RELATIONSHIP OF SPERM PROTAMINE CONTENT WITH BULL BREEDING SOUNDNESS EVALUATION AND SPERM DNA DAMAGE

By

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Chairman : Associate Professor Nurhusien Yimer Degu, PhD
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Protamine is a major nuclear component in mature spermatozoa known to be responsible for paternal genome protection through its roles in sperm chromatin condensation and DNA stability. Bull breeding soundness evaluation (BBSE) is the common method used to predict male fertility. This study aimed to assess the sperm protamine content using three different techniques and determine its correlation with BBSE and traditional semen quality parameters. Also to examine the role of sperm protamine in sperm DNA stability and resistance to cryo-damage. Five Brangus bulls from the University Putra Malaysia farm, between 3 – 8 years old and body weights ranging from 308 – 568 kg, were subjected to BBSE. A total of 35 semen samples, seven from each bull were collected using electro-ejaculation (EE) method. Protamine content was analysed using aniline blue (AB) test, chromomycin A3 stain and fluorescent microscope (CMA3-FLM), CMA3 and flow cytometry (CMA3-FCM). DNA damage was evaluated using acridine orange (AO) staining technique, and DNA fragmentation (SDF) analysed by FCM. The result of BBSE revealed that two of the five bulls examined were unsatisfactory due to low normal sperm percentages (< 70%). The same unsatisfactory bulls showed the highest level of protamine deficiency. One way ANOVA showed significant differences ($P < 0.05$) among the bulls in the means of protamine deficiency. A post hoc comparison disclosed that the mean values of bull B514 for protamine deficiency were significantly higher than the other bulls ($P < 0.05$). Negative significant relationship ($P < 0.05$) was observed between protamine deficiency and the percentages of normal sperm morphology (NS) and progressive motility (PM). Both AB and CMA3-FLM tests correlated significantly ($P < 0.01$) with CMA3-FCM (standard gold), but AB test exhibited the higher correlation. Therefore, AB test was used to investigate the relationship between sperm protamine deficiency and sperm morphological abnormalities. A significant ($P < 0.01$) positive correlation was found between AB positivity and proximal cytoplasmic droplets (PD), tail and head abnormalities (T/H), pyriform heads (Py), knobbed acrosome (KA), vacuoles and teratoid (V/T). No significant correlation was found between AB positivity and mid piece (MP)

or swollen acrosome (SA). Sperm protamine deficiency was significantly ($P < 0.01$) correlated with SDF. Sperm protamine deficiency assessed by CMA3-FCM showed the highest predictive value for SDF followed by AB test, while CMA3-FLM exhibited the lower predictive value. The effect of protamine content (CMA3-FLM) on sperm DNA integrity (AO staining) after thawing in three different times (0, 2, 4 hour) of incubation at 37°C was investigated. The results of sperm DNA damage were differed significantly across the three time points, affected by the sperm protamine content for each bull's group. Post hoc pairwise comparison showed an increased DNA damage in bulls with lower protamine values. The findings of this study concluded that sperm protamine can be used as a useful biomarker to predict semen quality and male potential fertility. Hence, it will be of benefit if included as additional parameter of the BBSE.

Keywords: Protamine content, Sperm DNA damage, Flow cytometry, Bull breeding soundness evaluation, Aniline blue.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains Veterinar

HUBUNGAN KANDUNGAN PROTAMIN SPERMA DENGAN PENILAIAN KESEMPURNAAN PEMBIAKAN LEMBU JANTAN DAN KEROSAKAN DNA SPERMA

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Protamina merupakan komponen nukleus utama dalam spermatozoa matang yang diketahui bertanggungjawab untuk perlindungan genom paternal melalui peranannya dalam kondensasi kromatin sperma dan stabiliti DNA. Penilaian kesempurnaan pembiakan lembu jantan (BBSE) merupakan kaedah biasa yang digunakan untuk meramal fertiliti jantan. Kajian ini bertujuan untuk menilai kandungan protamina sperma menggunakan tiga teknik yang berbeza dan untuk menentukan korelasinya dengan BBSE dan parameter kualiti semen tradisional. Di samping itu, untuk meneliti peranan protamina sperma dalam stabiliti DNA sperma dan ketahanan pada kerosakan krio. Lima lembu jantan Brangus dari ladang Universiti Putra Malaysia, berusia antara 3 – 8 tahun dan berat badan antara 308 hingga 568 kg, telah menjalani BBSE. Sebanyak 35 sampel semen, tujuh daripada setiap lembu jantan telah dikumpul menggunakan kaedah ejakulasi elektro (EE). Kandungan protamina telah dianalisis menggunakan ujian anilina biru (AB), pewarna kromomisin A3 dan mikroskop berpendarfluor (CMA3-FLM), CMA3 dan sitometri aliran (CMA3-FCM). Kerosakan DNA telah dinilai menggunakan teknik pewarnaan akridina jingga (AO), dan fragmentasi DNA (SDF) yang dianalisis oleh FCM. Dapatan BBSE memperlihatkan bahawa dua daripada lima lembu jantan yang diteliti adalah tidak memuaskan disebabkan peratusan sperma normal yang rendah (< 70%). Lembu jantan yang sama menunjukkan tahap kekurangan protamina yang tertinggi. ANOVA sehala menunjukkan perbezaan yang signifikan ($P < 0.05$) dalam kalangan lembu jantan dari segi min kekurangan protamina. Perbandingan pascahoc memperlihatkan bahawa nilai min lembu jantan B514 untuk kekurangan protamina secara signifikan adalah lebih tinggi daripada jembu jantan lain ($P < 0.05$). Hubungan signifikan yang negatif ($P < 0.05$) telah dikesan antara kekurangan protamina dan peratusan morfologi sperma normal (NS) dan kemotilitan progresif (PM). Kedua-dua ujian AB dan CMA3-FLM secara signifikan adalah berkorelasi ($P < 0.01$) dengan CMA3-FCM (piawai emas), tetapi ujian AB memperlihatkan korelasi yang lebih tinggi. Oleh sebab itu, ujian

AB telah digunakan untuk menyelidiki hubungan antara kekurangan protamina sperma dan keabnormalan morfologi sperma. Korelasi positif yang signifikan ($P < 0.01$) telah diperoleh antara positiviti AB dan titisan sitoplasmik proksimal (PD), keabnormalan ekor dan kepala (T/H), kepala piriform (Py), akrosom tombol (KA), vakuol dan teratoid (V/T). Tidak terdapat korelasi yang signifikan antara positiviti AB dan bahagian tengah (MP) atau akrosom bengkak (SA). Kekurangan protamina sperma secara signifikan berkorelasi ($P < 0.01$) dengan SDF. Kekurangan protamina sperma yang telah dinilai oleh CMA3-FCM menunjukkan nilai prediktif yang lebih tinggi bagi SDF diikuti oleh ujian AB, manakala CMA3-FLM memperlihatkan nilai prediktif yang lebih rendah. Kesan kandungan protamina (CMA3-FLM) ke atas integriti DNA sperma (Pewarnaan AO) selepas pencairan dalam tiga masa inbukasi yang berbeza (0, 2, 4 jam) pada 37°C telah diselidiki. Dapatan kajian bagi kerosakan DNA sperma secara signifikan adalah berbeza merentasi tiga titik masa, terjejas oleh kandungan protamina sperma bagi setiap kumpulan lembu jantan. Perbandingan berpasangan pascahoc menunjukkan peningkatan kerosakan DNA pada lembu jantan dengan nilai protamina yang lebih rendah. Dapatan kajian ini menyimpulkan bahawa protamina sperma dapat digunakan sebagai penandabio bagi meramal kualiti semen dan fertiliti potensi jantan. Oleh itu, dapatan kajian ini adalah bermanfaat sekiranya diambil kira sebagai parameter tambahan BBSE.

Kata kunci: Kandungan protamina, Kerosakan DNA sperma, Aliran sitometri, Penilaian kesempurnaan lembu jantan, Anilina biru

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Declaration by Members of the Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

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LIST OF ABBREVIATIONS

%	Percentage
AB	Aniline blue
ADCY10	Adenylyl cyclase 10
AI	Artificial insemination
AO	Acridine orange
ART	Assisted reproductive techniques
ATP	Adenosine triphosphate
BBSE	Bull breeding soundness evaluation
CASA	Computer assisted sperm analysis
CMA3	Chromomycin A 3
DTT	Dithiothreitol
EE	Electroejaculation
FCM	Flow cytometry
FLM	Fluorescent microscope
H2B	Histone 2 B
HSD	Honestly significant difference
ICSI	Intra cytoplasmic sperm injection
IZUMO1	Izumo sperm-egg fusion protein 1
KA	Knobbed acrosome
KK	Kedah Kelantan
LC-MS/MS	Liquid chromatography mass spectrometry
MP	Mid piece
NS	Normal sperm
PBS	Phosphate buffer saline

PD	Proximal droplets
PKC	Palm kernel cake
PM	Progressive motility
Prt1	Protamine 1
Prt2	Protamine 2
Prt3	Protamine 3
Prt4	Protamine 4
Py	Pyriform heads
RSA	Recurrent spontaneous abortion
RT-PCR	Real time polymerase chain reaction
SA	Swollen acrosome
SC	Scrotal circumference
SCD	Sperm chromatin dispersion
SCSA	Sperm chromatin structure assay
SDF	Sperm DNA fragmentation
SDS	Sodium dodecyl sulphate
Semi-QRT-PCR	Semi quantitative real time polymerase chain reaction
SFT	Society for Theriogenology
SIT	Sample injection tube
SPACA1	sperm acrosome associated 1
SPDA	Sperm protamine deficiency assay
T/H	Abnormal tails and detached heads
TB	Toluidine blue
TNE	A buffer composed of Tris-HCL, sodium chloride (NaCl), and ethylenediamine tetraacetic acid (EDTA)

TNP2	Transition protein 2
UPM	Universiti Putra Malaysia
V/T	Vacuoles and Teratoids
WHO	World health organization

CHAPTER 1

INTRODUCTION

1.1 Overview

In the recent decades, the demand of cattle production was noticeably increased due to the massive increase in human population worldwide. Accordingly, the fulfillment of the market's needs for beef and dairy products becomes a challenge. To achieve this task, considerable attention must be given to enhance the herd's fertility. Bull fertility is an essential factor in cattle reproduction which remarkably affects the productivity. The effect of a single sub-fertile bull on the overall herd fertility is paramount compared to the effect of one cow with fertility troubles. Traditionally, bull fertility potential can be estimated via bull breeding soundness evaluation (BBSE) and routine semen analysis. However, the obtained results may not provide an accurate prediction for male fertility. Additional methods to support the conventional evaluation might be a necessity. Recently, some studies suggest sperm protamine as a promising biomarker due to its relationship with male fertility (Talebi et al., 2016; Dogan et al., 2015; Fortes et al., 2014; Simon et al., 2011).

Protamine is the major protein in the nucleus of mature spermatozoa as it replaces histone during spermatogenesis (Oliva, 2006). It is responsible for chromatin condensation and DNA stability (Balhorn et al., 2000) which are important for the safe and fast delivery of paternal genome to the oocyte for fertilization (Brewer et al., 2002). Protamine is a positively charged and arginine rich residue. This factor enables it to bind strongly with the negative charge of the paternal DNA resulting in highly compact chromatin (Oliva & Dixon, 1990). There are two variants of protamine: protamine 1 (Prt1) the predominant variant in all studied vertebrates, and protamine 2 (Prt2) which is only found in certain species, such as humans and mice (Corzett et al., 2002). Protamine content can be evaluated directly using electrophoresis technique or indirectly following cytochemical methods using stains such as aniline blue (AB) and chromomycin A3 (CMA3) to discriminate between lysine-rich histone (immature spermatozoa) and arginine/ cysteine-rich protamine (mature spermatozoa) (Sellami et al., 2013). CMA3 stain could be used to detect sperm protamine deficiency via fluorescent microscope (FLM) or flow cytometry (FCM), while AB staining is assessed by light microscope.

It was documented that sperm protamine abnormalities are highly related to male infertility (Francis et al., 2014), but its relationship with basic semen quality parameters, particularly sperm morphology, is still uncertain. Although there are few studies which have been conducted in this regard, their findings were inconsistent. Boe-Hansen et al. (2018) and Kipper et al. (2017) from bulls; Akmal et al. (2016) from human; and Cho et al. (2001) from mice confirmed the relationship between sperm protamine deficiency and abnormal sperm

morphology. In the same line, Zandemami et al. (2012) stated that sperm protamine deficiency correlates negatively with normal morphology, motility, and concentration. However, Dehghanpour et al. (2020) found that there was no significant correlation between protamine deficiency and sperm morphology as much as sperm motility. In contrast, some studies found that sperm chromatin condensation is considered as a valuable marker in male fertility evaluation, independently from traditional semen parameters (Salsabili et al., 2006; Hammadeh et al., 2001).

BBSE is an economical, easy, and useful procedure to estimate a bull's potential fertility in order to manage herd operations. A complete BBSE consists of physical/reproductive examination, measurement of scrotal circumference (SC), and semen evaluation. The examined bull must pass the physical examination and exhibit the minimum standards of SC, normal sperm (NS), and progressive motility (PM) to be classified as satisfactory breeder. When serving capacity is involved in the evaluation, one of every four bulls will be classified as unsatisfactory (Barth, 2018). The term unsatisfactory includes degrees of fertility troubles that range from sterile to sub-fertile bulls. While sterile indicates that the bull is completely unable to reproduce, infertile is the bull that is temporarily unable to reproduce, and sub-fertile is the term for bulls with depressed reproductive ability (Barth, 2018). Despite the advantages gained when performing BBSE, the full acceptance by the producers and practitioners remains a challenge. The outcome of BBSE might be affected by several factors, such as environmental effects, the breeds in use, the length of breeding season, reproductive disease, and herd management. This may reduce its reliability and adequacy. Consequently, BBSE can indicate male potential but cannot provide a reliable prediction for male fertility. The addition of a more accurate parameter for BBSE to estimate bull fertility is required, provided that, the desired assay must be easy to conduct, rapid, and economical. Sperm protamine content is a suggested biomarker for male fertility. Several methods have been developed for protamine assessment and among them, the AB staining technique is considered as the cheapest and easiest (Sellami et al., 2013).

1.2 Problem statement

BBSE is an assessment method used widely to provide useful information which can help in predicting potential bull fertility. However, BBSE, particularly, semen evaluation can easily be affected by environmental factors, field condition, and semen handling. Some of these factors are uncontrollable although semen analysis constitutes an essential part in bull's evaluation. Up to date, BBSE didn't include or be related to an objective and advanced test, despite the disclosure and documentation of many molecular markers that linked to male fertility. The addition of an accurate parameter to the BBSE may serve to support its efficiency and reliability. This additional assessment must be accurate, easy to conduct, rapid, and inexpensive. We hypothesized that sperm protamine has a close relationship with BBSE in particular the main parameters of semen evaluation as well as sperm DNA integrity. Several methods were developed for protamine assessment and among them AB is the cheapest and easiest to conduct. This

study suggests sperm protamine assessed by AB staining as a suitable biomarker for bull fertility prediction to be included in BBSE.

1.3 Research hypothesis

Objective 1

1. H_o = Sperm protamine content is not related to BBSE.
2. H_1 = Sperm protamine content is highly related to BBSE.

Objective 2

1. H_o = Sperm protamine content has no significant effect on sperm DNA integrity.
2. H1 = Sperm protamine content has a significant effect on sperm DNA integrity.

Objective 3

1. H_o = The three methods used to assess the sperm protamine content in this study are not correlated.
2. H1 = The three methods used to assess the sperm protamine content in this study are closely correlated.

1.4 Research objectives

1. To investigate the relationship between sperm protamine content (evaluated using three different methods (AB test, CMA3-FLM, CMA3-FCM)) and BBSE parameters.
2. To determine the effect of sperm protamine content on sperm DNA stability after cryopreservation.
3. To evaluate the correlation between the three techniques used to assess sperm protamine content and to compare the efficiency of each method.

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