UNIVERSITI PUTRA MALAYSIA

REPRODUCTIVE EFFICIENCY THROUGH ESTRUS RESYNCHRONIZATION, TIMED ARTIFICIAL INSEMINATION AND SPERMATOZOA SEPARATION IN BEEF CATTLE

ABD MALIK

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REPRODUCTIVE EFFICIENCY THROUGH ESTRUS RESYNCHRONIZATION, TIMED ARTIFICIAL INSEMINATION AND SPERMATOZOA SEPARATION IN BEEF CATTLE

By

ABD MALIK

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

Februari 2012

Dedicated to my beloved Parents (Almarhum my father Munandar and my mother Tasmani), my wife Anis Latifa and my sons Mohammad Azarudin Malik and Masduki zaini Malik for their supplication, patience and understanding

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

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February 2012

Chairman : Professor Abd Wahid Haron, PhD

Faculty : Veterinary Medicine

The objectives of this study were to improve resynchronization of oestrus, timing of artificial insemination and natural mating, verification of X- and Y-chromosomes after separation, and to analyze the motility, membrane integrity and acrosome integrity of bull spermatozoa. In the first experiment, a total 140 Brangus cows were synchronized with CIDR. Thirty to thirty five days after artificial insemination (AI), the cows were checked for pregnancy using ultrasound and those that remained open (non pregnant) were divided into two groups and resynchronized with either CIDR or two injections of $PGF_2\alpha$ further at 11 days interval. All cows were observed visually for estrus response for a period of two hours at 12 h intervals, starting immediately after CIDR removalor after the second injection of $PGF_2\alpha$. Cows were in estrus when they were mounted at least 3 times during the period of observation. Following removal of CIDR and second injection of $PGF_2\alpha$, cows were inseminated at 60 and 70 h later, respectively. There were no significant differences

(P>0.05) in estrus response and pregnancy rate between cows with initial synchronization and resynchronization used CIDR protocol.

In Experiment 2, a total of 185 Brangus cows were randomly divided into two experiments. First, a total of 100 cows were randomly divided into three groups. Groups 1, 2 and 3 were artificially inseminated at 48-50 h (n=30), 53-55 h (n=29) and 58-60 h (n=41) after CIDR removal. The pregnancy rates were 26.6%, 24.1% and 36.6%., respectively. The pregnancy rate was higher in the G1 (36.6%) than the G1 (26.6%) and G2 (24.1%) groups, but not significantdifferent (P>0.05). Second, a total of 85 cows were divided into two groups. Group 1, consisted of 35 cows and further subdivided into seven sub-groups, consisting of 5 cows per group. Group 2 consisted of 50 cows which were artificially inseminated. At 58-60 h after CIDR removal, bulls were mixed in all sub-groups for one week (Group 1) and for Group 2, timed AI were conducted. The everage pregnancy rate in natural mating was 28.6%. The pregnancy rate in the timed AI group was 18.0%. However, the difference in the pregnancy rate in natural mating and timed AI groups were not statistically significant (P>0.05).

In Experiment 3, verification of X- and Y-chromosome bearing spermatozoa separation by nested PCR and the motility and membrane integrity of the bull spermatozoa were evaluated. Semen samples were collected using an electro ejaculator from four Brangus bulls. Spermatozoa separation was conducted using three spermatozoa separation methods; (1) swimming speed using cervical mucus from estrus cows, (2) Percoll discontinuous gradient (45-90%), and (3) swim up using TALP medium. The nested PCR was used to verify the presence of X- and Y- chromosome. The motility and membrane integrity were

also evaluated before and after spermatozoa separation. Primers were designed using amelogenin cDNA sequence with 329 bp and 266 bp X- and Y-bearing chromosome spermatozoa, respectively. Motility was analyzed using computer assist spermatozoa analysis (CASA) system, whereas the membrane integrity was analyzed using hypo-osmotic swelling test (HOST). These results were confirmed by the absence of a single band, either for X- or Y- chromosome. The double band indicating that the spermatozoa cannot be separated was observed, after separation. However, the percentage of X-chromosome spermatozoa in the swimming speed using cervical mucus from estrus cows media (58.33%), swim-up using TALP media (50%), while that in the Percoll gradient method was 44.33%. Statistically both percentage were significantly different ($P<0.001$) compared to the theoretical ratio (50:50). However, comparison of spermatozoa motility, membrane integrity, and concentration before and after separation showed significant differences ($P<0.05$) among them.

In Experiment 4,the effect of extenders on the spermatozoa quality after cryopreservation were studied. Four extenders were used in this study namely cervical mucus collected from cows at estrus, Tris buffer, Sodium citrate and Tyrode's albumin lactate and pyruvate (TALP).All of the extender aliquots were diluted to obtained sperm concentrations of 25 x 10^6 sperm/mL. Computer assisted semen analyses, hypo-osmotic swelling tests and fluorescence isothiocyanate-labelled peanut agglutinin (FITC-PNA) techniques were used to determine sperm motility, plasma membrane integrity and sperm acrosome integrity, respectively, in both fresh and frozen-thawed semen. The motility (74.9 ± 0.60%), membrane integrity (70.86 ± 0.81%) and acrosome integrity (67.31 ± 0.94%) of fresh ejaculates were higher than frozen-thawed semen. Sperm motility (47.93± 0.80%) was significantly higher

(P<0.05) in cervical mucus extender than in the Tris buffer and TALP extenders. Furthermore, the levels of membrane (43.09 ± 0.83%) and acrosome integrity (51.67 ± 0.80%) of post-thawed spermatozoa in the TALP extender were significantly lower (P< 0.05) compared to the other three extenders. In conclusion, the cervical mucus extender resulted in sperm motility, membrane integrity and acrosome integrity comparable to that of the other tested extenders. However, acrosome integrity after thawing was higher than that observed with the TALP extender.

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

KECEKAPAN PEMBIAKAN DENGAN SINKRONISASI SEMULA ESTRUS, WAKTU KAWIN DAN PENGASINGAN SPERMATOZOA DALAM LEMBU

Oleh

ABD MALIK

Februari 2012

Pengerusi : Profesor Abd Wahid Haron, PhD

Fakulti : Perubatan Veterinar

Objektif kajian ini adalah untuk meningkatkan pensinkronian semula estrus, masa permanian beradas atau pembiakan tabii pengesahan kromosom X dan Y, dan juga untuk menganalisis motiliti, kesempurnaan selaput dan kesempurnaan akrosom menggunakan kaedah pemisahan spermatozoa pada lembu . Dalam ujikaji pertama, 140 lembu Brangus disinkroni dengan CIDR. Tiga puluh hinggatiga puluh lima hari selepas permanian beradas (AI), lembu diperiksa untuk menentukan kebuntingan menggunakan ultrabunyi dan lembu yang tidak bunting dibahagikan kepada dua kumpulan dan disinkronisasi semula dengan CIDR atau dua suntikan PGF2α pada selang 11 hari. Semua lembu diperhati untuk menentukan tindakbalas estrus selama dua jam selang12 jam, bermula selepas CIDR dikeluarkanatau selepas suntikan kedua PGF2α. Lembu berada pada estrus apabila dipanjat sekurang-kurangnya 3 kali dalam tempoh pemerhatian. Selepas CIDR dikeluarkan dan suntikan kedua PGF2α, lembu diinseminasimasing-masing pada 60 dan 70 jam kemudian. Tiada perbezaan yang bererti (P> 0.05) dalam tindakbalas estrus dan kadar kebuntingan antara pensinkronian awal dan pensinkronian semula lembu menggunakan CIDR.

Dalam Ujikaji 2, sebanyak 185 lembu Brangus diasingkan secara rawak kepada dua ujikaji. Pertama, sebanyak 100 ekor lembu telah dibahagikan secara rawak kepada tiga kumpulan. Kumpulan 1, 2 dan 3 masing-masing diinseminasi pada 48-50 jam (n = 30), 53-55 jam (n = 29) dan 58-60 jam (n = 41) selepas CIDRdikeluarkan. Kadar kebuntingan adalah 26.6%,

24.1% dan 36.6%. Sungguhpun kadar kebuntingan ini adalah lebih tinggi bagi kumpulan G3 (36.6%) berbanding dengan G2 (24.1%) dan kumpulan G1 (26.6%), namun tiada perbezaan bererti (P>0.05). Kedua, sebanyak 85 ekor lembu telah dibahagikan kepada dua kumpulan.Kumpulan 1 terdiri daripada 35 ekor lembu dibahagikan lagi kepada tujuh sub-kumpulan, terdiri daripada 5 ekor lembu bagi setiap sub-kumpulan.Sementara kumpulan 2 terdiri daripada 50 ekor lembu yang dibiakkan secara AI. Pada 58-60 jam selepas CIDRdikeluarkan, lembu jantan telah dicampur dalam setiap sub-kumpulan selama seminggu (kumpulan 1) dan bagi kumpulan 2 pula, AI telah dijalankan. Purata kadar kebuntingan dalam kumpulan 1 adalah 28.6% dan kumpulan 2 adalah 18.0%. Walau bagaimanapun, perbezaan dalam kadar kebuntingan ini adalah tidak bererti (P> 0.05).

Dalam Ujikaji 3, pengesahanspermatozoa berkromosom X dan Y menggunakan kaedah nested-PCR, penilaian motiliti dan kesempurnaan selaput spermatozoa juga dijalankan dengan menggunakan tiga kaedah pemisahan spermatozoa; (1) kelajuan berenang menggunakan mukus cervik daripada lembu estrus, (2) Percoll kecerunan tak selanjar (45-90%), dan (3) berenang menggunakan media TALP. Nested PCR digunakan untuk mengesahkan kehadiran kromosom X dan Y. Penilaian motiliti dan kesempurnaan selaput juga dinilai sebelum dan selepas pemisahan spermatozoa. Primers direka dengan menggunakan jujukan cDNA amelogenin dengan 329 dan 266 bp masing-masing bagi spermatozoa berkromosom X dan Y. Motiliti dianalisis menggunakan penganalisisan semen berkomputer (CASA), manakala kesempurnaan selaput dianalisis menggunakan kaedah ujian bengkak hipo-osmosis (HOST). Keputusan ini telah disahkan oleh ketiadaan jalur tunggal, sama ada untuk kromosom X atau Y. Jalur berganda menunjukkan bahawa spermatozoaa tidak dapat dipisahkan juga diperhatikan, selepas pemisahan. Walau bagaimanapun, peratus

spermatozoa berkromosom X menggunakan media mukus servik daripada lembu estrus adalah 58.33%, manakala kaedah kecerunan Percoll adalah 44.33%.Statistik peratusan kedua-duanya berbeza (P<0.001) berbanding dengan nisbah teori (50:50).Walau bagaimanapun, perbandingan motiliti spermatozoaa, kesempurnaan selaput, dan kepekatan sebelum dan selepas pemisahan adalah mempunyai perbezaan yang bererti (P <0.05) di kalangan mereka.

Dalam Ujikaji 4, kesan pelarut pada motiliti, kesempurnaan selaput dan kesempurnaan akrosom spermatozoa lembu beku yang dicairkan juga dikaji. Empat pelarut digunakan dalam ujikaji ini iaitu mukus servik yang dikumpul daripada lembu estrus, penampan Tris, sodium sitrat dan media TALP.Kesemua alikuot pelarut telah dicairkan untuk menghasilkan kepekatan sebanyak 25 x 10^6 sperma/mL. Teknik CASA, HOST, dan florosent dilabel isothiocyanate kekacang agglutinin (FITC-PNA) digunakan untuk menentukan motili, kesempurnaan selaput dan kesempurnaan akrosom spermatozoa, masing-masing, dalam kedua-dua air mani yang segar dan beku yang dicairkan. Motiliti (74.9 ± 0.60%), kesempurnaan selaput (70.86 ± 0.81%) dan kesempurnaan akrosom spermatozoa (67.31 ± 0.94%) bagi air mani segar adalah lebih tinggi daripada air mani beku yang dicairkan.Motiliti spermatozoa (47.93 ± 0.80%) adalah berbeza (P <0.05) dalam mukus servik berbandiang penampan Tris dan TALP. Tambahan pula, kesempurnaan selaput (43.09 ± 0.83%) dan kesempurnaan akrosom (51.67 ± 0.80%) bagi spermatozoa beku yang dicairkandalam pengekal TALP adalah rendah (P<0.05) jika dibandingkan dengan pelarut lain. Kesimpulannya, pelarus mucus cervik memberikan keputusan motility spermatozoa, kesempurnaan selaput dan akrosom yang standing dengan pelarut lain. Walau bagaimanapun, kesempurnaan akrosom selepas dicairkan adalah lebih tinggi berbanding pelarut TALP.

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I certify that an Examination Committee met on 13 February 2012 to conduct the final examination of Abd Malik on his Doctor of Philosophy thesis entitled "Reproductive efficiency through estrus resynchronization, timed artificial insemination and spermatozoa separation in beef cattle" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The committee recommends that the candidate be awarded the Doctor of Philosophy.

Members of the Examination Committee are as follows:

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirements 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 institutions.

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ABD MALIK

Date: 13 February 2012

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LIST OF ABBREVIATIONS

AI	Artificial insemination
ANOVA	Analysis of variance
BCS	Body condition score
CASA	Computer assisted spermatozoa analysis
CI	Calving interval
CL	Corpus luteum/Corpora lutea
CIDR	Controlled Internal Drug Release
d	Day (s)
DNA	Deoxyribonucleic acid
EB	Estradiol benzoate
eCG	Equine chorionic gonadotropine
FITC	Fluorescein isothiocyanate
FSH	Follicle stimulating hormone
FTAI	Fixed-time insemination
GnRH	Gonadotropin releasing hormone
h	Hour (s)
HEPES	N-(2-hydroxyethyl) piperizine-N'-(2-ethanesulphonic acid)
hCG	Human chorionic gonadotropine
HOST	Hypo-osmotic swelling test
i.m.	Intramuscular
IU	International unit

IVF	In-vitro fertilization
kg	Kilogram (s)
LH	Luteinizing hormone
mOsmol	Milli-osmole
MGA	Melengestrol acetate
mg	Milligram (s)
mL	Milliliter (s)
mM	Millimole (s)
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
$PGF_2\alpha$	Prostaglandin $F_2\alpha$
pH	hydrogen ion concentration,-log 10 of
PNA	Peanut agglutinin
PSA	Pisum Sativum agglutinin
PRID	Progesterone releasing intra vagina device
RIA	Radioimmunoassay
RNA	Ribosome nucleate acid
ROS	Reactive oxygen species
SQA	Spermatozoa quality analyzer
S E M	Standard error of the mean
TALP	Tyrode's albumin lactate and pyruvate
Tris	Tris (hydroxymethyl) aminomethane
v/v	Volume: volume ratio

X sperm	X-chromosome bearing spermatozoa
Y sperm	Y-chromosome bearing spermatozoa

CHAPTER 1

INTRODUCTION

Reproductive efficiency is an essential tool that is used to evaluate beef cattle production (Dickerson, 1970). The target in cattle production is the maintenance of good reproductive performance, and it is also important to provide efficient management (Ball and Peters, 2004). Some of the reproductive technologies that can be used to increase reproductive efficiency include estrus synchronization, artificial insemination and spermatozoa separation.

Normally, estrus in cows are observed around days 21 of the estrus cycle if the cow is not pregnant. Failure of cows to become pregnant and calf losses at or immediatly after birth is the main reasons for reproductive failure (Bellows and Short, 1990; Wiltbank, 1994). Synchronization of estrus can be practiced to successive early pregnancy rate, which is essential to achieve a 12 month calving interval. This also allows more time for cows to resume estrus cyclicity before the beginning of the subsequent breeding season. Synchronization of estrus also improves homogeny of a calf crop (Dziuk and Bellows, 1983; Stagg *et al*., 1995).

Resynchronization of estrus is a new method for the improvement of calving intervals in cows. The advent of ultrasonography has allowed accurate early pregnancy diagnosis, at least 25 to 26 days after artificial insemination in cattle (Kastelic *et al*., 1988; Fricke, 2002; Ricardo *et al*., 2004; Bartolome *et al*., 2005;

Bartolome *et al*., 2009), which facilitated the resynchronization programs. Implementation of resynchronization of estrus programs was considering results of pregnancy diagnosis by ultrasonography or per rectal palpation of the uterus. If the pregnancy diagnosis shows that the cows are not pregnant, the continued estrus synchronization will reduce the calving interval in cattle (Bartolome *et al.,* 2002).

Development of reproductive biotechnology has made it possible to carry out separation of X- and Y-chromosome bearing spermatozoa. Sexing of spermatozoa, involving separation of X- from Y-chromosome bearing spermatozoa, implies its application in artificial insemination and *in vitro* fertilization (IVF). The result of sexing spermatozoa is essential for dairy and beef industry. Dairy farmers would prefer most of their calves to be female and thus potentially become replacement for the milking herd, whereas beef farmers may often prefer their cow to produce bull calves (Ball and Peters, 2004; Hamano *et al*., 2007). For a long time there had been attempts at spermatozoa sexing of X- and Y-chromosome on the basis of the physical, biological and immunological properties (Ball and Peters, 2004).

The spermatozoa separation has been accomplished by many different procedures such as the Percoll gradient, swim-up and modified swim-up (swimming speed), Sephadex columns, albumin gradient, sex specific antibody binding, free–flow electrophoresis, PCR, and flow cytometry (Iizuka *et al*., 1987; Khatamee *et al*., 1999; Madrid *et al*., 2003; Joerg *et al*., 2004; Kobayashi *et al.,* 2004). Sexed spermatozoa could be used in the management and insemination of cows with the aim of

producing calves of the required sex, and for the fertilization of oocytes *in vitro* to produce embryo of the required sex.

Cattle production in tropical countries could be described as multipurpose, with cows being used for milking, meat, clothing, fertilizer, fuel, draft power and sometimes for status or as a form of currency (Ball and Peters, 2004). Generally, in cattle production the main problem causing decreased reproductive efficiency, especially calving interval and delayed first estrus postpartum, is due to the high incidence of postpartum anestrus (Baruselli *et al*., 2004). The effects of a long calving interval have as a consequence, negative effects on reproductive performance.

The slow recovery of reproductive performance during postpartum is a limitation to the success of reproductive management programs that are implemented for beginning of inseminations. The synchronization, resynchronization of estrus and artificial insemination including spermatozoa separation program could be used to increase reproductive performance in beef cattle so that it will have a good economic impact for the farmer. Therefore, this study was conducted with these objectives:

1. to determine the effects of resynchronization with progesterone and prostaglandin $F_2\alpha$ on estrus response and pregnancy rate in beef cattle.
2. to compare between timed artificial insemination and natural mating on the pregnancy rate in postpartum estrus synchronized beef cattle.
3. to separate X- and Y-chromosome bearing spermatozoa using swim-up, swimming speed and Percoll gradient methods.

4. to verify X- and Y-chromosome bearing spermatozoa using nested PCR.
5. to determine the effects of cervical mucus from cows in estrus on the motility, membrane integrity and acrosome integrity of frozen-thawed bull spermatozoa.

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