UNIVERSITI PUTRA MALAYSIA

IMPROVED CRYOPRESERVATION OF BOER GOAT SEMEN

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IMPROVED CRYOPRESERVATION OF BOER GOAT SEMEN

By

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

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DEDICATIONS

This work is dedicated to my family and my country.

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

IMPROVED CRYOPRESERVATION OF BOER GOAT SEMEN

By

SOE WIN NAING

November 2010

Chairman : Abd Wahid Haron, PhD

Faculty : Veterinary Medicine

Recently, interest in the cryopreservation of spermatozoa as a potential source of valuable genes has escalated to improve reproductive efficiency and productivity in livestock such as goats. The objectives of this study were to improve the quality of semen cryopreservation media, technique of cryopreservation, and to analyse the effects of various factors on goat sperm survival after freezing and thawing.

To conduct this research, eleven Boer goats were used and semen was collected twice a week using an artificial vagina. For initial evaluation, the semen samples were assessed for volume, colour, consistency, mass activity, sperm concentration, sperm morphology, and percentage of motile spermatozoa. The qualified semen samples between one and two mL volume with a concentration of greater than 2.5 x 10^9 sperm/mL having >75%

progressively motile sperm and >85% of the sperm with normal morphology were selected for cryopreservation. The qualified ejaculates were then diluted with the semen extenders and packed in 0.25 mL French straws. After equilibration, cooling and freezing procedures were carried out in a cooling chamber and liquid nitrogen. Two days later, the semen was thawed at 37°C for 30 sec and evaluated for the semen qualitative parameters such as motility, acrosome integrity, membrane integrity, live and normal spermatozoa percentages. Data were analyzed using ANOVA, followed by Tukey's post hoc test to determine significant differences in all the parameters between groups using the SPSS software system.

The effects of four different sugars on semen quality were analyzed for the improvement of semen cryopreservation media in Boer goats. This study was conducted to analyze firstly the effect of two monosaccharides and two disaccharides in Boer goat semen cryopreservation, secondly, to investigate the combination effects of trehalose and other sugars, and finally to find out the most effective concentration of trehalose combination for Boer semen cryopreservation. The combination of 69.38 mM glucose and 198.24 mM trehalose conferred the practical and beneficial effects in cryopreservation of Boer goat spermatozoa.

The effects of four cryoprotectants, different concentrations of glycerol and three glycerolization procedures with two cooling times for Boer goat semen

evaluations, further fertility trials are required as ultimate test of improved cryopreservation.

Abstrak tesis disampaikan kepada Senat Universiti Putra Malaysia sebagai memenuhi sebahagian keperluan untuk ijazah Doktor Falsafah

PENINGKATAN KRIOPRESERVASI SEMEN KAMBING BOER

Oleh

SOE WIN NAING

November 2010

Pengerusi : Abd Wahid Haron, PhD

Fakulti :Fakulti Perubatan Veterinar

Baru-baru ini, kepentingan penyejukbekuan spermatozoa sebagai sumber potensi gen berharga telah bertambah bagi meningkatkan kecekapan pembiakan dan produktiviti ternakan seperti kambing. Tujuan kajian ini adalah untuk meningkatkan kualiti media penyejukbekuan semen, teknik penyejukbekuan, dan menganalisis kesan pelbagai faktor terhadap keupayaan hidup sperma kambing selepas pembekuan dan penyahbekuan.

Untuk melakukan kajian ini, sebelas ekor kambing Boer digunakan dan semen dikumpul dengan menggunakan vagina tiruan dua kali seminggu. Untuk penilaian awal, sampel semen dinilai untuk menentukan isipadu, warna, aktiviti massa, kepekatan sperma, morfologi sperma, dan peratusan spermatozoa motil. Sampel semen yang memenuhi syarat kemudian dicairkan dengan pencair semen dan dimasukan dalam 0.25 mL straw.

Setelah diekuliberasi, pendinginan dan prosedur pembekuan dilakukan dalam ruangan pendingin dan nitrogen cair. Dua hari kemudian, semen dinyahbeku pada suhu 37°C selama 30 saat dan dinilai untuk parameter kualitatif semen. Data dianalisis menggunakan ANOVA, diikuti dengan ujuan Tukey's pos hoc untuk menentukan perbezaan ketara dalam semua parameter antara kumpulan menggunakan sistem perisian SPSS.

Kesan empat jenis gula yang berbeza ke atas kualiti semen dianalisis untuk peningkatan media penyejukbekuan semen kambing Boer. Kajian ini mulanya dilakukan untuk menganalisis pengaruh dari dua monosakarida dan disakarida dalam dua penyejukbekuan semen kambing Boer, kedua, untuk menyiasat kesan gabungan trehalos dan gula lain, dan akhirnya untuk mengetahui kepekatan yang paling berkesan untuk kombinasi trehalos ke atas penyejukbekuan semen Boer. Kombinasi glukosa 69.38 mM dan 198.24 mM trehalos menunjukkan kesan praktikal dan bermanfaat dalam penyejukbekuan spermatozoa kambing Boer.

Kesan empat kriopelindung, kepekatan yang berbeza gliserol dan tiga prosedur pengliserolan dengan dua masa pendinginan untuk penyejukbekuan semen kambing Boer dinilai melalui analisis parameter motili. Penggunaan kriopelindung gliserol dan penambahan gliserol 7% menunjukkan kesan kriopelindungan terbaik untuk penyejukbekuan semen kambing Boer. Selain itu, dengan menggunakan tiga langkah kaedah

pencairan dan tiga jam masa pendinginan meningkatkan kualiti semen selepas penyejukbekuan.

Kesan empat asid amino (alanin, glisin, sistein dan glutamin) pada kepekatan 20, 40, dan 60 mM ke atas kualiti semen ditentukan dalam penyejukbekuan semen kambing Boer. Pengaruh kepekatan yang berbeza sistein (0, 5, 9.5, 15.5, 20 mM) ke atas penyejukbekuan semen kemudian dinilai untuk mengetahui kepekatan optimum bagi meningkatkan kualiti semen selepas penyejukbekuan. Sistein pada 5 mM memberikan perlindungan yang paling berkesan terhadap kecederaan akibat penyejukbekuan dengan meningkatkan kualiti semen selepas dinyahbeku semasa proses penyejukbekuan semen kambing Boer.

Tiga percubaan dilakukan untuk mementukan kesan dari pemisahan plasma seminal menggunakan dua pencair yang berbeza, kesan daripada tiga larutan mencuci yang berbeza dan kesan daripada rejim pengemparan yang berbeza pada cirri-ciri semen kambing Boer sebelum pembekuan dan selepas pencairan. Kajian ini menunjukkan bahawa pemisahan plasma seminal dengan pengemparan pada 3000 x g selama 3 minit dengan larutan mencuci tris glukosa asid sitrik dalam protokol penyejukbekuan semen kambing Boer boleh digunakan untuk meningkatkan keupayaan hidup sperma dalam penyejukbekuan semen kambing Boer. Namun, keputusan ini dibuat

berdasarkan penilaian in vitro. Ujian kesuburan lebih lanjut adalah diperlukan sebagai ujian sebenar terhadap peningkatan penyejukbekuan.

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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 that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

SOE WIN NAING

Date: 11 November 2010

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LIST OF ABBREVIATION

AI	Artificial insemination
ART	Assited reproductive techniques
ATP	Adenosine triphosphate
AV	Artificial vagina
BUSgp60	Bulbourethral gland secretion glycoprotein 60
CASA	Computer-assisted sperm analysis
CAT	Catalase
CFDA	Carboxyfluorescein-Diacetate
DMA	Dimethyl acetamide
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSO	Daily sperm output
DSP	Daily sperm production
EE	Electro-ejaculation
EG	Ethylene glycol
EYCE	Egg yolk coagulating enzyme
FAO	(UN) Food and Agriculture Organization
FC	Flow cytometry
FITC	Fluorescein isothiocyanate
GLUT	Glucose transporter proteins

g/l	Gram per litre
GSH	Glutathione
GSSG	Oxidized glutathione
HCG	Human chorionic gonadotropin
H_2O_2	Hydrogen peroxide
HOS	Hypo osmotic swelling
IU	International unit
IVF	In vitro fertilization
IVM	In vitro maturation
LDL	Low density lipoproteins
LN_2	Liquid nitrogen
LPC	Lysophosphatidylcholine
LPO	Lipid peroxidation
Me_2SO_4	Dimethyl sulfoxide
mL	Millilitre
mM	Millimole
NS	Normal saline
OPU	Ovum pick-up
PBS	Phosphate buffer saline
PC	phosphatidylcholine
PI	Propidium Iodide
PMSG	Pregnant mare serum gonadotropin
PNA	Peanut agglutinin

PSA	Pisum sativum agglutinin
ROS	Reactive oxygen species
TCG	Tris citric acid glucose
TGC	Tris glucose citric acid buffer
Tris	Tris (hydroxymethyl) aminomethane
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

Cryopreservation is a technique of preserving or stabilizing cells at cryogenic temperature usually referred to liquid nitrogen temperature of - 196°C. At this temperature, living cells are inactive metabolically and can be stored for a conceivably infinite period of time. Moreover, there is no formation of ice crystals and the rates of other biophysical process are too slow to affect cell survival. Therefore, cryopreservation in liquid nitrogen has subsequently become established as the standard medium for long term preservation of semen and, over the 40 years for which it has been practised, has maintained sperm fertility unscathed (Parkinson, 2008).

Sperm cryopreservation contributes to the expansion of assisted reproductive techniques (ART), such as artificial insemination (AI) and in vitro fertilization (IVF) which are important in genetic research and in the production of transgenic animals (Medeiros *et al.*, 2002). Semen cryopreservation is used for genetic improvement of domestic species to preserve rare breeds and in international germplasm exchanges. For efficient use of genetic selection schemes and improvement of goat production, AI with frozen semen is essential in goat breeding, especially in intensive systems of production to control reproduction and, in conjunction with accurate progeny testing. AI allows rapid and widespread diffusion of improved genotypes and the

exchange of genotypes to improve the production of milk, hair and meat without transmitting diseases (Parkinson, 2008). Boer goats have been known in many years as docile and fast maturing meat goats. Therefore, Boer goats have been promoted in a very professional way in several countries of the world (Coleby, 2002). In order to improve Boer goat production, the application of AI with the use of cryopreserved semen is relevant. Nevertheless, the success of an AI program depends on the proper management of semen collection, cryopreservation process and use (Barbas and Mascarenhas, 2009).

Cryopreservation of semen reduces the motility and disrupts the membrane integrity of spermatozoa. It is generally assumed that cryopreservation processes are detrimental and are associated with the loss of fertilising capacity. Many researchers have established different methods for processing, storage and insemination of spermatozoa. However, fertility is generally lower with cryopreserved semen than fresh semen after cervical insemination. One of the main causes of low fertility may be due to the components of spermatozoa especially in sperm plasma membrane (Maxwell and Watson, 1996). Therefore, it was suggested that the membranes of the motile spermatozoa are destabilised to the point where they may not survive further ageing in the female tract after cervical insemination while many spermatozoa remain motile after cryopreservation. The changes of sperm membrane during cryopreservation process are similar to the capacitation

and acrosome reaction of spermatozoa (Medeiros *et al.*, 2002). It is thus required to reduce the capacitation time in the female reproductive tract. On the other hand, it is possible to prevent or reverse some of these sperm membrane changes by using the proper methods of cryopreservation processes and cryopreservation diluents to improve fertility following insemination of cryopreserved semen. Therefore, it is important to understand an intricate knowledge of sperm physiology and seminal plasma as well as semen crypreservation media, cryoprotectants, anti-oxidants, semen dilution, cooling, freezing and thawing specific to the caprine species.

Cryopreservation consists of equilibration of spermatozoa in freezing solution, freezing, thawing, and diluting processes. Cryoinjury to spermatozoa could occur during all four processes. To maximize post-thawed recovery of spermatozoa, the single most important principle of semen cryopreservation is the removal of most of the water from spermatozoa before they freeze intracellularly. The intracellular ice crystal formation causes severe sperm damage, when such dehydration does not occur. Removal of too much water from spermatozoa is also detrimental (Barbas and Mascarenhas, 2009). Therefore, spermatozoa damages resulting from intracellular ice formation and the concentration of the solutes during the process are considered most important factors causing freezing injury. Attention should be focused greatly on the principle of moving water across sperm membranes, both to dehydrate spermatozoa prior to freezing and to

rehydrate them during dilution after thawing. In order to decrease spermatozoa damage and to maintain sperm survival during cryopreservation process, spermatozoa need to be extended in a diluent that contains not only substances that protect them against cold shock, but also cryoprotectants that protect them from the deleterious consequences of freezing (Parkinson, 2008). The use of cryoprotectant agent is very important to avoid intracellular ice formation because the permeable cryoprotectants have the ability to restrict the solution effect (Medeiros *et al.*, 2002). Therefore, the use of proper diluents, sperm dilution rate, cooling rate and thawing rate influence cryopreserved sperm survival (Purdy, 2006).

The use of assisted reproductive techniques (ART) has become increasingly widespread in livestock industry. The acceptability of artificial insemination, embryo transfer, and cryopreservation of sperm has lead to establishment of breeding organization, semen banks, ART courses, and sire stations. This is especially true in the dairy cattle industry, where semen technological advancements have been the most successful (Vishwanath and Shannon, 2000). However, the resulting quality of cryopreservation in many species is not remotely similar to that of the initial specimen collected. It is noteworthy that cryopreservation of goat semen differs from that of the other species, such as bull, boar, or ram. This is because the seminal plasma of goat contains an enzyme originating from the bulbourethral gland secretion and a protein fraction. It interacts with egg yolk resulting in coagulation of egg

yolk media and hydrolyses lecithin to fatty acids and spermicidal lysolecithins (Leboeuf *et al.*, 2000). Spermatozoa are particularly sensitive to osmotic changes and thermal changes leading to lipid reorganisation, oxidative stress and ice crystal damage (Barbas and Mascarenhas, 2009). Therefore, artificial insemination with the use of cryopreserved semen is limited by low fertility rates in goats. The cryopreserved semen are associated with a reduction in sperm motility, viability and fertilizing capacity (Evans and Maxwell, 1987; Holt, 1997; Atessahin *et al.*, 2008). In order to achieve the successful use of cryopreserved goat semen for the improvement in efficiency and productivity of goat, alternative semen cryopreservation media and exploitation of methodologies must be investigated further.

To accomplish this goal, this study was carried out to determine the effects of different sugars, amino acids, cryoprotectants, glycerolization procedures, cooling times, washing solutions, centrifugation regimes and removal of seminal plasma on goat semen quality following cryopreservation process.

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