UNIVERSITI PUTRA MALAYSIA

IMPROVEMENT OF THE MEDIUM AND PROCESSING PROTOCOL FOR CRYOPRESERVATION OF BOER GOAT SPERMATOZOA

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IMPROVEMENT OF THE MEDIUM AND PROCESSING PROTOCOL FOR CRYOPRESERVATION OF BOER GOAT SPERMATOZOA

By

AKEEL AHMED MEMON

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

November 2012

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DEDICATIONS

To those that have provided me with the love and support needed to accomplish this goal: my parents, for being there to encourage me every step of the way and for your willingness to give of yourselves so that your children can succeed; my wife, daughter and son for your unwavering desire to bring happiness, friendship, balance and laughter to my life; my sisters and brother, for being wonderful friends and being examples of how to find my strengths and use them to do great things; my grandparents, from whom I inherited my work ethic, ambition, and desire to help others; and finally, to the rest of an incredible support system composed of my family and friends.

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

IMPROVEMENT OF THE MEDIUM AND PROCESSING PROTOCOL FOR CRYOPRESERVATION OF BOER GOAT SPERMATOZOA

By

AKEEL AHMED MEMON

November 2012

Chairman: Professor Abd Wahid Haron, PhD

Faculty: Veterinary Medicine

Fertility rate is lower after AI in goats with cryopreserved semen. Low fertility with frozen semen is due to the procedures related with cryopreservation that leads to sperm damage, impairs its normal function and fertilizing potential. Therefore, improvements in the freezing media and processing protocols applied to the goat semen are needed to improve the quality of goat semen for AI.

Nine Boer male goats were used in the study. Semen was collected twice a week with the aid of an artificial vagina. Ejaculates were evaluated for volume, colour, consistency, mass activity, sperm motility, sperm concentration and sperm morphology and ejaculates qualified the standard criteria were processed according to the needs of each experiment. Sperm cytological characteristics were evaluated before freezing and post thawing.

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The effects of different buffers, egg yolk concentrations and sperm dilution rates were analysed to improve extending media for chilled and post thawed Boer goat spermatozoa. Tris buffer demonstrated practical and beneficial effects on sperm cytological characteristics before and after freezing. Significant ($P<0.05$) improvement was observed with Tris buffer in terms of motility and acrosome integrity of post thawed spermatozoa.

In the present study, significantly higher motility, membrane integrity and acrosome integrity were observed with the 18% egg yolk concentration compared to other egg yolk concentrations of 12% and 6%. A study was conducted to evaluate the effects of semen dilution rate on the characteristics of chilled and post thawed Boer goat semen. Results indicated that significant differences exist between low and high semen dilution rates. A significant improvement in the viability of pre and post thawed spermatozoa was observed with low dilution rate.

Four antioxidants at different concentrations were tested to determine their effectiveness in the preservation of Boer goat semen. Antioxidants (BHT, hypotaurine, cysteine and ascorbic acid) have improved the quality of chilled and frozen thawed stored Boer goat spermatozoa in terms of motility, membrane integrity, morphology, acrosome integrity and viability. Individual concentrations of each antioxidant were established in Tris fructose egg yolk glycerol extender. Significantly ($P<0.05$) better results were obtained for chilled and frozen thawed Boer spermatozoa quality characteristics with 2 mM, 10 mM, 5 mM and 8.5 mg/ml concentrations of butylated hydroxytoluene, hypotaurine, cysteine and ascorbic acid, respectively.

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Effects of antioxidants at the time of semen collection, and in the washing solution were subsequently evaluated to find out the best time of antioxidant addition to reduce oxidative stress, and improve chilled and post thawed quality of sperm. The results of these studies showed that the addition of antioxidants to the washing solution significantly improved the motility of chilled spermatozoa. Furthermore it was observed that motility and acrosome integrity of post thawed spermatozoa was significantly improved when washing solution was supplemented with antioxidants. However, the addition of the extender supplemented with antioxidants in collection tubes of the artificial vagina did not improve the quality of chilled and frozen thawed spermatozoa.

The results of the present study demonstrated that the rate of cooling significantly influenced the quality of chilled and frozen thawed Boer goat semen. Significantly ($P<0.05$) higher motility was observed with slow cooling rates and antioxidant supplementation.

The use of a programmable freezer for freezing spermatozoa compared to a polystyrene box method of freezing in a medium supplemented with antioxidants was investigated. In this experiment, significantly higher results were observed in motility, membrane integrity, morphology, acrosome integrity and viability of post thawed Boer goat spermatozoa extended with antioxidant in the programmable freezer compared to the polystyrene box method.

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The ability of antioxidants to reduce lipid peroxidation (LPO) after freeze thawing was measured using the thiobarbituric acid method. Results showed that addition of antioxidants significantly reduced ($P<0.05$) the rate of LPO in comparison to control. Ascorbic acid exhibited significantly lower values (1.27±0.28 nmol/2x10^8 spermatozoa), than butylated hydroxytoluene (1.32±0.42 nmol/2x10^8 spermatozoa), cysteine (2.27±0.16 nmol/2x10^8 spermatozoa) and hypotaurine (2.38±0.17 nmol/2x10^8 spermatozoa) than control (3.52±0.54 nmol/2x10^8 spermatozoa). However, differences among the supplements were non-significant. The overall pregnancy rate (%) was 34.38 %. Significantly higher ($P<0.05$) pregnancy rate was observed with ascorbic acid (42.85%) and butylated hydroxytoluene (35.71%) compared to those of hypotaurine (33.33%), cysteine (33.33%) and control (26.38%). In summary, this study pointed to the magnitude of improvement made possible when processing protocol and cryopreservation medium are optimised for Boer goat semen.

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

PENAMBAHBAIKAN MEDIUM DAN PROTOKOL PEMPROSESAN UNTUK KRIOAWETAN SPERMATOZOA KAMBING BOER

Oleh

AKEEL AHMED MEMON

November 2012

Pengerusi:Professor Dr Abd Wahid Haron, PhD

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Tahap kesuburan berikutan permanian beradas adalah lebih rendah apabila semen yang dikrioawet digunakan pada kambing.Keadaan ini berlaku kerana prosedur krioawetan spermatozoa mengakibatkan kerosakan spermatozoa yang menggugat fungsi lazim, serta potensi persenyawaan spermatozoa tersebut.Oleh itu, penambahbaikan ke atas media penyejukbekuan dan protokol pemprosesan semen kambing adalah perlu untuk meningkatkan kualiti semen untuk tujuan permanian beradas.

Sembilan kambing Boer jantan digunakan dalam kajian ini.Semen dikumpul dua kali seminggu dengan menggunakan vagina tiruan. Air mani dinilai dari segi jumlah, warna, keseragaman, aktiviti menyeluruh, motiliti sperma, kepekatan sperma, morfologi sperma dan kualiti ejakulasi kepada kriteria tertentu yang ditetapkan utuk

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pemprosesan bergantung kepada keperluan setiap ujikaji. Ciri-ciri sel sperma dinilai sebelum penyejukbekuan dan selepas pencairan.

Kesan bahan penampan yang berbeza, kepekatan kuning telur dan kadar pencairan sperma dianalisis untuk memperbaiki media bagi tujuan penyejukan dan selepas pencairan sperma kambing Boer. Penampan Tris menunjukan kesan yang baik ke atas ciri sel spermatozoa sebelum dan selepas penyejukbekuan.Penambahbaikan yang bererti ($P<0.05$) dicerap pada motiliti dan integriti akrosom spermatozoa selepas pencairan berikutan penggunaan penampan Tris.

Dalam kajian ini, motiliti, integriti membran dan akrosom adalah lebih tinggi dan berbeza dengan bererti apabila kuning telur digunakan pada kepekatan 18 % berbanding kepekatan kuning telur pada 12% dan 6%. Kajian juga dijalankan untuk menilai kesan kadar pencairan semen ke atas ciri penyejukkan dan pencairan semen kambing Boer. Keputusan menunjukkan perbezaan bererti di antara kadar pencairan semen yang rendah dan tinggi. Penambahbaikan yang signifikan ke atas spermatozoa sebelum dan selepas pencairan dicerap apabila kadar pencairan yang rendah digunakan.

Empat antioksidan pada kepekatan yang berbeza telah diuji untuk menentukan keberkesanan mereka dalam memelihara kualiti semen kambing Boer.Antioksidan (hidroksitoluen berbutil, hipotaurina, sisteina dan asid askorbik) berupaya memperbaiki kualiti spermatozoa kambing Boer sewaktu penyejukan, serta selepas pencairan.Aspek kualiti yang diperbaiki adalah dari segi motiliti, integriti membran, morfologi, integriti akrosom dan viabiliti.Kepekatan setiap antioksidan yang berkesan dalam penampan Tris-fruktosa-kuning telur-gliserol telah ditentukan.

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Keputusan bererti yang terbaik untuk spermatozoa Boer yang disejukkan, dan selepas pencairan adalah apabila hidroksitoluen berbutil, hipotaurina, sisteina dan asid askorbikmasing-masing digunakan pada kepekatan 2mM, 10mM, 5mM dan 8.5mg/ml.

Kesan antioksidan semasa pengumpulan semen, dan dalam larutan pembersihan juga dinilai untuk menentukan masa yang paling sesuai untuk menambah antioksidan bagi mengurangkan tegasan oksidasi, dan untuk memperbaiki kualiti sperma yang disejukkan dan selepas pencairan. Keputusan menunjukkan penambahan antioksidan ke dalam larutan pembersih telah membaiki kualiti sperma yang disejukkan dengan bererti. Tambahan pula, peningkatan motiliti serta integriti membrane akrosom yang bererti turut dicerap pada spermatozoa selepas pencairan apabila larutan pembersih ditambah antioksidan.Walaubagaimanapun, penambahan antioksidan pada cecair pengekal (extender) di dalam tiub pengumpul vagina tiruan tidak memberikan sebarang kesan ke atas kualiti spermatozoa yang disejukkan dan selepas pencairan.

Keputusan menunjukkan kadar penyejukkan memberi kesan bererti kepada kualiti semen Kambing Boer semasa penyejukkan dan selepas pencairan. Keputusan yang lebih baik dan bererti diperolehi apabila kadar penyejukkan yang perlahan digunakan dengan penambahan antioksidan.

Penggunaan mesin penyejukbekuan terprogram berbanding kaedah kotak polistirena untuk penyejukbekuan spermatozoa dalam media yang ditambah antioksidan telah dibuat dalam ujikaji seterusnya.Keputusan yang yang lebih baik dan bererti telah

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dicerap untuk motiliti, integriti membran, morfologi, integriti akrosom dan viabiliti spermatozoa kambing apabila mesin penyejukbekuan terprogram digunakan.

Keupayaan antioksidan untuk mengurangkan peroksidasi lipid (LPO) selepas pencairan diukur menerusi kaedah asid tiobarbiturik. Keputusan menunjukkan penambahan antioksidan menyebabkan pengurangan kadar LPO yang bererti berbanding kumpulan kawalan ($P<0.05$). Asid askorbik menujukkan keputusan yang lebih baik (1.27±0.28 nmol/2x10^8 spermatozoa) berbanding hidroksitoluen berbutil (1.32±0.42 nmol/2x10^8 spermatozoa), sisteina (2.27±0.16 nmol/2x10^8 spermatozoa), hipotaurina (2.38±0.17 nmol/2x10^8 spermatozoa) dan kawalan (3.52±0.54 nmol/2x10^8 spermatozoa).Walaubagaimanapun, perbezaan di antara bahan penambah ini adalah tidak bererti.Kadar kebuntingan keseluruhan adalah 34.38%.Kadar kebuntingan yang lebih tinggi secara bererti telah dicerap untuk asid askorbik (42.85 %), dan hidroksitoluen berbutil (35.71 %), berbanding hipotaurina (33.33 %), sisteina (33.33 %) dan kawalan (26.38 %).Secara keseluruhannya, kajian ini telah menunjukkan tahap penambahbaikan yang mungkin apabila protokol pemprosesan dan media krioawetan dioptimumkan untuk semen kambing Boer.

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I certify that a Thesis Examination Committee has met on 20.11.2012 to conduct the final examination of Akeel Ahmed on his thesis entitled: 'Improvement of the medium and processing protocol for cryopreservation of Boer goat spermatozoa' in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U. (A)106] 15 March 1998. The committee recommends that the student be awarded the Doctor of Philosophy.

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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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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

_____

Akeel Ahmed Memon

Date: 20 November 2012

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LIST OF ABBREVATIONS

AI	Artificial insemination
ANOVA	Analysis of variance
ART	Assisted reproductive technologies
AV	Artificial vagina
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
BUSgp60	Bulbourethral secreted glycoprotein
CAT	Catalase
EYCE	Egg yolk coagulating enzyme
GPx	Glutathione preoxidases
GSH	Glutathione
GSSG	oxidized glutathione
H_2O_2	Hydrogen peroxide
HNO	Nitroxyl anion
HNO_3	Peroxynitrious acid
HNO_3	Peroxynitrious acid
HOST	Hypo osmotic swelling test
IU	International unit
IVF	In vitro fertilization
LDL	Low density lipoproteins
LN_2	Liquid nitrogen
LPO	Lipid peroxidation
LSD	Least significant difference

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MDA	Malondialdehyde
mM	Millimole
N_2O	Nitrous oxide
NO	Nitric oxide
NO_3^-	Peroxynitrite
O_2^-	superoxide anion
$O_2^{-\cdot}$	superoxide radicals
•O2-	Superoxide anion
OH^-	hydroxyl radical
OH•	Hydroxyl radical
OHCl•	Hypochlorite radical
OPF	Oil palm frond
OS	Oxidative stress
Prx	Thioredoxin peroxidises
PUFA	polyunsaturated fatty acids
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SMEYG	Skim milk egg yolk glycerol
SOD	superoxide dismutase
SPSS	Statistical package for social sciences
TBARS	Thiobarbituric-acid-reactive substance
TEYG	Tris egg yolk glycerol
Tris	Tris (hydroxymeythyle) aminomethane
UPM	University Putra Malaysia
µg	microgram

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µL	microliter

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CHAPTER 1

INTRODUCTION

Domestic farm animals, including goats, are important to the world economy. They are very important livestock to small holders and farmers in developing countries (Kioumarsi *et al.,* 2011; Abdelrahman, 2009) for their meat and milk. The small ruminant industry has experienced a great expansion during the last two decades (Fonseca *et al.,* 2005). The population of this species is on the rise throughout the world increasing 26.4% between 1993 and 2003 (Boyazoglu *et al.,* 2005). Compared to other ruminants, the largest increase (56.3%) has also been seen in the population of goats from 1983 to 2003. During this period the sheep population was down 8.6% and cattle population only increased by 9.6% (Boyazoglu *et al.,* 2005). To date, it is estimated that the world population of goats is 879 million (FAOSTAT, 2011).

Most goats are found in the drier areas of the developing world. The two major repositories for goats are Asia and Africa and both are considered as home tracts for this species. There are about 545 and 245 million goats in Asia and Africa, respectively. This species is the principal source of meat and milk for human in these areas of the world (Devendra and Solaiman, 2010; Dubeuf and Boyazoglu, 2009).

There has been an increased market demand for goat cheese and yogurt, as it is now viewed as gourmet in some locations (Haenlein, 2007). Goat milk is also receiving elevated status due to its exceptional nutritional value (Haenlein, 2004). Goats are not

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only important for their meat and milk, but interest in fibres, skins, and brush control for fire prevention or to create better pasture for sheep and cattle to graze, has generated renewed interest in this species (Boyazoglu *et al.*, 2005; Haenlein, 2007). In general, goat products are often associated with agro-tourism in many mountainous regions and have a healthy and an ecological image (Dubeuf *et al.*, 2004). Opposite to purely economic interest the goat is also a culturally important animal that is used for religious and cultural events (Alexandre *et al.*, 2002).

Like other countries, the small ruminant population has been steadily increasing over the past ten years in Malaysia (DVS, 2011). The total population of goats in Malaysia was estimated at 336,000 heads in 2009 (FAOSTAT, 2011).

Imported Boer goats from South Africa and Australia are important contributors to the Malaysian goat and farming activity for several years since early 2000s. Substantial numbers of these animals were imported with the aim to meet the market demand of goat meat and improve the productivity of the local goats through advanced reproductive technologies (Ariff *et al.*, 2010).

Although the origin of the Boer goat is from South Africa (Malan, 2000) this breed has the ability to adapt toa wide range of climatic and feeding conditions. It has been also observed that this breed acclimatize well to the Malaysian environment which has a temperature range of 26 – 32 ^{o}C, relative humidity of 80 – 90%, and an average total annual rainfall of 2500 mm. Many of these goats are reared semi intensively in raised floor-houses and allowed to graze on native and cultivated pastures from late morning

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until early afternoon, and are fed with supplementary concentrate feed during the rest of the day (Ariff *et al.,* 2010).

Substantial numbers of these animals are imported and importation of purebred Boer goat is costly. Therefore, uses of assisted reproductive technologies (ARTs) are necessary to accelerate the efficiency of reproduction in this species. Amongst all ARTs, artificial insemination (AI) is the most effective technology for improving the reproductive rates in domestic animals and this could be a better option for increasing the reproductive performance and up gradation of existing Boer goat stock in this country.

Information regarding the semen characteristics of different breeds of livestock and their freezability reared in different geographically conditions is useful in developing a genetic improvement program. With the widespread application of artificial insemination in domestic animals, there has been a growing interest and necessity for more knowledge concerning the reproductive characteristics of farm animals reared all over the world in different climatic conditions.

Interest of goat farmers to breed does through artificial insemination necessities to establish and improve the current techniques for processing, cooling, packaging and freezing semen that may enhance the viability and fertility of spermatozoa. Many studies (Leboeuf *et al*., 2003; Dorado *et al*., 2007) reported inconsistent fertility results ranging 30% -70% with cryopreserved semen. Low fertility with frozen semen is due to the procedures related with cryopreservation (Luvoni, 2006) that leads to sperm damage, impairs its normal function and fertilizing potential. The main causes of sperm damage

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during cryopreservation are classified as (1) cold-shock (Watson, 2000), (2) osmotic stress (Watson, 2000) and (3) oxidative stress (Aitken and Krausz, 2001; Sikka, 2001; Agarwal *et al.,* 2003). The adverse effects of oxidative stress in the form of lipid per oxidation (LPO) in mammalian reproduction have been studied for over three to four decades and since then it has been established that mammalian spermatozoa are harmed by hydrogen peroxide and lipid peroxidation (Brzezinska-Slebodzinska *et al.,* 1995). They cause sperm cell damage, shortening its life span *in vivo* and affecting the preservation of semen for AI (Alvarez and Story, 1985). Artificial insemination provides a way to optimize animal reproduction efficiency but the additional oxidative stress due to processing steps involved with semen extension, cooling, and freezing may introduce extra oxidative stress and may limit the success. Although, the knowledge in this area has evolved immensely in recent years, a rising concern about the detrimental effects that can occur during the physiological processes and storage procedures still perseveres.

Use of AI is a very important tool for genetic improvement of livestock, the introduction of specialized breeds, development of selection programs and progeny testing. Although many investigations of goat semen preservation exist, still there is room for improvement, and several areas within the cryopreservation process require further investigation to establish the quality characteristics of semen and find better additives with simple, less sophisticated methods that minimize the damage and optimize chilled and frozen thawed sperm quality i.e., determination of appropriate extender, concentration of antioxidants and their method of addition, and cooling rates and freezing method.

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In the entire process of semen cryopreservation i.e., Semen collection, centrifugation, dilution, cooling, equilibration, freezing and thawing, spermatozoa may lose the ability to fertilize normally (Watson, 1995). Therefore, the process of cryopreservation needs improvements for obtaining the best quality frozen semen with a maximum number of structurally and functionally normal spermatozoa.

Maximizing the utilization of superior Boer goat germplasm, minimizing the losses due to poor freezability and improving the existing protocols for quality of frozen semen are the areas to be investigated with the aim to identify the reproductive potential of this very important genotype of goats in the Malaysian environment and to improve and validate the existing reproductive methods and technologies for increasing fertility, fecundity and total productivity of the flocks.

For AI, chilled or frozen thawed semen is used. The basic principle of chilling or freezing is similar for spermatozoa of most mammalian species, but sperm from different species may react differently due to their differences in morphology and certain biochemical constituents. Hence, a cryopreservation protocol developed for one species may not be ideal for sperm of other species (Sundararaman and Edwin, 2008). Various researchers reported successful cryopreservation of goat spermatozoa but the fertility rate with chilled and frozen stored goat spermatozoa is variable. Furthermore, the origin of the Boer goat is South Africa which has a temperate environmental condition different from Malaysia, therefore protocols for the cryopreservation of Boer goat semen needed to be investigated to identify the media and processing technique that minimize

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the freeze thaw damages and maximize the quality for the wider application of superior germplasm.

Specific Objectives

1. To determine the most effective buffers, egg yolk concentration and dilution rate for chilled and frozen stored Boer goat sperm.
2. To investigate the most effective concentrations of various antioxidants i.e., Ascorbic acid, butylated hydroxytoluene, hypotaurine and cysteine for protecting sperm cells from free radical damage.
3. To optimize the chilled and frozen thawed Boer goat sperm quality with the addition of antioxidants at various stages of dilution.
4. To determine the effectiveness of various cooling rates and methods of freezing for Boer goat sperm quality with extenders supplemented with antioxidants.
5. To examine the*in vivo* fertility with frozen semen obtained by optimized diluent, and cooling and freezing processes with antioxidants using artificial insemination technique.

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