UNIVERSITI PUTRA MALAYSIA

ASSESSMENT OF SPERM CRYOPRESERVATIVES AND OOCYTE VITRIFICATION FOR IN VITRO EMBRYO PRODUCTION IN CATTLE

SALMAN HAMMADI GHAREEB

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By

SALMAN HAMMADI GHAREEB

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

December 2017

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DEDICATION

Dedicated to my father (Almarhum) and my mother (Almarhuma), to my beloved wife and my kids Mayar, Acif and Layan for their supplication, patience and understanding

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

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Chairman : Professor Abd Wahid Haron, PhD
Faculty : Veterinary Medicine

Animal breeding programmes and assisted reproductive techniques (ART) such as artificial insemination (AI), embryo transfer (ET) and intra-cytoplasmic sperm injection (ICSI) are extremely dependant on the quality of sperm and oocytes used. Furthermore, fertility ratios still need to be improved particularly those related to the cryopreserved sperms and vitrified oocyte. In this study, a number of experiments were carried out to improve the quality of cryopreserved semen and vitrified immature oocytes and evaluation of *in vitro* embryo production as well. Seven bull semen extenders were prepared and used to extend a total of 102 ejaculates that were collected from twelve mature healthy fertile Brangus-Herford bulls by automatic electro-stimulation at the animal farm of the Universiti Putra Malaysia. On the other hand, 180 ovaries were collected from slaughtered local cattle at Banting Abattoir, Malaysia. Post-thaw semen evaluation were evaluated for the extending capacity of seven different types of extenders implemented in bull semen cryopreservation; these extenders were AndroMed®, BioXcell®, Triladyl®, Tris-egg yolk based extenders 20%, Tris-egg yolk plasma with concentration of 10%, Tris-egg yolk plasma with concentration of 15 % and Tris-egg yolk plasma with concentration of 20% on the sperm's motility, viability, morphology, DNA integrity, plasma membrane integrity and acrosome integrity. Also, the cryopreservation process was conducted in two regimes of fresh semen preparation prior to subjecting to cryopreservation. These were seminal plasma being removed from fresh semen samples and fresh semen samples with their seminal plasma. Semen samples were collected using electrical stimulation by an automatic electro-ejaculator (AEE), and semen quality was analysed using computer assisted semen analyser (CASA). The Eosin-Nigrosine staining technique was applied to evaluate the morphology, viability and acrosome intergrity. The DNA integrity was evaluated using a modified Acridine orange method. Amongst the three commercial extenders, AndroMed®, BioXcell®, Triladyl® and the Tris-egg yolk based extenders, the AndroMed® extender produced the highest extending capacity

to the sperm's motility, morphology, DNA integrity, plasma membrane integrity and acrosome integrity. BioXcell® and Tris-egg yolk based extenders had the best capacity to protect the sperm's viability. These results, therefore, favour AndroMed® over the rest of the other extenders used in post-thaw sperm parameters with seminal plasma. The AndroMed®, Triladyl® and Tris –egg yolk extender were highest in extending capacity than BioXcell® at the level of post-thaw semen evaluation and preserving semen without seminal plasma.

Among the egg yolk extenders, the egg yolk plasma extender 10% produced the highest extending capacity to the semen plasma membrane integrity, viability, and motility, while the Tris-egg yolk based extender 20% and Tris-egg yolk plasma 10% had the best capacity to protect the semen's morphology and acrosome integrity. These results, therefore, favour the concentration of Tris-egg yolk plasma 20% over the rest of the other extender concentration egg yolk used in extending ejaculated semen with seminal plasma. In the study that involved seminal plasma removal, the spermatozoa was also evaluated for its qualities using Tris-egg yolk based extenders 20%, Tris-egg yolk plasma with concentration 10% , Tris-egg yolk plasma with concentration 15% and Tris-egg yolk plasma with concentration 20%. The egg yolk plasma with concentration 15% extender gave a better extending capacity to the semen viability and acrosome integrity, while Tris-egg yolk based extender 20% had the best capacity to protect the semen's morphology and plasma membrane integrity. This result, therefore, favours the concentration of the egg yolk plasma with concentration 15% and Tris-egg yolk based extender 20% over the rest of the other extender concentrations of egg yolk plasma.

In the immature oocyte study, oocytes were harvested from bovine ovaries collected from local abattoir (Banting, Malaysia), then transported in thermos containers at 37°C (phosphate buffer saline) to the Theriogenology and Cytogenetic laboratory within 2-3 hours post-slaughtering. Slicing method of ovaries were applied to harvest the immature oocytes. Pipetting of the considered oocytes was done from the handling medium into the washing medium. This study aimed to study the effect of different vitrification exposure times of 15 sec, 30 sec and 45 sec of immature bovine oocytes and their ability to fertilise and produce an embryos after application of *In vitro* fertilisation technology. Ethylene Glycol (EG) and Dimethylsulfoxide (DMSO) were used as a combination of two cryoprotectants. Two main cryodevices were also used; they were open pulled straws (OPS) and closed French straws (CFS). OPS were modified locally by using hot plate and pulled manually. Vitrified-warmed immature oocytes were evaluated morphologically under epiflourocent microscopy. The viable oocytes were subjected into the maturation media, mainly composed of TCM199+HEPES supplemented with 10% FCS and 10 ng/ml epidermal growth factor (EGF). Polar body extrusion was observed 22 hr post IVM. *In vitro* fertilization (IVF) was applied using direct infusion of frozen-thawed bull semen extended in AndroMed® (as the best extender described in first experiment; Chapter Four). The frozen-thawed bull semen was collected at the bottom of Percoll solution (45% over 90%) then washed in Tyrodes medium. The viable spermatozoa were diluted in the appropriate volume of fertilization medium to achieve a final concentration of 2×10^6

sperm/ml. A 250 µl aliquot of this suspension was added to each well containing oocytes for a final concentration of sperm cells of 1×10^6 sperm/ml. Spermatozoa and oocytes were co-incubated for 18-22 h at 38.8 °C in a humidified atmosphere of 5% CO_2 in air. For *in vitro* culture (IVC) presumptive zygotes were vortexed and washed three times in HEPES-buffered TCM199 containing 0.4 g/l BSA and 4 µg/ml gentamicin, to remove cumulus cells and attached spermatozoa. Zygotes were washed two times in SOF medium supplemented with BME amino acids (50×), MEM amino acids (100×), 3 mM pyruvate, 6 mg/ml fatty acid free BSA and 5% FBS and transferred by groups of 25 into four-well petri dishes containing 50 µl drops of culture medium covered with 700 µl of mineral oil. This study showed that the immature bovine oocytes can be vitrified at the GV-stage with our optimized protocol and that the vitrified-warmed GV-oocytes could be matured and *in vitro* fertilized and subsequently in vitro embryo development. Also, the vitrification of bovine immature oocytes in the solutions composed of 15% EG + 15% DMSO using OPS method achieved the best viability, the highest maturation, and cleavage in our conditions after IVM and IVF despite a lower recovery rate than the other group. Data were analysed using SPSS software system (Version 12.0, SPSS, Chicago, IL). Study parameters were analysed using a 2-way factorial analysis of variance (ANOVA). Values were expressed as mean ± standard error of the mean (S.E.M). The level of statistical significance was at $P<0.05$.

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

PENILIAIAN UNTUK SPERMA KRIOAWETAN DAN OOSIT PENGEKAAN *IN VITRO* PRODUKSI EMBRIO LEMBU

Oleh

SALMAN HAMMADI GHAREEB

Disember 2017

Pengerusi : Profesor Abd Wahid Haron, PhD
Fakulti : Perubatan Veterinar

Program pembiakan haiwan dan teknik pembiakan dibantu (ART) seperti permanian beradas (AI), pemindahan embrio (ET) dan suntikan sperma intra-sitoplasma (ICSI) amat bergantung kepada kualiti sperma dan oosit yang digunakan. Tambahan pula, nisbah kesuburan masih perlu diperbaiki terutamanya yang berkaitan dengan sperma dikrioawet dan oosit divitfrifikasikan. Dalam kajian ini, beberapa eksperimen telah dijalankan untuk meningkatkan kualiti semen dikrioawet dan oosit divitrifikasikan yang tidak matang dan penilaian pengeluaran embrio *in vitro* juga. Semasa pembaikan sperma dikrioawet, empat eksperimen utama telah dijalankan. Tujuh penyambung semen lembu jantan telah disediakan dan digunakan untuk menyambungkan sejumlah 102 pancutan air mani yang dikumpul daripada dua belas lembu jantan Brangus-Herford sihat matang subur oleh elektro-rangsangan automatik di ladang haiwan Universiti Putra Malaysia. Sebaliknya, 180 ovari dikumpulkan dari lembu tempatan yang disembelih di Banting Abattoir, Malaysia. Penilaian air mani selepas-dicairkan telah dinilai untuk kapasiti menyambung tujuh jenis penyambung yang dilaksanakan untuk krioawetan semen lembu jantan; penyambung-penyambung ini ialah AndroMed®, BioXcell®, Triladyl®, penyambung berasaskan Tris-kuning telur penyambung 20%, plasma Tris-kuning telur dengan kepekatan 10%, plasma Tris-kuning telur dengan kepekatan 15% dan plasma Tris-kuning telur dengan kepekatan 20% ke atas motiliti, daya maju, morfologi, integriti DNA, integriti membran plasma dan integriti akrosom sperma. Juga, proses krioawetan telah dijalankan mengikut dua rejim penyediaan semen segar sebelum menjalankan krioawetan. Ini adalah pengeluaran plasma semen daripada sampel semen segar dan sampel semen yang segar dengan plasma semennya. Sampel semen dikumpul dengan menggunakan rangsangan elektrik dengan elektro-ejakulator automatik (AEE), dan kualiti semen dianalisis menggunakan penganalisis semen dibantu komputer (CASA). Teknik pewarnaan Eosin-Nigrosin digunakan untuk menilai morfologi, daya maju dan intergrity akrosom. Integriti DNA dinilai menggunakan kaedah oren Acridine

diubahsuai. Di antara tiga penyambung komersial, AndroMed®, BioXcell®, Triladyl® dan penyambung-penyambung berasaskan Tris-kuning telur, penyambung AndroMed® menghasilkan kapasiti penyambungan tertinggi kepada motiliti, morfologi, integriti DNA, integriti membran plasma dan integriti akrosom sperma. Penyambung berdasarkan BioXcell® dan Tris-kuning telur mempunyai keupayaan yang terbaik untuk melindungi daya maju sperma. Oleh itu, keputusan ini menyokong AndroMed® lebih dari penyambung yang lain yang digunakan untuk parameter sperma pasca-cair dengan plasma semen. Penyambung AndroMed®, Triladyl® dan Tris-kuning telur adalah paling baik dalam kapasiti penyambungan berbanding dengan BioXcell® pada tahap penilaian semen pasca-cair dan pemeliharaan semen tanpa plasma semen.

Di kalangan penyambung kuning telur, penyambung plasma kuning telur 10% menghasilkan kapasiti penyambung tertinggi untuk integriti membran plasma semen, daya maju, dan motiliti, manakala penyambung berdasarkan Tris-kuning telur 20% dan Tris-kuning telur plasma 10% mempunyai keupayaan terbaik untuk melindungi morfologi semen dan integriti akrosom. Oleh itu, keputusan ini menyokong kepekatan Tris-kuning telur plasma 20% berbanding kepekatan penyambung kuning telur lain yang digunakan untuk menyambung semen diejakulasi dengan plasma semen. Di dalam kajian yang melibatkan penyingkiran plasma semen, spermatozoa juga dinilai untuk kualitinya dengan menggunakan penyambung Tris-kuning telur 20%, Tris-kuning telur plasma dengan kepekatan 10%, Tris-kuning telur plasma dengan kepekatan 15% dan Tris-kuning telur plasma dengan kepekatan 20%. Penyambung plasma kuning telur dengan kepekatan 15% memberikan kapasiti penyambungan yang lebih baik kepada daya maju semen dan integriti akrosom, manakala penyambung berdasarkan Tris-kuning telur 20% mempunyai kapasiti yang terbaik untuk melindungi morfologi dan integriti membran plasma semen. Oleh itu, keputusan ini memihak kepada kepekatan plasma kuning telur dengan kepekatan 15% dan penyambung berdasarkan Tris-kuning telur 20% lebih berbanding dengan kepekatan pemanjang plasma kuning telur yang lain.

Di dalam kajian oosit yang tidak matang, oosit dituai dari ovari bovin dikumpul daripada rumah penyembelihan tempatan (Banting, Malaysia), kemudian diangkut di dalam bekas termos pada suhu 37°C (salina penampan fosfat) ke makmal Theriogenologi dan Citogenetik dalam masa 2-3 jam selepas penyembelihan. Kaedah penghirisan ovari digunakan untuk menuai oosit yang tidak matang. Penggunaan pipet bagi oosit yang dipertimbangkan telah dilakukan dari medium pengendalian ke dalam medium basuh. Kajian ini bertujuan untuk menyiasat kesan masa pendedahan vitrifikasi yang berbeza selama 15 saat, 30 saat dan 45 saat ke atas oosit bovin tidak matang dan keupayaan mereka untuk bersenyawa dan menghasilkan embrio selepas penggunaan teknologi persenyawaan *In vitro.* Etilena Glikol (EG) dan Dimethylsulfoxide (DMSO) telah digunakan sebagai gabungan dua kriopelindung. Dua krioperanti utama juga telah digunakan; ini ialah straw tertarik terbuka (OPS) dan straw Perancis tertutup (CFS). OPS diubahsuai secara tempatan dengan menggunakan plat panas dan ditarik secara manual. Oosit yang divitrifikasi-hangatkan yang belum matang telah dinilai dari segi morfologi di bawah

kemikroskopan epiflourosen. Oosit yang berdaya maju dimasukkan ke dalam media kematangan, yang terutamanya terdiri daripada TCM199+HEPES ditambah dengan 10% FCS dan 10 ng/ml faktor pertumbuhan epidermis (EGF). Penyemperitan badan kutub diperhatikan 22 jam pasca IVM. Persenyawaan *In vitro* (IVF) telah digunakan secara infusi langsung semen lembu jantan beku-dicairkan disambungkan dalam AndroMed® (sebagai penyambung terbaik digambarkan dalam eksperimen pertama Bab Empat). Semen lembu jantan beku-dicairkan dikumpulkan di bahagian bawah larutan Percoll (45% atas 90%) kemudian dicuci di medium Tyrodes. Spermatozoa berdaya maju telah dicairkan dalam isipadu medium persenyawaan yang sesuai untuk mencapai kepekatan muktamad sebanyak 2×10^6 sperma/ml. Suatu bahagian 50 µl ampaian ini telah ditambah kepada setiap telaga mengandungi oosit untuk kepekatan muktamad sel-sel sperma sebanyak 1×10^6 sperma/ml. Spermatozoa dan oosit telah dieram bersama selama 18-22 jam pada 38.8°C dalam atmosfera dilembapkan dan sebanyak 5% CO_2 di dalam udara. Kultur *invitro* (IVC) zigot andaian telah divortekskan dan dibasuh tiga kali di dalam TCM199 ditampan-HEPES mengandungi 0.4 g/l BSA dan 4 µg/ml gentamicin, untuk membuang sel-sel kumulus dan spermatozoa terlekat. Zigot dibasuh dua kali di dalam medium SOF ditambah dengan asid amino BME (50×), Asid amino MEM (100 ×), 3 mM pyruvate, 6mg/ml BSA bebas dari asid lemak dan 5% FBS dan dipindahkan secara 250 setiap kumpulan ke piring petri empat-telaga yang mengandungi 25 µl tetes medium kultur yang ditutupi dengan 700 µl minyak mineral. Kajian ini memperlihatkan bahawa oosit bovin yang tidak matang boleh divitrifikasi di peringkat-GV dengan protokol kami yang dioptimumkan dan bahawa oosit-GV divitrifikasi-hangatkan boleh dimatangkan dan disenyawakan in vitro dan seterusnya perkembangan embrio in vitro. Juga, vitrifikasi oosit tidak matang bovin dalam larutan terdiri daripada 15% EG + 15% DMSO menggunakan kaedah OPS mencapai daya maju yang terbaik, kematangan tertinggi, dan belahan dalam keadaan-keadaan kami selepas IVM dan IVF walaupun dengan Kadar pemulihan yang lebih rendah daripada kumpulan lain. Parameter dianalisis menggunakan 2-cara analisis (ANOVA) bagi varians. Nilai itu dinyatakan sebagai min piawai untuk min (S.E.M). Tahap kepentingan statistic adalah pada $P<0.05$.

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

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LIST OF ABBREVIATIONS

AO	Acridin Orange
AV	Artificial vagina
AEE	Automatic electro-ejaculator
CFS	Closed French straws
CPA	Cryoprotectants
EE	Electro-ejaculator
EG	Ethelen glycol
ES	Equilibrium solution
EYP	Egg yolk plasma
IVP	Invitro embryo production
LDL	Low density lipoprotein
OPS	Open pulled straws
PKC	Palm kernel cake
PMI	Plasma plasma integrity
SP	Seminal plasma
TCM	Tissue culture medium
TWEY	Tris whole egg yolk
TEYP	Tris egg yolk plasma
VS	Vitrification solution
WS	Warming solution

CHAPTER 1

INTRODUCTION

1.1 Background

Over the decades, history has recorded a tremendous revolution in animal breeding where difficulties in breeding certain desired breeds became inevitably exploited through the revolutionary scientific achievement in the field of Theriogenology where semen transportation became widely accepted (Aires et al., 2003). To achieve this , the application of cryopreservation was applied in breeding desired cattle breeds as farmers all over indulged in the practice of artificial insemination (AI) (Sathe & Shipley, 2015). In Malaysia to be precise, artificial insemination as a technique was practiced as early as 1963 when frozen semen was transported from Australia to Singapore and later railed into Malaysia (Zaman, 2013). Cattle breeding had become widely spread amongst animal farmers as a result of the hike in the per capita consumption of beef, in Malaysia it was reported by Abd Wahid Haron in 2013 to have elevated from about 5kg to 5.7kg in a space of a decade (2001-2010). The viability of semen became rested on the ability to lower its metabolic rates through reduced temperature. Extreme lower temperatures therefore provided a decline in semen metabolic activities thereby making the semen last longer if preserved (Parrish et al., 1986). Cremades in 2005 reported that apart from reduced temperatures, carbon dioxide also poses prolong preservatory property on semen but the success was however considered insignificant (Cremades et al., 2005). Lower temperatures of about 4-7°C have prolonged the survival span of semen and retained semen motility for almost seventy two hours with an appreciable fertility potentials. This technique explains how semen could be preserved; however, semen integrity, reduced semen motility, variation in viability and rates of conception became an outcry.

Cryopreservation was reported in 2000 to be affecting semen viability by almost 53% and eventually posing a detrimental effect on fertilisation to a tune of about seven ties. This necessitated a poll of experimental studies over the years as to the extent of the effects on the anatomy and physiology of semen particularly in cattle breeding. Researchers have pinned down factors like decondensation of chromatin, premature reaction of acrosome and impeded physiology of mitochondria to most of the in vitro experimental studies (Watson, 2000). About two decades ago, reduced extreme temperatures have been indicated to be directly proportional to semen stressing, this phenomenon was suggested to be a membrane disruptive factor which can lead to a condition called ‘cold shock’ (Kim et al., 2011). Semen cold shock syndrome has direct effect on the liveability of semen depending on the severity of the shock (Drobnis et al., 1993). This reason many trials involving addition some forms of cryoprotective agents like egg yolk, milk, bovine serum albumin, liposomes and glycerol in extenders have conferred some level of longitivity to the semen against the adverse effects of cryopreservation (Shoae & Zamiri, 2008). The advent of assisted reproductive biotechnology is another big achievement in the world of science

that gave a wider window to the improvement of breeding and reproduction (Haron, 2013) .

Immature egg cells known as the oocytes have also been cryopreserved over time for the purpose of breeding (Haron, 2013). Oocytes have been reported to be very difficult to preserve because it presents with a very low fertility results which is mostly associated with the physical changes in the composition of oocytes, expansion and contraction of the oocytes and careless handling of oocytes during cryopreservation or vitrification (Kuwayama, 2007). However positive reports have been recorded in the application of cryopreserved oocytes though a lot of augments have proven the report unacceptable (Vieira et al., 2002).

Cryopreserved sperm and vitrified oocytes as a gametes can proceed to produce embryos either *in vivo* or *in vitro* successfully. Embryo quality in cattle breeding is also a very important outlook that determines the success of any breeding industry. Records had it that almost 20% of bovine embryo are widely produced using the in vitro technology popularly known as the In vitro Embryo Production (IVP) (Wheeler et al., 2006). Embryo transfer is a widely accepted practice extremely dependent on the quality of cryopreserved sperms and vitrified oocytes. Initially surgery was the known mode of embryo transfer but in the recent years non-surgical transfer of embryo have been exploited. Therefore , preserving the semen quality and vitrified oocyte quality, fertility rate post-thaw will be a key factor to improving embryo transfer quality and hence cattle breeding thereby providing animal farmers with a better understanding and options to maximise profit through animal production.

1.2 Problem statement

A decline in cattle breeding had been experienced in the past years in Malaysia with a fluctuating pattern. Semen extenders as a media used to handle semen in cryopreservation have played important role in reproductive efficiencies in farm animals. In recent times, the bull semen quality due to anticipated variation in the protective efficiency of semen extenders have been worrisome coupled with the fact that seminal plasma have been argued to make negative effect on the cryopreserved semen. Egg yolk preserved extender had been of poor quality lately in many reports attributed to its microbial contamination, turbidity and granulation. Immature oocytes vitrification have a great value in oocytes preserving banks. Both sperm cryopreservation and oocyte vitrification still need to be improved to get more superior *in vitro* embryo production. Quality of the oocytes after vitrification-warming is still another challenge may decline the quality of oocyte and embryo as well. Cryodevice and of oocyte exposure time to vitrification solution still a matter of debate to optimise vitrified oocyte quality through reducing cryoprotectant-oocyte toxicity.

1.3 Significance of the study

The quality and fertility capacity of cryopreserved semen and vitrified immature oocytes is very crucial to any successful breeding program as it reflects directly the quality of embryos produced. Therefore, firstly, this study is significantly aims to provide information on using of seven different semen extenders, semen cryopreservation, effect of removal of seminal plasma on the sperm quality, immature oocyte vitrification process, vitrification exposure time, cryodevices used in preserving of vitrified oocytes, *in vitro* fertilization, *in vitro* maturation, *in vitro* embryo culture and *in vitro* embryo production in cattle . Secondly, our study aims to find out the causes resulting in declining sperm quality, oocyte quality and embryo quality as well. Lastly, the present study aims the improvement in cattle breeding knowledge.

1.4 Hypothesis:

A. $\mathbf{H_o}$ = Assessment of cryopreservatives and oocyte vitrification improve *in vitro* embryo production in cattle.

$\mathbf{H_a}$ = Assessment of cryopreservatives and oocyte vitrification doesnot improve *in vitro* embryo production in cattle

1.5 Study objectives

To evaluate the post-thaw bull spems pre-extended in: AndroMed®, BioXcell®, Triladyl® compared to Tris-egg yolk based extenders 20%.

1. To assess the effect of seminal plasma removal on the post-thaw bull sperms pre-extended in: AndroMed®, BioXcell®, Triladyl® compared to Tris-egg yolk based extenders 20%.
2. To explain the role of different concentration of egg yolk plasma (10%, 15% and 20%) on post-thaw bull sperm quality pre-extended with seminal plasma.
3. To esimate the role of different concentration of egg yolk plasma (10%, 15% and 20%) extenders on post-thaw bull sperm quality pre-extended without seminal plasma.
4. To illustrate the effect of different vitrification exposure time (15 sec, 30 sec and 45 sec) and different cryodevices (OPS/ CFS) on the quality of vitrified immature oocytes (harvested from slaughtered cattle) for *in vitro* embryo production in cattle.

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