UNIVERSITI PUTRA MALAYSIA

EFFECTS OF HORMONAL AND OOCYTE ACTIVATION TREATMENTS ON IN VITRO EMBRYO DEVELOPMENT AND INTERSPECIES HYBRIDIZATION OF BOVIDAE EMBRYOS FOLLOWING INTRACYTOPLASMIC SPERM INJECTION

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By

MOJTABA DASHTIZAD

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

September 2011

This thesis is exclusively dedicated to

My Parents,

Ahmad & Sakineh

My Wife and My Son,

Elham & Barbod

My Sister and My Brother,

Mitra & Mehrdad

My wife's Parents

Ghorban & Esmat

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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September 2011

Chairman: Professor Abd Wahid Haron, DVM, PhD

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In countries in the tropics such as Malaysia, local cattle are not suitable for the feedlot production system because of their poor meat production performance. Therefore, hybridization of domestic cattle with local wild bovids (*Bos gaurus hubbacki* and *Bos javanicus*) which are able to thrive very well in the tropics and at the same time develop a large muscular body and sturdy limbs may be an alternative way to improve livestock production system in Malaysia. *In vitro* embryo production (IVEP) technique plays a crucial role to manage hybridization and therefore may improve the desirable characteristics of livestock. Although IVEP techniques have received great attention in recent years, the rate of transferable blastocysts is quite low.

In the present study, to improve the yield of *in vitro* produced bovine embryos, the effect of different concentrations of insulin and ghrelin hormones on *in vitro* development of

bovine oocytes was evaluated. Insulin at 10 µg/ml significantly improved the proportion of immature bovine oocytes that reached the metaphase II stage. Furthermore, addition of 10 µg/ml insulin into the culture medium showed a positive effect on bovine embryo development until the morula stage. It was also found that supplementation with 50 ng/ml ghrelin in maturation and culture media enhanced nuclear maturation and blastocyst formation rates, respectively. Although inclusion of 50 ng/ml ghrelin in the culture medium strikingly increased the population of inner cell mass (ICM), trophoectoderm (TE) and total cell number cells of bovine blastocysts, no significant difference was detected in ratio of ICM: total cell number per blastocysts between the different treatment groups compared to the control.

In order to improve the *in vitro* bovine embryo development following intracytoplasmic sperm injection (ICSI), a variety of single and combined artificial oocyte activation treatments were assessed. Data analysis showed that single artificial oocyte activation by strontium chloride (20 mM) and combination of strontium chloride followed by ethanol (7%) significantly increased the cleavage rate and *in vitro* bovine embryo development ($p<0.05$) following ICSI. However, no significant difference was detected between treatments regarding the quality of the blastocysts evaluated by differential staining of ICM and TE cells.

Modified maturation and culture solutions and artificial oocyte activation (AOA) methods resulted from the present study were applied to assess the possibility and efficiency of advanced assisted reproductive techniques on *in vitro* production of hybrid

bovine embryos. Furthermore, the effects of different diameters of the sperm injection needle on *in vitro* development of bovine embryos were also evaluated.

The results indicated that frozen-thawed *Bos gaurus hubbacki* and *Bos javanicus* sperm were able to fertilize *in vitro* matured bovine oocytes and produced interspecies hybrid embryos following both *in vitro* fertilization (IVF) and ICSI methods at acceptable rates. In addition, the inner diameter of the injection pipette had significant effects on the *in vitro* production of hybrid embryos.

In conclusion, the *in vitro* mass production of hybrid embryos that were successfully achieved in the present study can be considered as the first step to introduce a novel source of meat for the future.

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

RAWATAN HORMON DAN PENGAKTIFAN OOSIT DALAM PEMBANGUNAN EMBRIO DAN HIBRIDASI INTERSPESIS EMBRIO LEMBU SELEPAS SUNTIKAN INTRASITOPLASMIK SPERMA

Oleh

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Bagi kawasan yang beriklim tropika seperti Malaysia, kebanyakan lembu ternakan tempatan tidak mempunyai keupayaan penghasilan daging yang sesuai yang boleh digunakan dalam pengeluaran makanan. Maka, penghibridan lembu ternakan domestik dengan lembu tempatan liar (*Bos gaurus hubbacki* dan *Bos javanicus*) yang mampu membiak dengan subur dan menghasilkan badan berotot besar dan tegap dalam iklim tropika mungkin boleh dijadikan sebagai alternatif dalam meningkatkan sistem pengeluaran ternakan di Malaysia. Teknik penghasilan embrio *in vitro* (IVEP) memainkan peranan penting dalam penghibridan dan secara tidak langsung meningkatkan ciri yang diingini dalam pengeluaran ternakan. Walaupun teknik IVEP telah mula diberi perhatian dalam tempoh beberapa tahun kebelakangan ini, namun kadar blastosista yang boleh dipindahkan masih lagi pada tahap yang rendah.

Dalam kajian ini, kesan kepekatan hormon insulin dan ghrelin yang berbeza dalam perkembangan *in vitro* bagi oosit lembu telah dikaji untuk meningkatkan penghasilan embrio secara *in vitro*. Kepekatan insulin pada 10 µg/mL berjaya meningkatkan kadar perpindahan oosit lembu tidak matang ke peringkat metafasa II. Disamping itu, penambahan 10 µg/mL insulin ke dalam media kultur juga menghasilkan kesan positif ke atas perkembangan embrio lembu sehingga ke peringkat morula. Manakala, penambahan 50 ng/mL ghrelin dalam media pematangan *in vitro* (IVM) dan kultur *in vitro* (IVC) berupaya meningkatkan kadar kematangan nukleus dan kadar pembentukan blastosista. Walaupun penambahan 50 ng/mL ghrelin dalam media IVC telah meningkatkan populasi jisim sel dalam (ICM), trofektoderm (TE) dan bilangan sel blastosis lembu dengan ketara, namun tiada perbezaan yang signifikan di antara nisbah ICM dengan bilangan sel blastosista antara kumpulan rawatan dengan kawalan.

Di samping itu, untuk meningkatkan perkembangan embrio lembu *in vitro* berikutan selepas suntikan intrasitoplasmik sperma (ICSI), pelbagai rawatan tunggal serta rawatan gabungan dalam pengaktifan oosit tiruan telah dikaji. Data menunjukkan pengaktifan oosit tiruan tunggal oleh strontium klorida (20 mM) dan kombinasi strontium klorida dengan etanol (7%) telah meningkatkan kadar pembelahan dan perkembangan embrio lembu *in vitro* ($p < 0.05$) dalam ICSI. Namun, tiada perbezaan signifikan dengan kumpulan rawatan berdasarkan pencerapan kualiti blastosis melalui pewarnaan pembezaan bagi sel ICM dan TE.

Larutan IVM dan IVC yang telah diubahsuai serta kaedah pengaktifan oosit buatan (AOA) berikutan kajian ini telah diaplikasi untuk menilai keupayaan dan kecekapan

bioteknologi reproduktif berbantu termaju dalam penghasilan embrio lembu secara *in vitro*. Selain itu, kesan diameter jarum suntikan sperma yang berbeza dalam perkembangan *in vitro* bagi embrio lembu juga telah dikaji. Hasil kajian menunjukkan sperma *Bos gaurus hubbacki* and *Bos javanicus* yang telah dibeku-cairkan berupaya bersenyawa dengan oosit lembu *in vitro* yang matang dan menghasilkan embrio hibrid antara spesies pada kadar yang bersesuaian menggunakan kaedah persenyawaan *in vitro* (IVF) dan ICSI. Diameter dalaman pipet suntikan memberi kesan terhadap penghasilan embrio hibrid *in vitro*.

Kesimpulannya, penghasilan embrio hibrid *in vitro* secara besar-besaran telah berjaya dicapai dalam kajian ini dan dijadikan sebagai satu permulaan bagi memperkenalkan sumber baru bagi mengisi keperluan daging untuk masa hadapan.

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I certify that a Thesis Examination Committee has met on 20 September 2011 to conduct the final examination of Mojtaba Dashtizad on his thesis entitled "Effects of Hormonal And Oocyte Activation Treatments On *In vitro* Embryo Development And Interspecies Hybridization of *Bovidae* Embryos Following Intracytoplasmic Sperm Injection" 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.

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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 institution.

MOJTABA DASHTIZAD

Date: 20 September 2011

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LIST OF ABBREVIATIONS

µm	Micrometer
µM	Micromolar
A I	Anaphase I (First anaphase)
AI	Artificial insemination
AOA	Artificial oocyte activation
ART	Assisted reproductive technology
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
Ca	Calcium
cAMP	Cyclic adenosine monophosphate
CC	Cumulus cells
CG	Cortical granule
COCs	Cumulus oocyte complexes
conc	Concentration
CS	Calf serum
CSF	Cytostatic factor
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
dpi	Day(s) post-insemination
DS	Denuding solution
E_2	Estradiol-17β
EGF	Epidermal growth factor
ERK	Extracellular-regulated kinase
FAF	Fatty acid free
FSH	Follicle stimulating hormone
g	Gram(s)
GAGs	Glycosaminoglycans

GH	Growth hormone
GHS-R	Growth hormone (GH) secretagogue receptor
GnRH	Gonadotrophin-releasing hormone
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
h	Hour(s)
Hepes	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
hpi	Hour(s) post-insemination
HS	Handling solution
ICM	Inner cell mass
ICSI	Intracytoplasmic sperm injection
ID	Inner diameter
IGF-I	Insulin-like growth factor I
IVC	*In vitro* culture
IVEP	*In vitro* embryo production
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
IVP	*In vitro* production
Kd	Kilo dalton
Kg	Kilogram
LH	Luteinizing hormone
M	Molarity/molar (moles per liter of solution)
M I	Metaphase I
M II	Metaphase II
MAPK	Mitogen-activated protein kinase
min	Minutes
ml	Milliliter (S)
mM	Millmolar
MM	Maturation medium
mOsm	Milliosmole

MPF	Maturation promoting factor
mRNA	Messenger ribonucleic acid
OD	Outer diameter
PB	Polar body
PB-1	First polar body
PBS	Phosphate-buffered saline
PHE	Penicillamine-hypotaurine-epinephrine
PN	pronucleus
PS	Pronase solution
PVA	Polyvinyl alcohol
PVP	Polyvinyl pyrolidone
RT	Room temperature
SOF	Synthetic oviductal fluid
SOFaaci	Synthetic oviductal fluid with amino acids, sodium citrate and myo-inositol
SS	Slicing solution
T I	Telophase I
TALP	Tyrode's albumin lactate pyruvate
TC	Total cell
TCM199	Tissue culture medium 199
TE	Trophoectoderm
TS	Transfer solution
WS	Working solution
ZP	Zona pellucida

CHAPTER 1

INTRODUCTION

The demand for livestock products continues to rise due to the steady growth of the world's population. Over the last two decades, various assisted reproductive methods have been acknowledged to increase the rate of transferable embryos and birth of healthy offsprings (Zhang et al., 1991; Ward et al., 2002). Nowadays, these techniques that are commonly applied in cattle are increasingly being used to enhance reproductive capacity, improve and preserve livestock genetics and develop new products such as transgenic or cloned animals. About 15% of bovine embryos are produced by *in vitro* embryo production (IVEP) throughout the world (Mapletoft and Hasler, 2005).

In tropical counties such as Malaysia, local cattle that are managed under the feedlot production system do not produce the expected dressing percentage. It is believed that heat stress as well as high temperatures and humidity, adversely affect livestock production performance (Johnston et al., 1994b). In addition, importing feeder calves for fattening is expensive. Furthermore, imported cattle that are introduced to tropical countries are susceptible to heat stress and parasite infestation (Owiny et al., 2009b). On the other hand, there are native wild bovine species which are able to thrive and develop a large muscular body and sturdy limbs in the tropical climate even with low-quality feed (Boediono *et al*., 2003). Unfortunately, these species are mostly listed as a critically endangered species according to the 2000 IUCN Red List (2010).

Therefore, hybridization of domestic cattle with local wild bovine may be an alternative way to improve livestock production system in Malaysia. Crossbreeding with domestic cattle is advocated as a means of enhancing tolerance to the hot and humid tropics and diseases. It means that the hybrids embrace the advantages of both vital factors; performance and resistance (Bradley *et al*., 1994). Advanced assisted reproductive techniques and gamete manipulation are becoming common ways to control hybridization and improvement of desirable properties and livestock production of both captive and natural population via heterosis (David, 1992; Wildt *et al*., 1992; Forsdyke, 2000; Boediono *et al*., 2003; Mastromonaco *et al*., 2007).

Development of efficient *in vitro* culture systems to support all stages of embryo development would be valuable towards producing high-quality embryos. Failure to achieve high success rates in bovine embryo cultures compared to other species may indicate the absence of vital factors in the culture medium which are normally present *in vivo*. Therefore, it is necessary to modify the culture medium and conditions to support higher percentage of blastocyst development *in vitro*. Several factors such as hormones, proteins and growth factors supplemented in the culture medium may have a crucial role on the outcome of IVEP (Bavister *et al*., 1992). Hence, the first part of the study carried out was to improve the yield of *in vitro* bovine embryo production by incorporation of insulin and ghrelin hormones in the culture system.

Insulin binds to the cell surface receptor and its action is receptor-mediated. It has been demonstrated that insulin stimulated glucose and amino acid uptake (Kaye and Harveyt, 1995) as well as protein synthesis (Harvey and Kaye, 1988).

Insulin receptors have also been detected in all stages of bovine embryos (Makarevich and Markkula, 2002). Therefore, it is expected that insulin have some beneficial effects on *in vitro* bovine embryo production.

Ghrelin is a novel hormone that is mainly secreted from the gastric mucosa into the blood circulation (Kojima *et al*., 1999; Kojima *et al*., 2001). Ghrelin increased the expression of mitogen-activated protein kinase (MAPK) in bovine oocytes (Popelkova, 2006). The MAPK pathway is involved in the regulation of microtubule organization during meiosis, spindle morphology, and maintenance of maturation promoting factor activity in bovine oocytes (Gordo *et al*., 2001). Ghrelin receptors also have been detected in mammalian ovaries. Therefore, it is rational to hypothesize that ghrelin affects the regulation of the reproductive system. Based on literature, information concerning the effects of ghrelin on nuclear maturation of mammalian oocytes and development of fertilized embryos *in vitro* are still at its infancy and thus, require further investigation.

Incubation of oocytes and sperm cells is a common procedure for fertilization in IVEP. However, this is not always the most effective method. For successful cleavage following *in vitro* fertilization (IVF), at least thousands of spermatozoa with adequate motility, morphology, and capacity are required. Usually, one of the reasons for IVF failure is associated with abnormal sperm having very poor motility and the ultimate result is inability of the sperm cells to penetrate the oocytes both *in vivo* and *in vitro*, while potentially they are able to contribute to embryonic development.

In order to eliminate preliminary and natural steps that occur during normal fertilization, mechanical injection of a single sperm into the cytoplasm of mature oocytes could be considered as an efficient alternative for common IVF procedure. Intracytoplasmic sperm injection (ICSI) can create an opportunity of using semen with poor motility or no *in vivo* fertility. Furthermore, in case of genetically and/or economically valuable animals, wild animals and endangered species, there are limited semen sources and their semen can be extremely expensive. Therefore, effective utilization of spermatozoa for livestock improvement and propagation of valuable animals by using ICSI technique seems worthwhile. In some species, such as mice and humans, ICSI by itself is able to act as a sufficient trigger to activate the oocyte, inducing sperm head decondensation, male pronucleus formation, resumption of meiosis and finally initiation of embryo development (Galli et al., 2003b). However, in cattle, sperm injection and presence of spermatozoon in the ooplasm cannot provide enough stimulation to induce oocyte activation (Simone *et al*., 2005).

In order to enhance fertilization and cleavage rates of bovine oocytes and increase the level of cytoplasmic calcium ions, the ICSI procedure can be accompanied by various artificial oocyte activation treatments such as electrical pulse (Hwang *et al*., 2000), ethanol (Hamano *et al*., 1999), calcium ionophore A23187 (Goto, 1993) or ionomicin (Rho *et al*., 1998). The mechanism for each regimen is different. For instance, ionomicin induces a single pulse to increase the level of calcium. Strontium chloride has shown to promote multiple free calcium oscillation similar to the method that takes place in normal fertilization (Okada *et al*., 2003). Although it has been reported that strontium chloride has been efficiently applied for parthenogenetic activation in mice (Wakayama

et al., 1998) and bovine (Simone Cristina *et al*., 2004) to produce intracellular calcium oscillations the same with that induced by the spermatozoa (Bos-Mikich et al., 1995b), there is no report regarding the use of strontium chloride to activate bovine oocytes following ICSI. Oocyte activation could be optimized by comparing different types of treatments to establish the most efficient one for reprogramming of the nucleus after fertilization (Marcus, 1990). Therefore, the second part of the present study was carried out to evaluate the performance of strontium chloride in artificial activation of ICSI-bovine oocytes compared with calcium ionophore A23187 and ethanol which are routinely used.

Based on all optimized protocols in this study, the last part of the study was carried out to investigate the possibility and efficiency of IVF and ICSI techniques, on *in vitro* development of hybrid embryos using bovine oocytes and sperm from two different wild bovine species, *Bos gaurus hubbacki* and *Bos javanicus*.

Bos gaurus hubbacki (Malayan gaur), locally known as seladang, is the heaviest species (grown male weigh over 1000 kg and stands up to 2.2 meters high at the shoulder) of extant wild cattle inhabiting deciduous and tropical rainforests throughout south eastern Asia (Johnston et al., 1994a). *Bos javanicus* (Banteng) is a vigorous animal which is able to tolerate a variety of climates, from warm and wet to hot and dry (Sansinena *et al*., 2005). Their genetic endowment for such tolerance could be considered as a valuable trait for hybridization program to improve livestock. The ultimate aim for this hybridization is to improve the desirable characteristics of original breeds and improve genetic characteristics of domesticated stock via heterosis.

Therefore, the objectives of this study were:

1. To determine the effects of insulin and ghrelin supplementation on nuclear maturation of immature bovine oocytes and *in vitro* development of bovine preimplantation embryos,
2. To evaluate the influence of ghrelin on viability and quality of bovine *in vitro* produced embryos,
3. To evaluate different activation regimens (strontium chloride, calcium ionophore, and ethanol) on *in vitro* developmental competence of ICSI-derived bovine embryos,
4. To produce hybrid embryos, using bovine oocytes with sperm from *Bos gaurus hubbacki* and *Bos javanicus* following *in vitro* fertilization and ICSI, and
5. To compare *in vitro* development and viability of hybrid and bovine cattle IVF and ICSI-derived embryos.

With the above objectives, the following major experiments were conducted:

1. Improvement of *in vitro* bovine embryo production by incorporation of insulin and ghrelin hormones in the culture system,
2. Effect of different oocyte activation treatments on development of preimplantation bovine embryos following intracytoplasmic sperm injection, and
3. Interspecies hybridization of bovidae embryos using advanced assisted reproductive biotechnologies.

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