



UNIVERSITI PUTRA MALAYSIA

***ASSESSMENT OF BALI CATTLE SEMEN USING DIFFERENT
COLLECTION METHODS, ULTRASTRUCTURAL CHANGES OF
SPERMATOZOA, AND RELATIONSHIP BETWEEN SEMINAL
PLASMA PROTEINS AND IN VITRO FERTILITY***

KAJAL SARSAIFI

FPV 2013 16



**ASSESSMENT OF BALI CATTLE SEMEN USING DIFFERENT
COLLECTION METHODS, ULTRASTRUCTURAL CHANGES OF
SPERMATOZOA, AND RELATIONSHIP BETWEEN SEMINAL
PLASMA PROTEINS AND IN VITRO FERTILITY**

By

KAJAL SARSAIFI

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

February 2013

COPYRIGHT

All material contained within the thesis, including without limitation text, logo, icons, photographs and all other artwork, is copyright material of Universiti Putra Malaysia unless otherwise stated. Use may be made of any material contained within the thesis for non-commercial purposes from the copyright holder. Commercial use of material may only be made with the express, prior, written permission of Universiti Putra Malaysia.

Copyright © Universiti Putra Malaysia



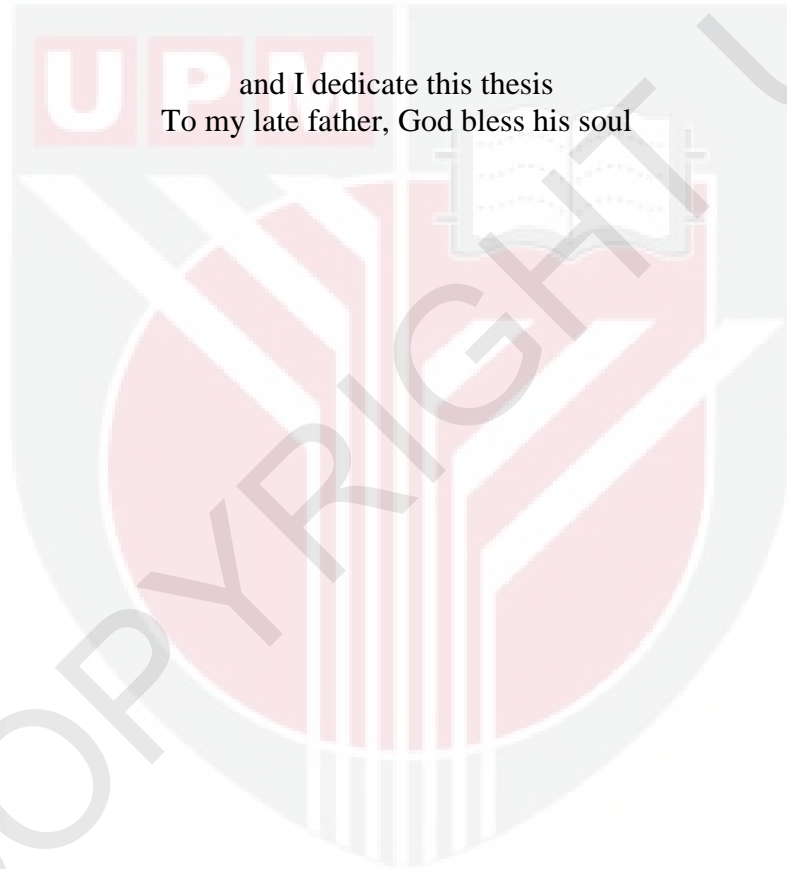
DEDICATION

I dedicate this thesis to my mother Fereshteh
Who has raised me to be the person who I am today. Thank you for all the
unconditional love, guidance and support that you have always given me

To my two dearest brothers Semko and Danial and to my lovely sister Delaram,
who have given lots of love and support to the continuance my education

To my beloved husband,
Homayoun Hani, for his immense support, patience, and encouragement.

and I dedicate this thesis
To my late father, God bless his soul



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

ASSESSMENT OF BALI CATTLE SEMEN USING DIFFERENT COLLECTION METHODS, ULTRASTRUCTURAL CHANGES OF SPERMATOZOA AND RELATIONSHIP BETWEEN SEMINAL PLASMA PROTEINS AND IN VITRO FERTILITY

By

KAJAL SARSAIFI

February 2013

Chairman: Prof. Abd. Wahid Bin Haron, D.V.M., PhD

Faculty: Veterinary Medicine

Male infertility is a major issue in mammalian reproduction and thus, it is important to ascertain that a bull is fertile prior to breeding. The potential fertility of a bull can be determined by field fertility rates. However, this procedure of determining fertility is expensive and time-consuming. Therefore, the present study was designed to develop a method for the prediction of frozen-thawed Bali cattle spermatozoa fertility using various sperm characteristics and heterologous *in vitro* fertilization (IVF) of zona-free hamster oocytes (ZFHOs).

The main objective of the study was to evaluate the protein composition of the seminal plasma of Bali bulls and the proteins related to bull fertility. Semen samples were collected from 25 untrained Bali bulls using three different collection methods namely electrical ejaculation (EE), rectal massage (RM) and a combination of RM and EE. The effects of these methods on pre- and post-

thawed semen characteristics were evaluated. All specimens were assessed for motility characteristics, capacitation, acrosome and membrane integrities, acrosome reaction, and ultrastructure. To assess the fertility of frozen-thawed bull spermatozoa, heterologous IVF using ZFHOs was conducted. To improve sperm penetration into ZFHOs, three sperm separation methods namely Swim-up, Percoll[®] and BoviPure[®], were used. Prior to co-incubation with ZFHOs, the spermatozoa were first incubated in four media containing 0, 25, 50 and 100 µg/ml heparin. The relationship between different motions of sperm characteristics semen assessed by CASA and the percentage of IVF success were also studied. In another study, the seminal plasma proteins (SPP) were separated using 2-dimensional SDS-PAGE followed by Coomassie blue staining and the polypeptide maps were analyzed using the Image Master Software. Proteins were identified by Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS/MS). The results of the study showed that EE and combination method (RM-EE) can be used to obtain semen from Bali cattle, with a 100% success rate with the EE method. Post-thawed semen collected using the EE method was also of acceptable quality that would increase the chance of conception, and subsequently can be used in the conservation of Bali cattle. Better fertilization percentages (FP) and fertilization indexes (FI) were observed in ZFHOs inseminated with spermatozoa separated by Swim-up and BoviPure[®] compared to the Percoll[®] method. In addition the higher heparin concentrations (50 and 100 µg/ml) with 30 min incubation time improved heterologous IVF rates (FP: 68.42-74.64 % and FI: 3.15-3.73). With regards to FP and FI, significant differences (P<0.05) were observed between bulls from heterologous IVFs of ZFHOs with Swim-up spermatozoa and

BoviPure® spermatozoa group. Among the spermatological and IVF parameters, FP ($r^2 = 0.930$) and FI ($r^2 = 0.875$) were identified as being the most predictive of bull fertility and have high correlations with acrosome reaction ($r^2 = 0.830$). The results suggest that heterogeneous IVF using ZFHOs is potentially an informative method for assessing *in vivo* fertilization ability of Bali bulls. Bulls of high fertility had significantly higher mean values of sperm characteristics compared to infertile bulls. Statistical analysis of percentage motility, morphology and FP showed that the correlation coefficient between sperm qualitative characteristics and overall bull fertility were significant ($P < 0.05$). Based on these findings, the proteomics profiling of seminal plasma of Bali bulls have significant merit. An average of 116 ± 8 spots was detected on the SPP gels. An interesting finding in this study is that there were three spots, namely 9, 13 and 16 (MW: 14 to 16 kDa, and PI: 4.9 to 5.8), which were generally more significantly intense in the infertile group than in the fertile group. Upon MALDI-TOF-MS/MS analysis, these three spots were identified as a type of bovine SPP (PBSA1/A2) known as PDC-109 that was up-regulated ($P < 0.05$) in the infertile Bali cattle group. The PDC-109 is involved in sperm capacitation and fertilization and its abundance is associated with fertility of Bali bulls. Spots 3 and 5, identified as sperm-adhesion proteins (MW: 13 to 15 kDa and PI: 5 to 5.8) were not significant ($P > 0.05$) up-regulated in the infertile group. Spots 18 and 68, known as seminal ribonuclease (MW; 14 to 16 kDa and PI; 8.4 to 9.3), were significantly down-regulated ($P < 0.05$) in the infertile group. However, spot 68 was found at the edge of the acidic site, so it may not serve as a good potential marker due to the limitation of pI range (3 to 10) in the ReadyStrip™. Spot 79, which is serum albumin, was also down-regulated in the

infertile group. This is the first report on the PDC-109 abundance related to bull fertility. This study is also a first comprehensive description of Bali cattle semen and seminal plasma proteome. These findings suggest that there are several proteins in the Bali cattle seminal plasma fluid which could be related to the fertility of this species.



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

**PENILAIAN SEMEN LEMBU BALI MENGGUNA KAEDAH
PENGUMPULAN BERBEZA, PERUBAHAN ULTRASTRUKTUR
SPERMATOZOA DAN PERKAITAN DI ANTARA PROTEIN PLASMA
SEMEN DAN KESUBURAN *IN VITRO***

Oleh

KAJAL SARSAIFI

Februari 2013

Pengerusi : Prof. Abd. Wahid Bin Haron, D.V.M., PhD

Fakulti : Perubatan Veterinar

Kesuburan jantan adalah suatu isu utama dalam pembiakan mamalia dan justeru itu adalah penting untuk memastikan lembu jantan itu subur sebelum melakukan pembiakbakaan. Kesuburan potensi seekor lembu jantan boleh ditentukan daripada kadar kesuburan di padang. Bagaimanapun, prosedur untuk menentukan kesuburan ini adalah mahal dan memakan masa. Dengan demikian, kajian ini direka bentuk untuk mengembangkan suatu kaedah untuk meramalkan kesuburan spermatozoa sejuk beku-nyahsejuk lembu Bali beku mengguna pelbagai ciri sperma dan pensenyawaan *in vitro* (IVF) heterologous untuk oosit hamster bebas-zona (ZFHOs). Objektif utama kajian ini ialah menilai komposisi protein dalam plasma semen lembu jantan Bali dan protein berkaitan kesuburan lembu jantan. Sampel semen dikumpul daripada 25 ekor lembu jantan Bali yang tidak terlatih, mengguna tiga kaedah pengumpulan iaitu pemancutan elektrik

(EE), urut rektum (RM) dan gabungan EE-RM. Kesan kaedah ini terhadap ciri semen pra- dan pasca-nyahsejuk beku dinilai. Semua spesimen dinilai untuk ciri kemotilan, pengkapasita, integriti akrosom dan membran, tindak balas akrosom dan ultrastruktur. Untuk menilai kesuburan spermatozoa lembu jantan nyahsejuk beku, IVF heterologus mengguna ZFHOs dilakukan. Untuk meningkatkan penembusan sperma masuk ZFHOs, tiga kaedah pengasingan sperma iaitu renang-naik, Percoll® dan BoviPure® digunakan. Sebelum dieramkan bersama ZFHOs, spermatozoa terlebih dahulu dieramkan dalam empat media mengandungi 0, 25, 50 dan 100 µg/ml heparin. Perkaitan di antara pelbagai pergerakan ciri sperma dinilai dengan CASA dan peratusan kejayaan IVF juga dikaji. Dalam suatu kajian lain, protein plasma semen (SPP) dipencil mengguna SDS-PAGE 2-dimensi, diikuti dengan pewarnaan Coomassie biru dan peta polipeptida dianalisis mengguna perisian Image Master. Protein dikenal pasti melalui spektrometri jisim masa-terbang nyahjerapan/pengionan terbantu-matriks (MALDI-TOF-MS/MS). Hasil kajian menunjukkan yang kaedah EE dan gabung kaedah RM-EE boleh diguna untuk memperolehi semen daripada lembu Bali, dengan kadar kejayaan 100% melalui kaedah EE. Kualiti semen pascanyahsejuk beku dikumpul mengguna kaedah EE boleh diterima dan akan meningkatkan kemungkinan konsepsi, dan seterusnya boleh diguna dalam pemuliharaan lembu Bali. Peratusan pensenyawaan (FP) dan indeks pensenyawaan (FI) yang lebih baik dilihat pada ZFHOs dipermani dengan spermatozoa yang diasing melalui kaedah renang-naik dan BoviPure® daripada kaedah Percoll®. Disamping itu, kepekatan heparin lebih tinggi (50 dan 100 µg/ml) dengan tempoh pengeraman 30 minit meningkatkan kadar IVF heterologus (FP: 68.42-74.64% dan FI: 3.15-3.73). Merujuk kepada FP dan FI,

perbezaan tererti ($P < 0.05$) dilihat di antara lembu jantan daripada kumpulan IVF heterologus ZFHOs dengan spermatozoa renang-naik dan kumpulan spermatozoa BoviPure®. Di kalangan parameter spermatologi dan IVF, FP ($r^2 = 0.930$) dan FI ($r^2 = 0.875$) dikenal pasti sebagai yang paling baik dalam meramal kesuburan lembu jantan dan ianya mempunyai korelasi tinggi dengan tindak balas akrosom ($r^2 = 0.830$). Hasil ini menyarankan yang IVF heterologus mengguna ZFHOs merupakan kaedah yang berpotensi memberi matlumat banyak dalam penilaian keupayaan pensenyawaan *in vivo* lembu jantan Bali. Lembu jantan tinggi kesuburannya mempunyai nilai min ciri sperma lebih tinggi tererti ($p < 0.05$) berbanding lembu tak subur. Analisis statistik terhadap peratusan kemotilan, morfologi dan FP menunjukkan bahawa pekali korelasi di antara ciri kualiti sperma dengan kesuburan keseluruhan lembu jantan adalah tererti ($P < 0.05$). Berdasarkan penemuan ini, pemprofilan proteomik plasma semen lembu jantan Bali ada merit yang nyata. Secara purata 116 ± 8 bintik dapat dikesan pada gel SPP. Penemuan yang menarik dalam kajian ini ialah wujudnya tiga bintik iaitu 9, 13 dan 16 (berat molekul: 14 hingga 16 dan PI: 4.9 hingga 5.8) yang umumnya lebih terang tererti dalam kumpulan tidak subur daripada kumpulan subur. Apabila analisis MALDI-TOF-MS/MS dilakukan, tiga bintik tersebut dikenal pasti sebagai sejenis SPP bovin (PBS A1/A2) dikenali sebagai PDC-109, yang ternaik kawal ($P < 0.05$) dalam kumpulan lembu Bali tidak subur. PDC-109 terlibat dalam pengkapasitan dan pensenyawaan sperm dan wujudnya dengan banyak adalah berkaitan dengan kesuburan lembu jantan Bali. Bintik 3 dan 5 yang dikenal pasti sebagai protein lekatan sperma (berat molekul: 13 hingga 15 dan PI: 5 hingga 6.8) ternaik kawal secara tidak tererti ($P > 0.05$) dalam kumpulan tidak subur. Bintik 18 dan 68 yang dikenali

sebagai ribonuklease semen (berat molekul: 14 hingga 16 kDa dan PI: 8.4 hingga 9.3) yang terturun kawal secara tererti ($p < 0.05$) dalam kumpulan tidak subur. Bagaimanapun, bintik 68 terdapat pada pinggir tapak berasid, justeru itu ia tidak boleh diguna sebagai penanda berpotensi baik kerana adanya batasan julat pI (3 hingga 10) pada ReadyStrip™. Bintik 79 yang merupakan albumin serum juga terturun kawal dalam kumpulan tidak subur. Ini adalah laporan pertama mengenai banyaknya PDC-109 yang berkaitan kesuburan lembu jantan. Kajian ini juga merupakan huraian komprehensif pertama terhadap semen dan proteom plasma semen lembu Bali. Penemuan ini menyarankan bahawa ada beberapa protein dalam bendalir plasma semen lembu Bali yang mungkin boleh dikaitkan dengan kesuburan spesies ini.

ACKNOWLEDGEMENTS

With sincere and deep gratitude, I would like to acknowledge my supervisor Prof. Dr. Abd. Wahid Haron for his time, effort, constructive advice, encouragement and support during my Ph.D. program. I would like to appreciate the kindness of Associate Prof. Dr. Rosnina Hj. Yusoff, for her invaluable guidance, patience and support in preparation of this dissertation. My sincere thanks are to Dr. Abas Mazni Othman (MARDI) and Dr. Jaya Vejayan (Monash University) for their invaluable advice, suggestions and their support which were really helpful towards completion of my study. I was fortunate to have such a great supervisory committee. I also wish to express my gratitude to Prof. Dr. Mohamed Ariff Omar, Prof. Dato Dr. Tengku Azmi Tengku Ibrahim, Prof. Dr. Rasedee Abdullah and Associate Prof. Dr. Goh Young Meng for their invaluable advice, suggestions and efforts spent to improve the quality of the thesis, are very much appreciated.

I am grateful to staff members of the Theriogenology and Cytogenetic Laboratory, Faculty of Veterinary Medicine, UPM especially Mr. Yap Keng Chee, Dr. Nurhusien Yimer, Meat Laboratory, Faculty of Agriculture, Electron Microscopy Department, Institute of Biosciences, Mr. Ho Oi Kuan, Animal Laboratory, Agro-Biotechnology Institute Malaysia (ABI), Mr. Lai Wei Hong , Mrs. Tan Ying Ju and Dr. Fazly Ann, Federal Land Development Authority farms (FELDA) and National Institute of Biodiversity and Veterinary (IBVK), Dr. Hafizan and Mr. Zawawi B. Ismail for their valuable critical and also technical assistances. Here, I would like to express my especial thanks to Laila

Zarei, Ph.D. Candidate of Plant Genetics, for her great assistance and consulting in statistical analysis of this investigation data.

Also, my special thanks to the Agro Biotechnology Institute (ABI), Ministry of Science Technology and Innovation (MOSTI) Malaysia, that provided financial support. This dissertation was a part of the research project, *Rapid Multiplication of Bali Cattle and Its Crossbred Using Reproductive Biotechnology (Research Project Number: 08/05/ABI-ab032)*.

Last but not least, my deepest gratitude goes to my family. I would like to thank my mother, brothers Semko and Danial and lovely sister, Delaram, for their blessings, prayers from far away, their support and unconditional love all the time. I would like to thank my mother-in-law and father-in-law for their love, encouragement and blessings, thank you with all my heart. Most importantly, my special thanks go to my beloved husband, Homayoun Hani, for his understanding, patience and steadfast support that have made the task of completing this Ph.D. project possible, I could not have done this without him. Finally, I thank all those who helped me directly or indirectly during the period of my study.

I certify that a Thesis Examination Committee has met on 27th of Jun 2013 to conduct the final examination of Kajal Sarsaifi on her thesis entitled "Assessment Of Bali Cattle Semen Using Different Collection Methods, Ultrastructural Changes Of Spermatozoa, And Relationship Between Seminal Plasma Proteins And In Vitro Fertility" 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.

Members of the Thesis Examination Committee are as follows:

Mohd Zamri Saad, PhD

Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Mohamed Ariff Bin Omar 1, PhD

Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

Mohamed Ali Rajion 2, PhD

Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

Muhammad Azam Kakar, PhD

Professor
Faculty of Veterinary and Animal Sciences
Lasbela University of Agriculture, Water and Marine Sciences, Uthal,
Balochistan
(External Examiner)

NORITAH OMAR,, PhD

Assoc. Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia
Date: 16 Agust 2013

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of Supervisory committee are as follows:

Abd. Wahid Bin. Haron, PhD

Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Rosnina Hj Yusoff, PhD

Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

Abas Mazni Othman, PhD

Department of Biotechnology
Malaysia Agriculture Research and
Development Institute (MARDI)
(Member)

Jaya Vejayan, PhD

Lecturer
Monash University Sunway Campus
Bandar Sunway
(Member)

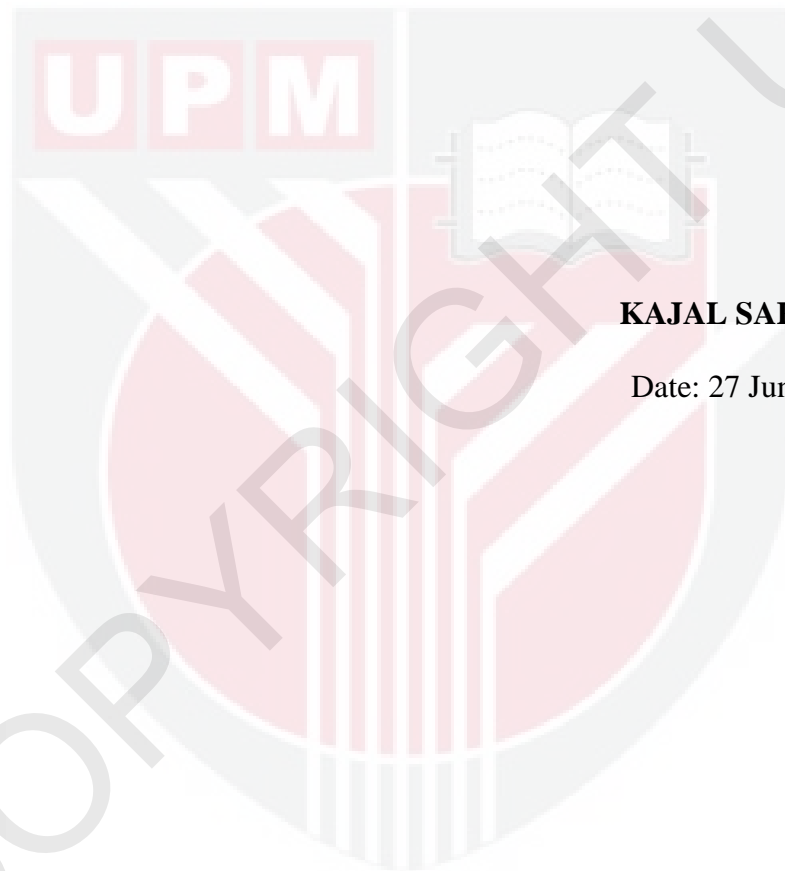
BUJANG BIN KIM HUAT, PhD

Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 12 September 2013

DECLARATION

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



KAJAL SARSAIFI

Date: 27 Jun 2013



TABLE OF CONTENTS

	Page
ABSTRACT	iv
ABSTRAK	viii
ACKNOWLEDGEMENTS	xii
APPROVAL	xiv
DECLARATION	xvi
LIST OF TABLES	xxiv
LIST OF FIGURES	xxiv
LIST OF APPENDICES	xxv
LIST OF ABBREVIATIONS	xxvii
CHAPTER	
1 INTRODUCTION	1
1.1 Background	1
1.2 Problem statement	1
1.3 Justification	4
1.4 Objectives of the study	7
1.5 Null hypotheses	8
2 LITERATURE REVIEW	9
2.1 Bali cattle	9
2.2 Reproductive biology of Bali cattle	11
2.3 Bali cattle in Malaysia	12
2.4 Bull effect on fertility	14
2.5 Reproductive performance and management in Bali cattle	14
2.6 Anatomy and physiology of the Bali cattle reproductive system	16
2.6.1 Testes	17
2.6.2 Secondary sex organs of the Bali bull	18
2.6.2.1 Epididymis	18
2.6.2.2 Vas deferens and urethra	19
2.6.3 Accessory sex glands	20
2.6.3.1 Vesicular glands	21
2.6.3.2 Prostate gland	22
2.6.3.3 Bulbourethral glands	22
2.6.4 Spermatogenesis	23

2.7	Spermatozoon	24
2.7.1	Head of sperm	25
2.7.2	Sperm tail	26
2.7.3	Surface of the spermatozoon	27
2.8	Seminal fluid	29
2.9	Semen Collection	30
2.9.1	Semen collection by artificial vagina (AV)	30
2.9.2	Semen collection by electro-ejaculation (EE)	30
2.9.3	Semen collection by transrectal massage (RM)	32
2.10	Prediction of bull fertility	33
2.11	Conventional parameters to predict bull fertility	33
2.11.1	Breeding soundness examination	34
2.11.2	Semen quality	35
2.11.3	Sperm morphology	35
2.11.4	Sperm motility	36
2.11.5	Acrosome integrity	38
2.11.6	Membrane integrity	40
2.12	Sperm functional test	41
2.12.1	Hyperactivation	42
2.12.2	Acrosome reaction	44
2.12.3	Sperm capacitation	46
2.12.4	In vitro capacitation with heparin	47
2.12.5	Sperm separation methods	48
2.12.6	Sperm penetration assay (heterologous <i>in vitro</i> fertilization)	50
2.13	Heparin-binding proteins in semen	52
2.14	Protein markers in semen for bull fertility	57
2.14.1	Osteopontin and lipocalin-type prostaglandin-D Synthase	57
2.14.2	Fertility-associated antigen (FAA) and Tissue inhibitor of metalloproteinases-2 (TIMP-2)	60

3 EFFECT OF SEMEN COLLECTION METHODS ON THE QUALITY OF BALI CATTLE (*BOS JAVANICUS*) SPERMATOZOA 62

3.1	Introduction	62
3.2	Materials and methods	65
3.2.1	Animals and location	65
3.2.2	Breeding soundness examination (BSE)	66
3.2.3	Semen collection methods	66
3.2.3.1	Semen collection by electro-ejaculator	67
3.2.3.2	Semen collection by transrectal massage	68
3.2.3.3	Semen collection by combination of RM and EE method	68

3.2.4	Semen evaluation	68
3.2.4.1	Sperm motility	69
3.2.4.2	Sperm viability	70
3.2.4.3	Sperm morphology	71
3.2.5	Semen cryopreservation	71
3.2.6	Motility pattern of spermatozoa evaluation by CASA	71
3.2.7	Assessment of acrosome integrity	72
3.2.8	Statistical analysis	74
3.3	Results	74
3.3.1	Response of bulls to collection methods	74
3.3.2	Effect of collection methods on semen quality	76
3.3.3	Effects of collection methods on fresh and frozen-thawed semen quality	78
3.4	Discussion	80
4	DEVELOPMENT OF A HETEROLOGOUS <i>IN VITRO</i> FERTILIZATION TEST TO PREDICT FERTILITY OF BALI CATTLE SEMEN	85
4.1	Introduction	85
4.2	Materials and methods	87
4.2.1	Semen collection and experimental design	87
4.2.2	Sperm separation methods	88
4.2.2.1	Swim-up method	89
4.2.2.2	Percoll® gradient	89
4.2.2.3	BoviPure® gradient	90
4.2.3	Effectiveness of sperm separation method	90
4.2.4	In vitro sperm capacitation with heparin	91
4.2.5	Acrosome reaction	91
4.2.6	Simplified triple-stain technique	92
4.2.7	Assessment of acrosome reaction	94
4.2.8	Hypo-osmotic swelling tests (HOST)	94
4.2.9	Sperm penetration assay (heterologous in vitro fertilization)	95
4.2.10	Definitions of sperm motion characteristics by CASA	96
4.2.11	Statistical analysis	98
4.3	Result	98
4.3.1	Effect of sperm separation method	98
4.3.2	Bull Effect and different sperm separation methods	101
4.3.3	Heterologous <i>in vitro</i> fertilization	107

4.3.4	CASA evaluation	110
4.3.5	Sperm capacitation	111
4.3.6	CASA evaluation of capacitated spermatozoa	113
4.3.7	Relationship of spermatological characteristics with heterologous IVF parameters	114
4.3.8	Regression analysis	115
4.3.9	Pearson correlation	116
4.4	Discussion	118
4.4.1	Effectiveness of sperm separation method	118
4.4.2	Effectiveness of sperm separations methods on heterologous in vitro fertilization	123
4.4.3	Movement characteristics assessed by CASA system	124
4.4.5	In vitro capacitation of frozen-thawed Bali spermatozoa	125
4.4.6	Acrosome reaction	126
4.4.7	Heterologous in vitro fertilization	127
5	ULTRASTRUCTURE STUDY ON BALI BULL SPERMATOZOA	132
5.1	Introduction	132
5.2	Materiel and methods	135
5.2.1	Experimental design	135
5.2.2	Preparation of Bali bull spermatozoa for SEM and TEM	135
5.2.3	Sample primary fixation, washing, and post-fixation for SEM and TEM	136
5.2.4	Ultra-thin section of Bali spermatozoa for TEM	137
5.2.5	Uranyl acetate and lead citrate staining for TEM	137
5.2.6	Scanning electron microscopy (SEM) study on Bali spermatozoa	138
5.2.7	Statistical analysis	139
5.3	Results	139
5.3.1	General morphology of Bali bull spermatozoa	139
5.3.2	Acrosome	140
5.3.3	Post-acrosomal border and post-acrosomal cap	141
5.3.4	Mid-piece of sperm tail	142
5.3.5	End-piece of sperm tail	142
5.3.6	TEM results	150
5.4	Discussion	160

6	CHARACTERIZATION OF SEMINAL PLASMA PROTEINS FROM BALI BULLS USING 2D-ELECTROPHORESIS	171
6.1	Introduction	171
6.2	Material and method	173
6.2.1	Chemicals and reagents	173
6.2.2	Sample collection and processing	174
6.2.3	Seminal plasma protein preparation	174
6.2.4	Determine seminal plasma protein concentration	175
6.2.5	Iso electro focusing (1D; IEF)	175
6.2.6	Second electrophoresis (2D-PAGE)	176
6.2.7	Gel staining, scanning and analysis	177
6.2.8	MALDI-TOF-MS/MS identification of seminal plasma proteins	178
6.3	Results	178
6.3.1	Proteomics analysis of seminal plasma proteins	179
6.3.2	Protein identification by MALDI-TOF-MS/MS	186
6.4	Discussion	187
7	GENERAL DISCUSSION	200
8	SUMMARY, GENERAL CONCLUSION AND RECOMMENDATION	207
	BIBLIOGRAPHY	217
	APPENDICES	259
	BIODATA OF STUDENT	290
	LIST OF PUBLICATION	291