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IMPROVING QUALITY OF FROZEN-THAWED BULL SPERMATOZOA VIA IN VITRO SUPPLEMENTATION WITH ALPHA-LINOLENIC AND DOCOSAHEXAENOIC ACID

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By

ASMATULLAH KAKA

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DEDICATION

I would like to dedicate this thesis with love and gratitude to my grandfather ALLAH WARAYO KAKA and my father GHULAM QADIR KAKA always guided, and encouraged me throughout my life.

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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Chairman: **Professor Abd Wahid Haron, PhD**
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Animal production has been modernized through cryopreservation. However, cryopreservation decreases the quality and fertility of bull sperm by impairing its normal functions due to thermal, oxidative and osmotic changes and re-distribution of lipid bonding. Therefore, the present study was conducted to test, if supplementation of semen extenders with fatty acids and antioxidants could improve the quality of frozen-thawed of bull semen.

Semen samples from three fertile beef bulls were collected twice a week by an electro ejaculator. After collection, semen samples were brought to the laboratory for initial evaluation. Ejaculates with motility ≥ 70% and normal morphology and viability ≥ 80% were processed for cryopreservation. Ejaculates were extended in Tris and BioXcell® extenders containing 0 (control), 3, 5, 10 and 15ng/ml of docosahexaenoic acid (DHA) and α-linolenic acid (ALA). As the fatty acids are insoluble in water, 0.05% ethanol was added as a solvent. Extended samples were initially incubated at 37^{o}C for 15 minutes to allow absorption of ALA by the sperm membrane, and then chilled for 2 hours, followed by packaging into 0.25ml straws with 20 x 10^{6} sperm per straw. The straws were placed 3cm above the surface of liquid nitrogen for 10 minutes and finally immersed into liquid nitrogen for storage. After 24 hours, straws were thawed and evaluated for sperm motility using a computer assisted semen analyzer (CASA), membrane functional integrity (hypo-osmotic swelling test), viability, morphology, acrosome integrity (eosin-nigrosin stain), DNA integrity (comet assay), fatty acid composition (gas chromatography), lipid peroxidation (thiobarbituric acid reactive substances, TBARS) and superoxide dismutase (SOD assay). Data of all the parameters was analyzed with SAS 9.2 version with the general linear model (GLM) and Duncan multiple range test (DMR).

Results showed that adding ALA into BioXcell® and Tris semen extender improved post- thawed quality of bovine semen. Frozen-thawed sperm motility, morphology, viability, morphology, acrosome integrity, DNA integrity and ALA concentration were improved significantly in treated groups compared to control. Uptake was observed to be linear in relation to ALA concentration added. A concentration of 5ng/ml of ALA was found to be the optimum level for improved semen cryopreservation using

Bioxcell® and Tris extender along with tolerable Lipid peroxidation (LPO) reactions and amount of MDA production. DHA supplementation into BioXcell® and Tris extenders also produced positive effects on freezing quality of bull sperm. Frozen-thawed sperm motility, morphology, viability, morphology, acrosome integrity, DNA integrity and DHA concentration were significantly improved at 3ng/ml and 10ng/ml of DHA in BioXcell® and Tris extenders, respectively. Docosahexaenoic acid supplementation produced higher lipid peroxidation rate as compared to ALA but it did not affect sperm quality. Superoxide dismutase enzyme was also improved in both ALA and DHA supplementations. A combined effect of DHA and ALA into BioXcell® and Tris extenders however decreased frozen-thawed quality of the bull sperm. Frozen-thawed sperm motility, morphology, viability, morphology, acrosome integrity and DNA integrity were decreased in treated groups compared to control. Fatty acid and SOD improved positively but MDA was produced in large quantity that decreased quality of sperm.

In the last experiment 5ng/ml of ALA level from Experiment 1 and 3 and 10ng/ml of DHA from Experiment 2 were combined with 0.2, 0.4 and 0.8mM of α-tocopherol to evaluate the effect of fatty acids and antioxidant combination on post thawed quality of bull sperm. Results showed that combination of ALA and α-tocopherol improved frozen-thawed quality compared to control in both BioXcell® and Tris extenders. Significantly higher values were obtained at 5ng/ml of ALA and 0.2mM of α-tocopherol in BioXcell® extender and 5ng/ml ALA with 0.4mM α-tocopherol in Tris extender. DHA also improved frozen-thawed quality in both BioXcell® and Tris extenders, with significant improvement at 3ng/ml of DHA with 0.5mM α-tocopherol and 10ng/ml of DHA with 0.8mM α-tocopherol in BioXcell® and Tris extenders respectively. Fatty acid level was improved, MDA and superoxide dismutase production was decreased. In conclusion, combination of ALA and DHA decreased quality of frozen-thawed bull semen. However, addition of ALA at 5ng/ml and DHA at 3 and 10ng/ml in combination with α-tocopherol improved quality of frozen-thawed of bull semen in Tris and BioXcell® extenders respectively.

Keywords: Bull, semen, ALA, DHA, α-tocopherol and cryopreservation.

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

MENINGKATKAN KUALITI FROZEN DICAIRKAN BULL SPERMATOZOA VIA DALAM VITRO MENGAMBIL SUPLEMEN ALFA-LINOLENIK DAN DOCOSAHEXAENOIC ACID

Oleh

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Pengeluaran haiwan telah dimodenkan melalui krioawetan. Walau bagaimanapun, krioawetan mengurangkan kualiti dan kesuburan sperma lembu jantan dengan merosakkan fungsi normal disebabkan oleh haba, pengoksidaan dan perubahan osmotik dan pengagihan semula ikatan lipid. Oleh itu, kajian ini dijalankan untuk diuji, jika suplemen mekanisma air mani dengan asid lemak dan antioksidan dapat meningkatkan kualiti air mani lembu beku yang dicairkan.

Sampel air mani dari tiga ekor lembu daging yang subur telah diambil dua kali seminggu menggunakan ejakulator elektro. Selepas pengumpulan, sampel air mani telah dibawa ke makmal untuk penilaian awal. Proses ejakulasi dengan motiliti 70% dan morfologi biasa dan daya maju 80% telah diproses untuk krioawetan. Proses ejakulasi telah disambung dengan mekanisma Tris dan BioXcell® yang mengandungi 0 (kawalan), 3, 5, 10 dan 15ng / ml asid docosahexanoic (DHA) dan asid alfa-linolenik (ALA). Data telah dianalisis menggunakan SAS versi 9.2 dengan model general linear (GLM) dan Ujian Duncan multiple range test (DMR).

Sebagai asid lemak tidak larut dalam air, 0.05% etanol telah ditambah sebagai pelarut. Sampel dilanjutkan pada mulanya dieram pada suhu 37°C selama 15 minit dengan membenarkan penyerapan ALA oleh membran sperma, kemudian disejukkan selama 2 jam diikuti dengan pembungkusan ke dalam tiub kecil bersaiz 0.25ml dengan 20 x 106^{6} sperma per tiub. Tiub diletakkan 3 cm di atas permukaan cecair nitrogen selama 10 minit dan akhirnya tenggelam ke dalam cecair nitrogen untuk simpanan. Selepas 24 jam, tiub telah dicairkan dan dinilai untuk motiliti sperma menggunakan komputer untuk analisa sperma (CASA), integriti fungsi membran (tidak menimbulkan osmosis ujian bengkak), daya maju, morfologi, integriti akrosome (noda eosin-nigrosin), integriti DNA (ujian komet), komposisi asid lemak (gas kromatografi), peroksidaan lipid (bahan reaktif asid thiobarbiturik, TBARS) dan superoxide dismutase (SOD kit).

Keputusan menunjukkan bahawa penambahan ALA ke dalam mekanisma BioXcell® dan Tris air mani bertambah baik selepas dicairkan kualiti air mani lembu beku. Motiliti sperma beku yang dicairkan, morfologi, daya maju, morfologi, integriti

akrosome, integriti DNA dan kepekatan ALA telah meningkat dengan ketara dalam kumpulan dirawat berbanding dengan kawalan. Pengambilannya diperhatikan menjadi selari berhubung dengan kepekatan ALA yang ditambah. Kepekatan 5ng / ml ALA didapati tahap optimum untuk krioawetan air mani bertambah baik menggunakan mekanisma Bioxcell® dan Tris dan juga dengan reaksi peroksidaan lipid yang boleh diterima (LPO) dan jumlah pengeluaran MDA. Penambahan DHA ke dalam mekanisma BioXcell® dan Tris juga menghasilkan kesan positif terhadap kualiti sperma beku lembu. Motiliti sperma beku yang dicairkan, morfologi, daya maju, morfologi, integriti akrosome, integriti DNA dan kepekatan DHA telah bertambah baik dengan ketara dalam 3ng / ml dan 10ng / ml DHA dalam mekanisma BioXcell® dan Tris masing-masing. Suplemen DHA menghasilkan kadar peroksidaan lipid yang lebih tinggi berbanding ALA tetapi ia tidak menjejaskan kualiti sperma. Penambahan asid docosahexaenoic telah juga bertambah baik pada kedua-dua tambahan DHA dan ALA.

Gabungan DHA dan ALA ke dalam mekanisma BioXcell® dan Tris bagaimanapun menurunkan kualiti sperma lembu yang beku yang dicairkan. Motiliti sperma beku yang dicairkan, morfologi, daya maju, morfologi, integriti akrosome dan DNA integriti telah menurun dalam kumpulan dirawat berbanding dengan kawalan. Asid lemak dan SOD bertambah baik secara positif tetapi MDA dihasilkan dalam kuantiti yang besar yang menurunkan kualiti sperma.

Dalam penyelidikan terakhir 5ng / ml tahap ALA daripada eksperimen 1 serta 3 dan 10ng / ml DHA dari eksperimen 2 telah digabungkan dengan 0.2, 0.4 dan 0.8mM daripada α-tokoferol untuk menilai kesan asid lemak dan gabungan antioksidan pada kualiti sperma lembu beku yang dicairkan. Hasil kajian menunjukkan bahawa kombinasi ALA dan α-tokoferol membaiki lebih baik kualiti sperma lembu beku yang dicairkan berbanding kawalan dalam mekanisma BioXcell® dan Tris. Nilai-nilai yang lebih tinggi diperolehi dalam 5ng / ml ALA dan 0.2mM α-tokoferol dalam mekanisma BioXcell® dan 5ng / ml ALA dengan 0.4mM α-tokoferol dalam mekanisma Tris. DHA juga meningkatkan kualiti sperma beku yang dicairkan dalam mekanisma BioXcell® dan Tris, dengan peningkatan yang ketara dalam 3ng / ml DHA dengan 0.5mM α-tokoferol dan 10ng / ml of DHA dengan 0.8mM α-tokoferol dalam mekanisma BioXcell® dan Tris masing-masing. Tahap asid lemak juga telah bertambah baik dan MDA telah menurun dan pengeluaran superoxide dismutase. Kesimpulannya, kombinasi ALA dan DHA menurunkan kualiti air mani lembu beku yang dicairkan. Walau bagaimanapun, penambahan ALA dalam 5ng / ml dan DHA pada 3 dan 10ng / ml, dan gabungan tahap terbaik dengan α-tokoferol meningkatkan kualiti air mani lembu jantan beku yang dicairkan dalam mekanisma Tris dan BioXcell® masing-masing.

Tahap asid lemak telah ditingkatkan, MDA dan penghasilan superoxidae dismutase telah diturunkan. Secara kesimpulannya, kombinasi ALA dan DHA telah menurunkan kualiti semen lembu beku yang cairkan. Walaubagaimanapun, penambaahn ALA pada 5ng/ml dan DHA pada 3 dan 10ng/ml dalam kombinasi dengan α-tocopherol telah membaiki kualiti semen lembu beku yang dicairkan dalam Tris dan BioXcell® masing-masing.

Kata Kunci: Lembu jantan, air mani, ALA, DHA, α-tokoferol dan krioawetan

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TABLE OF CONTENTS

		Page
ABSTARCT		i
ABSTRAK		iii
ACKNOWLEDGEMENT		v
APPROVAL		vi
DECLARATION		viii
LIST OF TABLES		x
LIST OF FIGURES		xiv
LIST OF APPENDICES		xvi
LIST OF ABBREVIATIONS		xviii
CHAPTER		
1	**GENERAL INTRODCUTION**	1
	1.1 Background	1
	1.2 Objectives	2
	1.3 Hypothesis	3
2	**REVIEW OF LITERATURE**	4
	2.1 Male reproductive system of the bull	4
	2.1.1 Testes	4
	2.1.2 Structure of testes	5
	2.1.3 Spermatogenesis	5
	2.1.4 Epididymis	7
	2.1.4.1 Changes in spermatozoa membrane and lipid profile	8
	2.1.4.2 Motility	9
	2.1.4.3 Storage	9
	2.1.5 Accessory sex glands	9
	2.1.6 Seminal plasma	10
	2.2 Semen Extenders	11
	2.2.1 Component of semen extenders	11
	2.2.2 Egg yolk	11
	2.2.3 Sugars	11
	2.2.4 Buffers	12
	2.2.5 Cryoprotectants	12
	2.2.6 Antibiotics	13
	2.3 Lipids and Fatty Acids	13
	2.3.1 Saturated, monounsaturated and polyunsaturated fatty acids	13
	2.4 Role of omega-3 fatty acids in fertility of bull semen	15
	2.5 Fatty acid composition of mature sperm	16
	2.6 Lipid peroxidation and oxidative stress in sperm	17
	2.6.1 Effects of lipid peroxidation on spermatozoa	17
	2.7 Role of Antioxidants	18
	2.7.1 Enzymatic antioxidants	19
	2.7.2 Non-enzymatic antioxidants	19

2.8 Cryopreservation 20
2.8.1 Cold Shock 20
2.8.2 Formation of ice crystal 20
2.8.3 Lipid Peroxidation or oxidative damages 21
2.8.4 DNA Damage 21
2.9 The Role of Fatty Acids in Improving the Lifespan of sperm 21

3 GENERAL MATERIALS AND METHODS 22
3.1 Selection and management of animals 22
3.2 Semen collection 22
3.3 Semen evaluation of fresh and frozen samples 22
3.3.1 Colour 22
3.3.2 Volume 23
3.3.3 Motility and concentration 23
3.3.4 Spermatozoa morphology and viability 23
3.3.5 Acrosome integrity 23
3.3.6 Spermatozoa membrane integrity 23
3.3.7 DNA integrity 24
3.3.8 Fatty acid evaluation 24
3.3.9 Lipid peroxidation (LP) 25
3.3.10 Superoxide dismutase test (SOD) 25
3.4 Experimental design 26
3.5 Statistical analysis 27

4 EFFECT OF ALPHA-LINOLENIC ACID ON FROZEN-THAWED SEMEN QUALITY, FATTY ACID COMPOSITION, AND SUPEROXIDE DISMUTASE (SOD) IN TRIS AND BIOXCELL® EXTENDERS 28
4.1 Introduction 28
4.2 Material and Methods 29
4.3 Experimental Design 29
4.3.1 Effect of supplementation of ALA on quality of frozen-thawed bull sperm in BioXcell® extender 29
4.3.2 Effect of supplementation of ALA on quality of frozen-thawed bull sperm in Tris extender 29
4.4 Results 30
4.5 Discussion 34
4.6 Conclusion 35

5 EFFECTS OF DOCOSAHEXANOIC ACID (DHA) ON FROZEN-THAWED SEMEN QUALITY, FATTY ACID COMPOSITION AND SUPEROXIDE DISMUTASE (SOD) IN TRIS AND BIOXCELL® EXTENDERS 36
5.1 Introduction 36
5.2 Materials and methods 38
5.3 Experimental Design 37
5.3.1 Effect of supplementation of DHA on quality of frozen-thawed bull sperm in BioXcell® extender 37
5.3.2 Effect of supplementation of DHA on quality of frozen-thawed bull sperm in Tris extender 37

5.4 Results 38
5.5 Discussion 42
5.6 Conclusion 43

6 EFFECT OF COMBINATION OF DHA AND ALA ON FROZEN-THAWED SEMEN PARAMETERS, DNA INTEGRITY, FATTY ACID COMPOSITION, SUPEROXIDE DISMUTASE (SOD) AND LIPID PEROXIDATION IN TRIS AND BIOXCELL® EXTENDERS 44
6.1 Introduction 44
6.2 Materials and Methods 45
6.3 Experimental Design 45
6.3.1 Combine effect of DHA and ALA on quality of frozen-thawed bull sperm in BioXcell® extender 45
6.3.2 Combine effect of DHA and ALA on quality of frozen-thawed bull sperm in Tris extender 45
6.4 Results 46
6.5 Discussion 50
6.6 Conclusion 51

7 EFFECT OF COMBINATION OF DHA AND ALA WITH ALPHA-TOCOPHEROL ON FROZEN-THAWED PARAMETERS, DNA INTEGRITY, FATTY ACID COMPOSITION, SUPEROXIDE DISMUTASE (SOD) AND LIPID PEROXIDATION IN TRIS AND BIOXCELL® EXTENDERS 52
7.1 Introduction 52
7.2 Materials and Methods 53
7.2.1 Combine effect of DHA with α – tocopherol on quality of frozen-thawed bull sperm in BioXcell® extender 53
7.2.2 Combine effect of DHA with α – tocopherol on quality of frozen-thawed bull sperm in Tris extender 53
7.2.3 Combine effect of ALA with α – tocopherol on quality of frozen-thawed bull sperm in BioXcell® extender 54
7.2.4 Determine the effect of ALA with α – tocopherol into Tris extender on quality of frozen-thawed bull sperm 54
7.3 Results 54
7.7 Discussion 63
7.8 Conclusion 64

8 GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS 65
8.1 Discussion 65
8.2 Conclusions 67
8.3 Recommendations 68

REFERENCES 69
APPENDICES 94
BIODATA OF STUDENT 108
LIST OF PUBLICATIONS 109

LIST OF TABLES

Table		Page
4.1	Effects of different α-linolenic acid (ALA) concentrations in BioXcell® extender on frozen-thawed spermatozoa parameters of bulls (Mean%±SEM; n=24).	30
4.2	Comparison of fatty acid composition of frozen-thawed spermatozoa treated with different concentrations of α-linolenic acid (ALA) in BioXcell® extender (Mean % ± SEM; n=24).	31
4.3	Effect of different α- linolenic acid (ALA) concentration in Tris extender on frozen-thawed bull spermatozoa parameters (Mean%±SEM; n=24).	32
4.4	Comparison of fatty acid composition of frozen-thawed spermatozoa treated with Tris extender containing different concentrations of α-linolenic acid (ALA) (Mean % ± SEM; n=24).	33
5.1	Effects of different docosahexaenoic acid (DHA) concentration in BioXcell® extender on frozen-thawed spermatozoa parameters of bulls (Mean%±SEM; n=24).	38
5.2	Comparison of fatty acid composition of frozen-thawed spermatozoa treated with different concentrations with docosahexaenoic acid (DHA) in BioXcell® extender (Mean % ± SEM; n=24).	39
5.3	Effects of different docosahexaenoic acid (DHA) concentration in Tris extender on frozen-thawed spermatozoa parameters of bulls (Mean%±SEM; n=24).	40
5.4	Comparison of fatty acid composition of frozen-thawed spermatozoa treated with different concentrations with docosahexaenoic acid (DHA) in Tris® extender (Mean % ± SEM; n=24).	41
6.1	Effects of combination of docosahexaenoic acid (DHA) and α-linolenic acid (ALA) in BioXcell® extender on frozen-thawed spermatozoa parameters in bulls. (Mean%±SEM; n=24).	46
6.2	Comparison of fatty acid composition in different concentrations of docosahexaenoic acid (DHA) and α-linolenic acid (ALA) combination in BioXcell® extender on frozen-thawed bull spermatozoa (Mean % ± SEM; n=24).	47
6.3	Effects combination of docosahexaenoic acid (DHA) and α-linolenic acid (ALA) in Tris extender on frozen- thawed spermatozoa parameters of bulls (Mean%±SEM; n=24).	48
6.4	Comparison of fatty acid composition with different concentrations of docosahexaenoic acid (DHA) and α-linolenic acid (ALA) combination in Tris extender of frozen-thawed bull spermatozoa (Mean % ± SEM, n=24).	49

7.1	Effect of combination of 3ng/ml docosahexaenoic acid (DHA) with α-tocopherol on frozen–thawed Semen characteristics in BioXcell® extender. (Mean % ± SEM, n=20).	55
7.2	Effect of 3ng/ml docosahexaenoic acid (DHA) with α-tocopherol on fatty acid composition of frozen-thawed bull semen in BioXcell® extender (Mean % ± SEM, n=20)	56
7.3	Effect of combination of 10ng/ml docosahexaenoic acid (DHA) with α-tocopherol on frozen-thawed semen characteristics in Tris extender. (Mean % ± SEM, n=20).	57
7.4	Effect of 10ng/ml docosahexaenoic acid (DHA) with α-tocopherol on fatty acid composition of frozen-thawed bull spermatozoa in Tris extender. (Mean % ± SEM, n=20)	58
7.5	Effect of combination of 5ng/ml α-linolenic acid (ALA) with α-tocopherol on frozen–thawed semen characteristics in BioXcell® extender. (Mean % ± SEM, n=20).	59
7.6	Effect of 5ng/ml α-linolenic acid (ALA) with α-tocopherol on fatty acid composition of frozen-thawed bull semen in BioXcell® extender. (Mean % ± SEM, n=20).	60
7.7	Effect of combination of 5ng/ml α-linolenic acid (ALA) with α-tocopherol on Frozen –thawed semen characteristics in Tris extender. (Mean % ± SEM, n=20).	61
7.8	Effect of combination of 5ng/ml α-linolenic acid (ALA) with α-tocopherol on frozen–thawed fatty acid composition of bull in Tris extender. (Mean % ± SEM, n=20).	62

LIST OF FIGURES

Figure		Page
2.1	Reproductive system of the bull	4
2.2	Cross section of a testis.	5
2.3	Phase of spermatogenesis.	7
2.4	Phases of spermiogenesis; Golgi phase, cap phase, acrosome phase, tail transformation phase and maturation phase.	7
2.5	Structure of α- linolenic acid (ALA), docosapentanoic acid (DPA), eicosapentanoic (EPA) and docosahexanoic acid (DHA), respectively.	14
2.6	Metabolism of parent fatty acids ALA (n-3) and LA (n-6) into longer carbon chain fatty acids with relevant enzymatic reactions to form the fatty acids (Lenzi et al., 1996).	15
4.1	Melondialdehyde (MDA) production in frozen-thawed bovine semen treated with α-linolenic acid (ALA) in Bioxcell® extender	31
4.2	Melondialdehyde (MDA) production in frozen-thawed bovine semen supplemented with different levels of α-linolenic acid (ALA) in Tris extender	33
5.1	Melodialdehyde (MDA) production in frozen-thawed bovine semen treated with docosahexaenoic acid (DHA) in BioXcell® extender.	39
5.2	Melondialdehyde (MDA) production in frozen-thawed bovine semen treated with docosahexnoic acid (DHA) in Tris extender.	41
6.1	Melondialdehyde (MDA) production in frozen-thawed bovine semen treated with combination of α-linolenic acid (ALA) and docosahexaenoic acid (DHA) in BioXcell® extender.	47
6.2	Melondialdehyde (MDA) production in frozen-thawed bovine semen treated with combination of docosahexaenoic acid (DHA) and α-linolenic acid (ALA) in Tris extender.	49
7.1	Melondialdehyde (MDA) production in frozen-thawed bovine semen 3ng/ml docosahexaenoicacid (DHA) treated with α-tocopherol in BioXcell® extender.	56
7.2	Melondialdehyde (MDA) production in frozen-thawed bovine semen 3ng/ml DHA treated with α- tocopherol in Tris extender.	58
7.3	Melondialdehyde (MDA) production in frozen-thawed bovine semen 5ng/ml ALA treated with α- tocopherol in BioXcell® extender	60

7.4 Melondialdehyde (MDA) production in frozen-thawed bovine semen supplemented with 5ng/ml ALA and α- tocopherol in Tris extender 62

LIST OF APPENDICES

Appendix		Page
A	Preparation of solution	94
A .1	Eosin nigrosin stain	94
A.2	Hypo osmotic swelling test assay (HOST)	94
A.3	Composition of extender	95
A.4	Comet assay	95
A.5	Malondialdehyde assay	96
A.6	Super oxide dismutase assay	97
B	Detailed tables of fatty acid evaluation	98
B.1	Comparison of fatty acid composition in different concentrations of α-linolenic acid (ALA) in BioXcell® extender frozen-thawed spermatozoa (Mean % ± SEM; n=24)	98
B.2	Comparison of fatty acid composition of frozen-thawed spermatozoa treated with Tris extender containing different concentrations of α-linolenic acid (ALA) (Mean % ± SEM; n=24)	99
C	Detailed tables of fatty acids evaluations	100
C.1	Comparison of fatty acid composition in different concentrations of docosahexaenoic acid (DHA) in BioXcell® extender frozen-thawed spermatozoa. (Mean % ± SEM; n=24)	100
C.2	Comparison of fatty acid composition in different concentrations of docosahexaenoic acid (DHA) in Tris® extender frozen-thawed spermatozoa (Mean % ± SEM; n=24)	101
D	Detailed tables of fatty acids evaluations	102
D.1	Comparison of fatty acid composition in different concentrations of docosahexaenoic acid (DHA) and α-linolenic acid (ALA) combination in BioXcell® extender frozen-thawed spermatozoa (Mean % ± SEM; n=24)	102
D.2	Comparison of fatty acid composition in different concentrations of docosahexaenoic acid (DHA) and α-linolenic acid (ALA) combination in BioXcell® extender frozen-thawed spermatozoa (Mean % ± SEM, n=24)	103
E	Detailed tables of fatty acids evaluations	104
E.1	Effect of 3ng/ml docosahexaenoicacid (DHA) with α-tocopherol on fatty acid composition of frozen-thawed bull	104

semen in BioXcell® extender (Mean % ± SEM, n=20)

E.2 Effect of 10ng/ml docosahexaenoic acid (DHA) with α-tocopherol on fatty acid composition of frozen-thawed bull semen in Tris extender. (Mean % ± SEM, n=20) 105

E.3 Effect of 5ng/ml α-linolenic acid (ALA) with α-tocopherol on fatty acid composition of frozen-thawed bull semen in BioXcell® extender. (Mean % ± SEM, n=20) 106

E.4 Effect of combination of 5ng/ml α-linolenic acid (ALA) with α-tocopherol on frozen–thawed fatty acid composition of bull in Tris extender. (Mean % ± SEM, n=20) 107

LISIT OF ABBREVIATIONS

AA	Arachidonic acid
ADP	Adenosine diphosphate
ALA	Alpha Linolenic acid
AI	Artificial insemination
AOAC	Association of official analytical chemists
ATP	Adenosine triphosphate
BCS	Body condition score
BF_3	Boron Tri-fluride
BSP	Bovine seminal protein
BTB	Blood testes barrier
CASA	Computer assisted semen analyzer
Ca++	Calcium
DHA	Docosahexanoic acid,
DPA	Docosapentanoic acid
DMF	Dimethyl formamide
DNA	Deoxyribonucleic acid
DVS	Department of Veterinary Services
EG	Ethylene glycol
EPA	Eicosapentaenoicacid
FAO	Food and Agriculture organization
FA	Fatty acid
FAME	Fatty acid methyl esters
FID	Flame ionization detector
FMP	Forward motility protein
GLM	General linear model
GPX	Glutathione peroxidase
GPR	Glutathione reductase
HOST	Hypo osmotic swelling test
HO_2•	Hydroperoxyl radical
H_2O_2	Hydrogen peroxide
K^+	Potassium
KOH	Potassium Hydrooxide
LA	Linoleic acid
LDL	Low density lipoprotein
LPO	Lipid peroxidation
MDA	Melondialdyde
MF	Methyl formamide
ML	Mililiter
mM	Milimoles
MMP	Mitochondrial membrane potential
MUFAs	Monounsaturated fatty acids
NG	Nanogram
OA	Oleic acid
$OH^{\bullet}$	Hydroxyl radical
OS	Oxidative stress
PKC	Palm kernel cake
PBS	Phosphate buffer solution
PUFAs	Polyunsaturated fatty acids

PA	Palmitic acid
PGE	Prostaglandins E
PSA	Phosphatase and prostate-specific antigen
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SA	Stearic acid
SDS	Sodium dodecyl sulfate
SFA	Saturated fatty acids
SEM	Standard error of the mean
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
UPM	Universiti Putra Malaysia

CHAPTER 1

INTRODUCTION

1.1 Background

Cattle, domesticated largely throughout the world, belong to the subfamily bovine, genus Bos and are generally classified as *Bos primigenius*. Cattle are domesticated for several purposes including meat, milk, drought power, production of leather and breeding. The estimated population of cattle is about 1.467 billion in the world (FAO, 2015). Demand of livestock products are increasing throughout the world, due to the shifting of consumption patterns towards livestock products and increase in human population. Meat and dairy consumption over the last decade increased at a rate of 3-5% annually in Asian countries (FAO, 2013). In Malaysia, the total population of cattle is about 784,684, which is 23.5% of its total animal population (DVS, 2012). Although there is a slight decline in the total number of cattle during recent years, the country is improving its self-sufficiency in milk and meat production (DVS, 2012).

Artificial insemination (AI) has modernized animal production through genetic improvement. Cryopreservation supports beneficial uses of AI in terms of short and long term storage and easy transportation of semen throughout the globe (Baily *et al*., 2000; Kaka *et al*., 2012). However, cryopreservation has some disadvantages such as reduction in the viability of frozen spermatozoa compared to fresh semen by increasing spermatozoa deaths and impairing the functions of live spermatozoa (Watson, 2000; Celeghini *et al*., 2008). Cryopreservation mainly affects motility and integrity of plasma membrane of spermatozoa (Hammerstedt *et al*., 1990; Parks and Graham, 1992, Watson, 1995; Yoshida, 2000). Therefore, a higher concentration of frozen semen is needed to obtain the fertilization rate that is comparable with fresh semen (Watson, 2000). Furthermore, both freezing and thawing have thermal, oxidative and osmotic effects, which cause remarkable mechanical damage to the spermatozoa membrane (Hammerstedt *et al*., 1990; Holt, 2000; Thuwanut *et al*., 2008). Cryopreservation also causes redistribution of membrane lipids and alters lipid-lipid and lipid-protein bonds (Royere *et al*., 1996: Marti *et al*., 2003; Chakrabarty *et al*., 2007). It also reduces head size of bull spermatozoa and cuases other irreversible alterations on membrane and acrosome structures (Gravance *et al*., 1998; Chakrabarty *et al*., 2007). Cooling rapidly to 0^{o}C causes severe damage to the spermatozoa (cold shock). However, sensitivity to cold shock varies with animal species and ratio of unsaturated and saturated fatty acid present in the spermatozoa plasma membrane.

Fatty acids are vital components of phospholipids and glycerides. Fatty acids may be saturated or unsaturated; unsaturated fatty acids can be divided as monounsaturated (MUFAs) or polyunsaturated (PUFAs). Polyunsaturated fatty acids are further classified as omega-3, 6 and 9 unsaturated fatty acids depending on the site of the first double bond from the methyl terminal (Lenzi *et al*., 1996). Bulls, boars and humans obtain them from their diet and are synthesized in the body by *de novo* synthesis (Lenzi *et al*., 1996).

Bull, ram, and boar spermatozoa have high concentrations of omega-3 fatty acid (White, 1993) which maintain the structure and function of the plasma membrane during freezing and thawing, improve fluidity of the plasma membrane, prevent formation of ice crystals, osmotic and chilling injuries, cytoplasmic fractures, as well as cytoskeleton and genomic abnormalities (Isachenko, 2003; Robinson *et al*., 2006). Cryopreservation changes the fatty acid structure from crystalline phase to rigid (gel) structure (Watson, 2000) and decreases omega-3 fatty acid concentration (Chakrabarty *et al*., 2007; Nasiri *et al*., 2012). Previous studies focused to maintain omega-3 fatty acid concentration by including fatty acids in the diet of different animals species (Comhaire and Mahmoud, 2003; Keirnan *et al*., 2013). Supplementation of dietary oils (alternative sources of omega-3 fatty acids) have successfully modified the fatty acid profile of the spermatozoa plasma membrane in many species with varying levels of success (Kelso *et al*., 1997a; Comhaire *et al*., 2000; Rooke *et al*., 2001; Castellano *et al*., 2010; Gholami *et al*., 2010).

As the fatty acids are considered susceptible of lipid peroxidation, therefore, different antioxidant is being used to to diminish the lipid peroxidation (LPO). α-tocopherol is lipid soluble and considered most effective antioxidant. It decreases formation of free radical in the semen and spermatozoa of bull, in results reduces lipid peroxidation and improves frozen thawed quality of bull spermatozoa (Nasiri *et al*., 2012).

In vitro models provide an opportunity to study the effect of exogenous fatty acids on frozen-thawed bull spermatozoa. In humans, the *in vitro* addition of unsaturated fatty acids (arachidonic, linoleic, docosahexaenoic, palmitoleic and oleic) have increased lipid peroxidation of spermatozoa (Aitken and Baker, 2006; Koppers *et al*., 2010). However, in boars, motility, viability and acrosome reactions were improved after exogenous unsaturated fatty acid (oleic and linoleic, combination of oleic and arachidonic acid) *in vitro* supplementation to boar spermatozoa (Hossain *et al*., 2007). In bulls, spermatozoa viability and motility were maintained with addition of α-linolenic acid (ALA) but declined with the supplementation of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in semen chilled at 5^{o}C for seven days (Kiernan *et al*., 2013).

There is limited research work published on the effect of unsaturated fatty acids on the quality of frozen-thawed bull spermatozoa. Therefore, the main aim of this study was to investigate if exogenous α-linolenic acid (ALA) and docosahexaenoic acid (DHA) with and without α-tocopherol can ameliorate the quality of frozen-thawed bull spermatozoa.

1.2 Objectives

The specific objectives of this study were

1. To determine the effects of exogenous α-linolenic acid (ALA) and docosahexaenoic acid (DHA) added into semen extenders on the quality of frozen-thawed bull spermatozoa.
2. To determine the effects of combined supplementation of ALA and DHA on the quality of frozen-thawed bull spermatozoa.
3. To enhance the quality of frozen-thawed bull spermatozoa with addition of α-tocopherol in combination with ALA and DHA.

1.3 Hypothesis

Hypothesis for this study is that the supplementation of ALA and DHA with or without α-tocopherol would improve motility, viability, membrane integrity, acrosome integrity, fatty acid composition, reduce DNA damage, and diminish lipid peroxidation of frozen-thawed bull semen.

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