, dan chi kuasa dua telah digunakan untuk menganalisis data. Hasil kajian menunjukkan bahawa tanda-tanda ketidakselesaan juga telah dikurangkan (P<0. 05) apabila menggunakan Kaedah II berbanding Kaedah I. Jumlah masa yang diambil untuk pengumpulan semen adalah sama bagi kedua-dua kaedah. Juga, tidak terdapat perbezaan yang signifikan bagi parameter semen segar. Ekstrak akueus *Panax ginseng* tidak memperbaiki parameter fungsi semen lembu jantan yang disejukkan dan dibeku-cairkan. Tambahan lagi, integriti DNA sperma semen yang dibeku-cairkan juga tidak bertambah baik. Dos yang rendah, 0.25 mg/mL dan 0.5 mg/mL tidak signifikan berbanding dengan kumpulan kawalan. Walau bagaimanapun, dos lebih daripada 0.5 mg/mL menunjukkan penurunan ketara dalam ciri-ciri sperma (P<0.01). Ekstrak akueus Tongkat Ali dengan ketara memperbaiki semen yang disejukkan dan yang dibeku-cairkan berbanding kawalan. Dos Tongkat Ali yang terbaik bagi semen disejukkan adalah 1 mg/mL dan 5 mg/mL bagi semen dibeku-cairkan. Integriti DNA dan peroksidaan lipid bertambah baik dengan ketara di dalam 5 mg/mL berbanding dengan kumpulan kawalan semen dibeku-cairkan. Di samping itu, kepekatan yang berbeza bagi gabungan antara PGe dan TAe ke dalam pengekal Tris-kuning telur tidak memperbaiki kualiti semen dibeku-cairkan lembu jantan.

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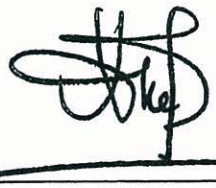

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LIST OF ABBREVIATIONS

AI	Artificial insemination
ALH	The amplitude of lateral head displacement
ATP	Adenosine triphosphate
AV	Artificial vagina
BCF	The beat cross frequency
BHT	Butylated hydroxytoluene
CASA	Computer-assisted sperm analysis
CAT	Catalase
CFDA	Carboxyfluoroscein di-acetate
COMET	Single cell gel electrophoresis
CR	Rate of calving
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulphoxide
dsDNA	Double-strand DNA
EE	Electro-ejaculator
EG	Ethylene glycol
EL	*Eurycoma longifolia* jack
FCM	Flow-cytometry
FTAI	Fixed-time artificial insemination
GR	Glutathione reductase
GSH-Px	Glutathione peroxidase
h	Hour
HDL	High density lipoproteins

HOST	Hypo-osmotic swelling test
IAV	Internal artificial vagina
IgY	Immunoglobulin Y
IVF	*In vitro* fertilization
LDL	Low-density lipoproteins
LIN	The line of the spermatozoa motility
LPO	Lipid peroxidation
m	Meter
MDA	Malondialdehyde
min	Minute
PAF	Platelet-activating factors
PAFA	Platelet activating factor acetyl-hydrolase
PGe	*Panax ginseng* aqueous extract
PUFAs	Poly unsaturated fatty acids
ROS	Reactive oxygen species
SCSA	Sperm chromatin structure assay
s	Second
SEM	Standard error of the mean
SOD	Super oxide dismutase
ssDNA	Single-strand DNA
STR	The straightness of the sperm's movement
TA	Tongkat Ali
TAe	Tongkat Ali aqueous extract
TBARS	Thiobarbituric acid reactive substance

VAP	Velocity average pathway
VCL	Velocity curvilinear
V	Volume
VSL	Velocity straight line
ZP	Zona pellucida

CHAPTER 1

INTRODUCTION

1.1 General

Bull semen can be collected by different methods. An artificial vagina (AV) is the commonest method to collect semen samples from bulls because AV is very close to natural mating (Palmer, 2016). However, AV requires bulls to be trained to mount a cow or a dummy (Palmer, 2005). Training of bull requires a substantial amount of time and it is unsuitable for aggressive bulls. Sometimes, a bull abstains to mount a cow or a dummy because the operator holding the AV is standing close to the bull. With this reason, an alternative method of collection uses an electro-ejaculator (EE). Nowadays, EE is the most popular method to obtain semen samples from wild and domestic animals due to its ease of use and reliability in obtaining a semen sample and safety (Palmer, 2016).

The earliest account on artificial insemination (AI) was in 1322 when an Arab chieftain pinched some semen from a stallion owned by his foe and used it to impregnate his prized mare. Whether that story is a fact or fiction is immaterial, but it does emphasise the fact that AI had been around for a good many years (Foote, 2002). Nowadays, artificial breeding through AI in dairy and beef cattle is practiced in many countries around the world (Thibier and Wagner, 2002). Artificial insemination uses diluted and extended semen which can be preserved by either chilled (4-5℃) or frozen (-196℃). Chilled semen can be used to inseminate females for a few days only, while frozen semen can be used for a very long period when stored properly. Thus, 95% of semen that are used in an AI programme is frozen-thawed semen. In addition, 95% of mature cows are artificially inseminated using either chilled or frozen semen, due to many factors, such as the usage of semen obtained from high-quality sires, safety purposes, as some bulls are known to be aggressive require careful handling. Moreover, genetic material of top sires can be imported or exported as frozen semen. In addition, AI also can reduce the risk of spreading diseases (Salisbury *et al*., 1978). Although, the use of frozen-thawed semen in AI is highly popular, however, the fertility rate of frozen-thawed semen is quite low than fresh semen. This is because of the lower post-thawed survival rate of spermatozoa (Yimer *et al*., 2014). Approximately, 50% of spermatozoa are dead during freezing-thawing course (Watson, 2000). Thus, it is still very important to enhance the method of semen collection and semen handling, improve the component of extenders and the process of freezing-thawing and achieve better quality of spermatozoa after thawing. Temperature variation, ice formation and oxidative stress are the main challenges in preparation of frozen-thawed semen (Watson, 2000; Sariözkan *et al*., 2009).

In addition, semen extenders should contain buffers, source of energy, antibiotics to prevent the bacterial growth, and cryoprotectant to protect against physical and chemical changes that occur during chilling, freezing and thawing processes (Salisbury *et al*., 1978). Egg yolk is an ingredient in the extender to reduce cold shock effect (Moussa *et al*., 2002), while glycerol reduces the harmful effect of ice formation (Taşdemir *et al*., 2013) Antibiotics prevent bacterial infection (Gloria *et al*., 2014), and liquid nitrogen allows the sperm to be preserved infinity (Foote, 2002).

1.2 Problem statement

Currently, as it was mentioned earlier, EE is the most popular method to collect semen samples from domestic animals (Palmer, 2016). Reliability in obtaining semen samples, requires minimal facilities and safety were the main benefits of this method (Palmer, 2016). However, EE is accompanied with discomfort and stress because this method works on electrical stimulation in a very sensitive area (ductus deferens, accessory genital glands and nerves around). Thus, this action may be associated with struggling, vocalizing and attempting to lie down of bulls during collection (Ohl, 1993).

In spite of the aim of sperm cryopreservation is to preserve sperm functional parameters and fertility; the cryopreservation course surely causes impairment to spermatozoa (Tvrdá *et al*., 2016), so that reducing fertility. To date, various amount of studies have been done to elevate and enhance chilled and frozen-thawed semen quality using some additives to the extender of semen such as, unsaturated fatty acid (Kaka *et al*., 2015; Khoshvaght *et al*., 2015), anti-oxidant (Khumran *et al*., 2015; Eidan, 2016), or amino acids (Holt *et al.*, 2015; Kumar *et al.*, 2015; Sarıözkan *et al*., 2015). Furthermore, some plants' extract have demonstrated as anti-oxidant additives to semen or semen extender (Sapanidou *et al*., 2015; Tvrdá *et al*., 2016), to study the toxic effect of extract on sperm (Kaefer *et al*., 2013) and to dilute semen (Abul Rashid and Nurin Qistina, 2015). However, most of these additives cannot achieve satisfactory results, and also some of these additives make the extender costly. Therefore, there is no natural material, rich with bioactive components has been investigated to improve chilled and frozen-thawed semen quality.

1.3 Justification

As mentioned above, there are some limitations to use AV for semen collection, such as bulls must be trained to mount a cow or a dummy, training of bull requires time, and AV is unsuitable for aggressive bulls. However, EE can overcome these limitations and has been reported that EE is associated with pain (Ohl, 1993). Few studies were conducted to reduce and relieve pain such as using a narcoleptic tranquilizer (pipothiazine palmitate) for Bison bulls (Toosi *et al*., 2013), hormones such as oxytocin to reduce time of ejaculation (Palmer *et al*., 2004) and segmented the probe of EE (Etson *et al*., 2004). However, the use of a tranquilizer may cause animals to lie down, and heavy weight sires may be injured during the process.

Hormones, e.g. oxytocin may increase the pain because it increases the contractions of smooth muscles. Segmented probe has no significant effect compared to normal probes (Etson *et al*., 2004). Thus, there is a need to find out a modified method that can be used to collet semen samples from bulls with minimal discomfortness.

Panax ginseng and Tongkat Ali (*Eurycoma longifolia*) are very well studied medicinal herbal plants. They have many beneficial effects. *Panax ginseng* can improve the awareness in rising energy levels and overall strength (Won *et al*., 2014). Principally, *Panax ginseng* gives an amusing diversity of energetic elements, minerals and nutrients for health benefits to the body (Kim *et al*., 2006). Conventionally, *Panax ginseng* is recognized to fight weaknesses, provide additional energy, increase psychological effectiveness and relieve exhaustion (Yun, 2001). Numerous reports pointed out that *Panax ginseng* has anti-oxidative effects (Lim *et al*., 1998; Yokozawa *et al*., 1998; Hu and Kitts, 2001; Yokozawa *et al*., 2004; Jung *et al*., 2005; Li *et al*., 2008; Kim *et al*., 2011; Wei *et al*., 2012; Yun *et al*., 2016).

Tongkat Ali is presumed to be a treatment for numerous problems such as ulcer, fatigue, poor endurance, to improve physical and mental performance, enhance the immune system and boost energy levels (Hool *et al*., 1997; Kuo *et al*., 2003; Abdullah *et al*., 2004; Ismail *et al*., 2012; Tambi *et al*., 2012; George and Henkel, 2014; Hai *et al*., 2016; Han *et al*., 2016). Many studies have been conducted to examine the benefits of Tongkat Ali, such as to improve overall well-being (Ismail *et al*., 2012), anti-oxidant (Panjaitan *et al*., 2013; Varghese *et al*., 2013; Lulu *et al*., 2015), and anti-stress benefits (Talbott *et al*., 2010, 2013). Husen *et al*. (2004) reported a substantial anti-hyper glycaemic effect of Tongkat Ali in a rat model.

AI is widely used to inseminate females with diluted semen. Semen that is used for AI can be stored either chilled or frozen. Chilled semen should be used within three days because after three days of the quality of semen is diminished leading to a reduction in fertility rate (Bucher *et al*., 2009; Crespilho *et al*., 2014). Frozen-thawed semen can be stored for a very long time in liquid nitrogen (Vishwanath and Shannon, 2000). However, about 50% of spermatozoa die during the freezing-thawing process (Shannon and Vishwanath, 1995; Curry, 2000; Lessard *et al*., 2000; Vishwanath and Shannon, 2000; Watson, 2000). Moreover, the lifespan of frozen-thawed semen is very short as compared to fresh and chilled semen. In addition, the motility of frozen-thawed semen is not as good as fresh semen (Thomas *et al*., 1998; Watson, 2000; Chaveiro *et al*., 2006; Yimer *et al*., 2014, 2015; Papa *et al*., 2015); and thus, the fertility rate of frozen-thawed semen is lesser than fresh semen (Shannon and Vishwanath, 1995; Holt, 2000; Amirat-Briand *et al*., 2010; Büyükleblebici *et al*., 2014).

At present, there is no plant, rich with bioactive components that has been investigated to improve chilled and frozen-thawed semen quality. Thus, it is important to investigate the effects of these two plants extracts (PGe and TAe) which are rich with bioactive components on chilled and frozen-thawed semen.

1.4 Hypothesis:

Ho:

i. The modified EE method will not reduce the discomfort signs during semen collection in bulls.
ii. There is no beneficial effect on chilled and frozen-thawed bull sperm quality that was supplemented with *Panax ginseng*, Tongkat Ali (*Eurycoma longifolia*) and their combination.

Ha:

i. The modified EE method reduces the discomfort signs during semen collection in bulls**.**
ii. Bioactive components of *Panax ginseng* and Tongkat Ali (*Eurycoma longifolia*) extender improve quality of chilled and/or frozen-thawed semen. Aqueous extraction of these plants used as supplements to semen extenders separately or in combination could protect spermatozoa and might increase the viability, provide energy for sperm motility, protect the membranes of spermatozoa, improve DNA integrity, and reduce lipid peroxidation**.**

1.5 Objectives:

Thus, the present study was carried out with the following objectives:

i. To minimize the discomfort signs during semen collection using electro-ejaculator.
ii. To evaluate the effect of *Panax ginseng* aqueous extract on the quality of chilled and frozen-thawed bull semen.
iii. To evaluate the effect of Tongkat Ali aqueous extract on the quality of chilled and frozen-thawed bull semen.
iv. To assess the synergistic effect of Tongkat Ali and *Panax ginseng* extracts on the quality of frozen-thawed bull semen.

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