UNIVERSITI PUTRA MALAYSIA

EFFECTS OF BUTYLATED HYDROXYTOLUENE ON PROTEIN P25b, CHILLED AND CRYOPRESERVED BULL SEMEN IN BIOXCELL®, TRIS- AND CITRATE-EGG YOLK EXTENDERS

KHUMRAN ARMIYA'U MADA

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By

KHUMRAN ARMIYA'U MADA

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

November 2016

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Dedicated to my beloved parents; Armiya'u Mada, Aishatu Armiya'u, my parents in-law; Muhammad Inuwa, Safiya Muhammad, my wife Sadiya Muhammad and my children; Fatima, Asma'u and Abubakar.

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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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KHUMRAN ARMIYA'U MADA

November 2016

Chairman : Nurhusien Yimer Degu, PhD
Faculty : Veterinary Medicine

Semen quality drops after cryopreservation, which is caused by high oxidative stress exerts on the sperm during freezing and thawing procedures. The consequenceof this effect is that the protein composition of the spermatozoa especially P25b is adversely affected, thereby hindering the protein's role in fertilization. The sperm surface P25b is considered a bull fertility marker, very important in zona pellucida binding during fertilization. Thus, the fertility of spermatozoa may be preserved if P25b is protected. The aim of this study is to investigate the effects of various concentrations of antioxidant butylated hydroxytoluene (BHT) in three semen extenders with the view to preserving the spermatozoa quality and protecting the P25b from damage during chilling as well as cryopreservation. Four bulls were initially selected and their ejaculates were assessedfor color, volume, concentration, pH, general and progressive motilities, morphologically normal spermatozoa, acrosome and DNA damage, and lipid peroxidation. Transmission electron microscopy (TEM) was also performed to evaluate the ultrastructures of the spermatozoa.The assessment revealedthat the semen color varied from creamy-white in bull #1, to milky white in bulls #2 and 4, then cloudy in bull #3. Highest sperm concentration, lipid peroxidation and pH were recorded from bull #4. Highest volume, progressive motility, morphology, less acrosome damage and viability were from bull #2. While best values for general motility and DNA damage were obtained from bull #1. TEM revealed 92.5, 90.0 and 82.5 percent of intact heads for bulls #1, 2 and 3, respectively, significantly higher than bull # 4 (62.5%). In addition, TEM also showed 32.5, 25.0, and 37.5% of total defective spermatozoa in the respective bulls #1, 2 and 3, significantly lower than 80.0% in bull # 4. Bulls #1, 2 and 3 were therefore consistently satisfactory in most parameters evaluated and hence considered havinghigh semen freezability potential. On the other hand, bull #4 expressed higher ($p < 0.05$) sperm concentration but yet unsatisfactory in most other parameters assessed, including the low live/dead ratio and high percentage of abnormalities, manifesting poor potential of freezability.The three bulls with high semen freezability potential were then usedfor the study. Semen samples were collected by electroejaculation, diluted with Bioxcell®, tris- or citrate- egg yolk

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extenders supplemented with 0.0 (control), 0.5, 1.0, 1.5, 2.0 and 3.0 mM/mL concentrations of BHT. Thereafter, the dilutedsamples were either chilled at 4^{o}C for three days or frozen at -196^{o}C in liquid Nitrogen for 14 days. At the end of storage time, four straws were randomly pooled from each group and evaluated. Results showed that addition of BHT at 0.5 mM/mL in Bioxcell®, 1.0 mM/mL in tris- and 1.0 to 1.5 mM/mL in citrate egg yolk extenders have improved ($p<0.05$) spermatozoa morphology, viability and protection against acrosomal damage when compared with the control after chilling. After cryopreservation, general motility, progressive motility, morphology, protection against acrosomal damage, DNA damage and lipid peroxidation (LP) of the spermatozoa were significantly improved compared with the control at the same BHT concentrations as in the chilledsamples for the various extenders. The appropriate BHT concentration in each extender as demonstrated in the chilling and freezing were also consistent in the protection of P25b ($p<0.05$) than in the control after SDS-PAGE gel densitometry. The spermatozoa quality parameters assessed in both the chilling and freezing samples deteriorated ($p<0.05$) against control at higher BHT concentrations of 2.0 and 3.0 mM/mL in all the extenders. However, the deterioration of the concentration of P25b at 2.0 and 3.0 mM/mL BHT were the same as the control in all the extenders. The outcome of this study strongly suggests a better bull sperm fertility potential after BHT supplementationof 0.5 mM/mL in Bioxcell®, 1.0 mM/mL in tris- egg yolk and 1.0 to 1.5 mM/mL in citrate- egg yolk extenders.

Key words: butylated hydroxytoluene, chilling, cryopreservation, extender, P25b, semen.

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KESAN HIDROKSITOLUEN BERBUTIL KE ATAS PROTEIN P25b,SEMEN LEMBU JANTAN DINGIN DAN KRIOAWET DALAM BIOXSEL®, TRIS- DAN SITRAT –PELUAS KUNING TELUR

Oleh

KHUMRAN ARMIYA'U MADA

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Kualiti semen menurun selepas pengawetankrio disebabkan oleh tekanan oksidatif tinggi yang dikenakan ke atas sperma ketika prosedur penyejukbekuan dan pencairan. Akibatnya, komposisi protein spermatozoa terutama P25b teruk terjejas, oleh itu menghalang peranan protein dalam fertilisasi. Permukaan sperma P25b yang dianggap sebagai penanda fertilitilembu jantan, sangat penting dalam zona pengikatan pelusida ketika fertilisasi. Oleh sebab itu, fertiliti spermatozoa mungkin diawet sekiranya P25b dilindungi. Tujuan kajian ini adalah untuk menyelidiki kesan pelbagai konsentrasi antioxidan hidroksitoluen berbutil (BHT) dalam tiga pengekal semen dengan pandangan untuk mengawet kualiti spermatozoa dan melindiungi P25b daripada rosak ketika pendinginan juga ketika pengawetankrio. Empat lembu jantan pada awalnya telah dipilih dan ejakulasi lembu tersebut telah dinilai untuk warna, isi padu, konsentrasi, pH, motiliti umum dan progresif, spermatozoa normal secara morfologi, akrosom dan kerosakan DNA, dan pemperoksidaan lipid.Mikroskopi elektron transmisi (TEM) juga telah dijalankan bagi menilai ultrastruktur spermatozoa. Penilaian tersebut menunjukkan bahawa warna semen bervariasi dari putih kuning bagilembu jantan #1, kepada putih susu bagilembu jantan #2 and 4, kemudian warna keruhbagi lembu jantan #3. Konsentrasi sperma tertinggi, pemperoksidaan lipid dan pH telah direkodkan daripada lembu jantan #4. Isi padu tertinggi, motiliti progresif, morfologi, kerosakan akrosom yang kurang dan viabilitiadalah dari lembu jantan #2. Manakala nilai yang baik dari motiliti umum dan kerosakan DNA telah diperoleh dari lembu jantan #1. TEM menunjukkan 92.5, 90.0 dan 82.5 peratus kepala utuh bagi lembu jantan #1, 2 dan 3, masing-masing,adalah lebih tinggi secara signifikan dari lembu jantan bull # 4 (62.5%). Di samping itu, TEM juga menunjukkan 32.5, 25.0, dan 37.5% daripada keseluruhan spermatozoa yang rosak bagi lembu jantan #1, 2 dan 3 tersebut, 80.0% lebih rendah secara signifikan bagi lembu jantan # 4. Lembu jantan #1, 2 dan 3 oleh itu adalah memuaskan secara konsisten dalam kebanyakan parameter yang dinilai dan oleh sebab itu dianggap mempunyai potensi kebolehsejukbekuan semen yang tinggi. Sebaliknya, lembu jantan #4 memperlihatkan konsentrasi sperma yang lebih tinggi ($p < 0.05$) tetapi masih tidak memuaskan dalam kebanyakan parameter lain yang

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dinilai, termasuk jangka hayat hidup/ratio kematian dan peratusan abnormaliti yang tinggi, manifestasi potensi kebolehsejukbekuan yang lemah.Ketiga-tiga lembu jantan dengan potensi kebolehsejukbekuan semen yang tinggi kemudiannya telah digunakan dalam kajian ini. Sampel semen telah dikumpulkan melalui elektroejakulasi, dicairkandengan Bioxsel®, tris- atausitrat- pengekal kuning telur yang disuplemenkan dengan 0.0 (kawalan), 0.5, 1.0, 1.5, 2.0 dan 3.0 mM/mLkonsentrasi BHT. Kemudian, sampel yang disimpan sama ada didinginkan pada 4^{o}Cuntuk tiga hari atau disejukbekukan pada -196^{o}Cdalam cecair Nitrogenuntuk 14 hari. Pada penghujung masa penyimpanan, empat straw telah diambil secara rawak daripada setiap kumpulan dan dinilai. Dapatan menunjukkan bahawa penambahan BHT pada 0.5 mM/mLdalam Bioxsel®, 1.0 mM/mLdalam tris- dan 1.0 to 1.5 mM/mLdalam sitrat pengekal kuning telurtelah menambah baik morfologi spermatozoa ($p<0.05$), viabilitidan perlindungan daripada kerosakan akrosomal apabila dibandingkan dengan kawalan selepas dibekukan.. Selepas pengawetankrio, motiliti umum, motiliti progresif, morfologi, perlindungan daripada kerosakan akrosomal ,kerosakan DNA danpemperoksidaan lipid (LP)bagi spermatozoa adalah memuaskan secara signifikan berbanding dengan kawalan pada konsentrasi BHT yang sama seperti sampel yang disejukbekukan bagi pelbagai pengekal.Konsentrasi BHT yang sesuai dalam setiap pengekal sebagaimana yang didemonstrasikan dalam pendinginan dan penyejukbekuan juga didapati konsisten dalam perlindungan P25b ($p<0.05$) daripada kawalan selepas densitomerti gel SDS-PAGE. Parameter kualiti spermatozoa yang dinilai dalam kedua-dua sampel yang didinginkan dan disejukbekukan merosot ($p<0.05$) daripada kawalan pada konsentrasi BHT yang tinggi, iaitu 2.0 dan 3.0 mM/mL dalam semua pengekal. Walau bagiamanapun, kemerosotan konsentrasi P25b pada 2.0 dan 3.0 mM/mL BHT adalah sama seperti kawalan dalam semua pengekal. Dapatan kajian ini mencadangkan sekuat-kuatnya potensi fertiliti lembu jantan yang lebih baik selepassuplementasi BHT 0.5 mM/mLdalam Bioxsel®, 1.0 mM/mLdalam tris-kuning telur danantara 1.0 hingga 1.5 mM/mLdalam sitrat- pengekal kuning telur.

Kata kunci:hidroksitoluen berbutil, pendinginan, pengawetankrio, pengekal, P25b, semen.

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This thesis was submitted to the Senate of 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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TABLE OF CONTENTS

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LIST OF TABLES

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LIST OF FIGURES

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LIST OF ABBREVIATIONS

Ab	Antibody
AI	Artificial Insemination
ART	Assisted Reproductive Technology
ATP	Adenosine Triphosphate
AV	Artificial Vagina
BHT	Butylated Hydroxytoluene
BSA	Bovine Serum Albumin
BSE	Breeding Soundness Examination
BSP	Binding Sperm Proteins
CASA	Computer Assisted Semen Analyzer
CBB	Coomasie Brilliant Blue
CEY	Citrate-Egg Yolk
CIR	Cosmetic Ingredient Review
ddH_2O	Double Distilled Water
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic Acid
D-PBS	Dulbecco's Phosphate Buffer Saline
EDTA	Ethylenediaminetetraacetic acid
EE	Elactro Ejaculator
EtBr	Ethidium bromide
FDA	Food and Drug Administration
GAG	Glycosaminoglycan
GPX	Glutathione Peroxidase
HRP	Horseradish Peroxidase
kDa	Kilo Dalton

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LP	Lipid Peroxidation
MDA	Malondialdehyde
MW	Molecular Weight
NCI	National Cancer Institute
PBS	Phosphate Buffer Saline
PHE	Penicillamine Hypotaurine Epinephrine
PS	Ponceau-S
ROS	Reactive Oxygen Species
SC	Service per Conception
SCGE	Single Cell Gel Electrophoresis
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate- Polyacramine Gel Electrophoresis
SOD	Superoxide Dismutase
SP	Seminal Plasma
Spp	Species
SP-TL	Sperm-TALP
TALP	Tyrode's Albumin Lactate and Pyruvate
TBARS	Thiobarbituric Acid-Reactive Substances
TBS	Tris-Buffer Saline
TBS-T	Tris-Buffered Saline and Tween 20
TEM	Transmission Electron Microscopy
TEY	Tris-Egg Yolk
TPU	Taman Pertanian Universiti
UPM	Universiti Putra Malaysia
WB	Western Blotting

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CHAPTER 1

INTRODUCTION

Artificial insemination (AI) is an effective biotechnological tool for genetic improvement in cattle. Since its inception in Malaysia in 1963 (Musa, 1974), it has had a tremendous effect on both dairy and beef industries, producing cross breeds with higher productivity than the local breeds, reasonably acclimatized to the tropical environment and sufficiently better disease resistant compared to the pure temperate breeds (Raymond and Saifullizam, 2010). Nevertheless, when compared to the AI coverage in other developed countries of the world, whichhas risen to 64% in dairy cattle and 7.8% in beef cattleas reported by Chupin and Schuh(1993). The AI coverage in most Asian countries including Malaysia stands at a very low (1-5%)level. There has been an increase in the trend of AI services in Malaysia from 2000 to 2009 (Azizah et al., 2014)although the proportion of the AI coverage remains similar (4.2 – 4.3%) due to an increase in the number of breeder females. In order to rapidly transform the genetic composition of the cattle population in Malaysia with higher impact in the industry, the breeding service needs to be heightened by increasing the existing AI coverage to 10% per annum (Raymond and Saifullizam, 2010). This can be achieved by effective delivery of AI services, increasing the number of bull stations, semen storage facilities, AI centers and technicians. However, increasing the AI coverage alone would not bring the desired change in the cattle industry unless the frozen-thawed semen used for AI is of high quality that subsequently determines the number of services per conception (SC) and pregnancy success. Several factors can influence the quality or fertility of frozen-thawed sperm. These include cryopreservation/freezing and thawing processes, type of semen extender, and seminal plasma components such as sugars, antioxidantsand free radicals. Cryopreservation procedures affect many aspects of sperm physiology and morphology with most damages occurring during freezing and thawing. These steps have major consequences on the fertilizing ability. Generally, viability is decreased by 50% whereas fertilizing capacity is affected by seven folds(Lessardet al., 2000). Subtle sperm damages such as injuries to sperm surface proteins can also be induced by cryopreservation (Lasso et al., 1994). The deterioration in the quality and fertilizing ability of fresh semen due to cryopreservation can be minimized by using cryoprotectants such as glycerol, adding antioxidants and nutrients into best-suited extenders.

Looking for the best extender for semen cryopreservation for improved fertility outcome in AI has been the focus of many researchers in the last decade. However, findings on types of extenders and their effects on cryopreservation of sperm remained a debate and hence research has continued through several modifications. Comparison among different semen extenders is usually done by conventional andrological tests such as assessment of sperm motility, viability and morphology before and after cryopreservation (Asadpour and Tayefi-Nasrabadi, 2010; Ijazet al., 2009). These may not fully explain the quality and fertilizing capacity of the spermatozoa. This is because, although the spermatozoa are morphologically normal andare capable of propelling to reach the site of fertilization, damage to factors such

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as sperm surface proteins that play a key role in the process of interaction with the oocytewill adversely affect thesuccess of fertilization. Therefore, comparison among different semen extenders based on the level of sperm surface protein that is known to have effect on fertility, apparently gives a more detailed and better explanation on the quality of the spermatozoa after cryopreservation. Hence, the use of sperm surface proteins as a parameter provides a more detailed means of evaluation to identify the most suitable extender for sperm cryopreservation or the one that provides the highest protection to the spermatozoa against cryopreservation injuries.

As mentioned earlier, several different types of semen extenders have been evaluated byresearchers for bull semen cryopreservation based on conventional andrological tests. Among the most commonly tested extenders include egg yolk-based Citrate and Tris extenders(Leite et al., 2010). Studies are rare on comparison of extenders based on theirlevel of protection to sperm surface proteins from damage during cryopreservation. Different proteins have been identified and proposed to be involved in the steps leading to fertilization (McLeskey et al., 1997). Among the important sperm surface proteins proven to be related to fertility index of bull spermatozoa used for AI include, P25b sperm surface protein.The loss of P25b during cryopreservation is sufficient to decrease frozen-thawed semen fertilizing ability, a decrease that cannot be evaluated by conventional andrological tests (Lessardet al., 2000).

The quality of post-thawed sperm is affected by oxidants like reactive oxygen species (ROS) which are produced physiologically in living cells during respiration as well as by abnormal or dead sperm and phagocytic cells of both the ejaculate and the female reproductive tract (Asadpour and Tayefi-Nasrabadi, 2010; Ijaz et al., 2009). These ROS can inhibit sperm motility, capacitation and acrosome reaction mediated by lipid peroxidation of sperm membrane. Lipid peroxidation has been correlated with exposure of spermatozoa to ROS and it has been demonstrated that spermatozoa undergoing freeze-thaw cycles produced ROS (Alvarez and Storey, 1992). In mammals, seminal plasma contains a number of antioxidants which include superoxide dismutase (SOD), catalase, glutathione peroxidase (GPX), free radical scavengers such as vitamins C and E, hypotaurine, taurine and albumin (Ijaz et al., 2009). The presence of these antioxidants in semen helps to counteract the oxidants and protect the spermatozoa from damage. Semen dilution using extenders for the purpose of having more doses from a single ejaculate and cryopreservation however decreases the concentrations of natural components of antioxidants. The decrease in the components of antioxidants due to dilution coupled with an increase in production of ROS during cryopreservation exacerbates the condition of spermatozoa and further degrades its post-thaw quality and fertilizing ability. To minimize the effect of oxidants in diluted semen, researchers have tested the impact of adding antioxidants into extenders in many species of animals including cattle and have observed improvement in the quality of post-thawedspermatozoa compared to control based on conventional andrological tests (Beconiet al., 1991).

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Addition of natural antioxidant like alphatocopherol and ascorbate, have been reported to have protective effect on metabolic and cellular viability of cryopreserved bovine spermatozoa (Beconi et al., 1991). More recently, butylated hydroxytoluene (BHT) has been tested for its antioxidant effect oncattle(Asadpour and Tayefi-Nasrabadi, 2010) and buffalo bull spermatozoa (Ijaz et al., 2009). These studies investigated the impact of adding BHT into semen extenders on post-thaw Holstein bull semen and Nili-Ravi buffalo semen quality based on conventional andrological tests such as motility and viability. Results of their study indicated improvement of semen quality following cryopreservation compared to untreated controls. However, Ijaz et al. (2009) worked on buffalo semen, while Asadpour and Tayefi-Nasrabadi, (2010) evaluated effect of BHT only on motility and viability of Holstein semen. The present study intends to extensively assess BHT effects on motility, viability, morphology, acrosome damage and DNA damage. Furthermore, effective optimum BHT level have not been agreed upon and none of the above mentioned studies either assessed the impact of adding BHT on the protective level of sperm surface proteins P25b or its effects in lecithin (non-egg yolk) based semen extender, which is included in this project. Butylated hydroxytoluene is a synthetic analogue of Vitamin E that checks the auto-oxidation reaction which is lipid soluble and has been successfully tested to preserve liquid semen in other species of animals such as turkey, to minimize cold shock damage in ram, boar and goat spermatozoa (Ijaz et al., 2009). Investigation on the protective level of P25b surface proteins using different extenders with or without addition of antioxidants like BHT would provide a detailed explanation on the better semen extender and antioxidant combination best suited for cryopreservation. This study would also generate information and new knowledge as to the mechanism of protection of using the different extenders and addition of antioxidant.

Therefore the objectives of this study were:

1. To evaluate the physical and ultrastructural attributes offresh bull's semen parameters
2. To evaluate the effects of BHT combined with different semen extenders on chilled bull semen stored for3 days.
3. To evaluate the effects of BHTcombined with different semen extenders on frozen-thawed bull semenstored for 14 days.
4. To compare the effects of different semen extenders treated with different concentrations of BHT on bull sperm surface protein P25b.

Hypothesis of the study

$\mathbf{H_o}$= Treatment of semen extenders with low levels of BHTconcentrationwill not exert any positive effect onsurface protein P25b, chilled andpost thawedbull semen.

$\mathbf{H_a}$ = Treatment of semen extenders with low BHTconcentration will improve surface protein P25b, chilled andpost thawedbull semen.

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