IN VITRO EMBRYO PRODUCTION FROM VITRIFIED MATURE BOVINE OOCYTES

By

HANY MOHAMED AMIN LAITHY
B.Sc. Agric. Sc. (Animal Production), Ain Shams Univ.,(1991)
M.Sc. Agric. Sc. (Animal Production), Ain Shams Univ.,(2003)

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This thesis for Ph.D. degree has been approved by:

Dr. Mokhtar Ibrahim Yousef ……..
Prof. of Environmental Animal Physiology and Reproductive Toxicology, Institute of Graduate Studies and Research, Alexandria University

Dr. Ahmed Mohammed El- Sherbiny ……..
Prof. of Animal Physiology, Faculty of Agriculture, Ain Shams University

Dr. Farouk Abdalla El- Sayed Khalil ……..
Prof. Emeritus of Animal Physiology, Faculty of Agriculture, Ain Shams University

Dr. Essmat Bakry Abdalla ..……..
Prof. Emeritus of Animal Physiology, Faculty of Agriculture, Ain Shams University

Date of Examination: 05/06/2016

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Under the supervision of:

Dr. Essmat Bakry Abdalla
Prof. Emeritus of Animal Physiology, Department of Animal Production, Faculty of Agriculture, Ain Shams University, Egypt (Principal Supervisor)

Dr. Farouk Abdalla El- Sayed Khalil
Prof. Emeritus of Animal Physiology, Department of Animal Production, Faculty of Agriculture, Ain Shams University, Egypt

Dr. Alaa El-Sayed Bellasy Zeidan
Head Research of Animal Reproduction and Artificial Insemination, Animal Production Research Institute, Dokki, Giza, Egypt

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ABSTRACT

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Cryopreservation of animal oocytes will permit germplasm of valuable or unique females to be preserved for extended times. The objective of this research was to derive a procedure to cryopreserve bovine oocytes by vitrification to be used as recipients for somatic cell nuclear transfer (SCNT). The present study was conducted to investigate the impact of different cryoprotectants like Ethylene glycol (EG), Propanediol (PROH) and Dimethyl sufoxide (DMSO) alone or thier combinations, cryodevices, Straw, open pulled straw (OPS), solid surface vitrification (SSV), and L-carnitine supplementation in oocytes maturation medium on morphology, survival, fertilization rate and developmental competence of mature bovine oocytes using vitrification. The vitrification of bovine oocytes was performed by six experiments. The first, second and third experiment were conducted using (10, 20 and 40%) concentration of EG, (10, 20 and 40%) DMSO, (2.5, 5 and 10%) of PROH. The fourth experiment was conducted using combinations of the best results from the first three experiments as follow (5%PROH + 20%EG), (5%PROH + 20%DMSO), (20%EG + 20%DMSO), (5%PROH + 20%EG + 20%DMSO). The fifth experiment was conducted using different cryodevices (OPS-Straw-SSV) on vitrfication with best results from fourth experiment. The sixth experiment was conducted using L-carnitine supplementation in maturation medium for control and treated groups at different concentrations (0.1,0.3,0.5 mM/ml), with best cryoprotectant from fourth experiment and best cryodevices from fifth experiment. It was found that combination of cryoprotectants (20% EG + 20% DMSO), proved to be more efficient than using other combinations or Propanediol (PROH), Dimethyl sulfoxide (DMSO) and ethylene glycol

(EG) alone. The morphological damages were founded to be less (10.09%) using combination of (20% EG + 20% DMSO) compared with 5% PROH or 20% DMSO alone which were (34.14%) and (27.27%), respectively. The survival rate was found (92.45%) compared with 20% EG or 20% DMSO which was (91.54% and 90.63%), respectively. The fertilization rate was significantly (P<0.05) higher using combination (20%EG + 20% DMSO) (57.14) compared with PROH, DMSO and EG which recorded (40.0%, 44.83%, 44.61%) respectively. The developmental rate cleavage, morula to the blastocyst stage were significantly (P<0.05) higher (10.03%, 8.73%, 5.98%) using combinations of (20% EG + 20% DMSO) than using 5% PROH or 20% DMSO alone. PROH and DMSO when used alone found to induce adverse effect on the morphology, fertilization rate and developmental competence of bovine oocytes. However, combination of 20% EG + 20% DMSO found better to maintain the integrity and internal structure of bovine oocytes by vitrification. Using cryodevice (OPS, SSV techniques) was significantly (P<0.05) higher viability rate (94.73% and 95.23%), fertilization rate (61.11% and 67.50%), and cleavage rate (24.32 and 31.25%) than 0.25ml semen straw technique. It was found that 0.3 concentration of L-Carnitine resulted in significantly higher normal morphology, viability and fertilization rate (70.18%, 92.11% and 62.86%) respectively than control and (0.1,0.5) concentration. Also supplementation of maturation medium of immature cow oocytes with different concentrations of L.C (0.1, 0.3 and 0.5 mM /ml) for 24 h., in the presence of 0.3 mM LC, resulted in an increase of percentages of cleavage rate (40.48%), morula (19.05%) and blastocyst rate (11.90%) compared with control and other treatments.

Keywords: *In vitro* fertilization, Vitrification, Cryoprotectants, Cryodevice, L-Carnitine.

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LIST OF ABBREVIATIONS

ABBREVIATIONS	Mean
ART	Assisted reproductive technologies
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BZ	Brocken zona pellucida
CF	Fluorescent dye
CFDA	Carboxyfluorescein diacetate
CON	Conventional straw
CPAs	Cryoprotectant agents
CO_2	Carbon dioxide
COA	Acyl-COA esters
COCs	Cumulus oocyte complexes
CR1aa	Charles Rosenkran1 containing amino acids
DPBS	Dulbecco's phosphate buffer saline
DMSO.	Dimethyl sulfoxide
DNA	Deoxy ribonucleic acid
EDFS	Ethylene glycol,dimethyl sulfo.,ficoll ,sucrose
EDFSF	Ethylene glycol,dimeth sulfo.,ficoll ,sucrose,
EDM	Embryo development medium
EG	Ethylene glycol
EGF	Epidermal Growth Factor
ES	Equilibration solution
FBS	Fetal bovine serum
FCS	Fetal calf serum
FSH	Follicle stimulating hormone
GV	Germinal vesicle
HEPES	Germinal vesicle breakdown

IVC	*In vitro* competence
IVD	*In vitro* development
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
LC	L-carnitine
LH	Luteinizing hormone
LN_2	Liquid Nitrogen
LP	Lactate and sodium pyruvate
M II	Metaphase II
OPS	Open pulled straws
SC	Shrinkage cytoplasm
SCNT	Somatic cell nuclear transfer
SOF	Synthetic oviductal fluid
SSV	Solid-surface vitrification
S-TALP	Swim-up procedure
PBS	Phosphate buffer saline
PROH	Probanediol
P^H	Hydrogen number
PN	Pronucleus
PVP	Polyvinyl pyrrolidone
TCM 199	Tissue culture media
VS	Vitrification solution
ZP	Zona pellucida

1. INTRODUCTION

In vitro embryo production through *in vitro* maturation (*IVM*) and *in vitro* fertilization (*IVF*) has been possible in domestic animals, which have been widely utilized for embryo transfer, splitting, sexing, freezing banks, cloning nuclear transfer and transgenic animals by micromanipulation of embryos **(Gordon and Lu, 1990; and Fukui *et al*. 1997)**.

Cryopreservation of germplasm was performed either by slow freezing **(Gautam *et al*. 2008a),** or vitrification **(Yamada *et al*. 2007)**.

The conventional slow freezing method for cryopreservation of oocytes and embryos often causes intracellular ice crystallization and osmotic shock, resulting in cell damage **(Ledda *et al*. 2006).** Cryopreservation using vitrification is an advantageous method which cells are exposed to high concentrations of cryoprotectants and ultra-rapid cooling rate, resulting in ice-crystal free solid glass-like structures **(Pereira and Marques, 2008)**.

Vitrification minimize chilling injury by rapidly passing through the danger temperature zone (15 to -5ºC) **(Dobrinsky, 1996; Martino *et al*., 1996a; Isachenko *et al*., 1998 and Zeron *et al*. 1999)**. Moreover, vitrification is a simple and fast technique of cryopreservation, requires less specialized or expensive equipment, it allows the operator to observe the cells during vitrification process **(El-Danasouri, 2005)**.

Unfortunately, the sudden exposure to high concentrations of cryoprotectants during vitrification is toxic to oocytes **(Succu *et al*. 2007 and Sripunya *et al.* 2010)**.

There are several methods have been used to reduce cytotoxic and osmotic effect of the cryoprotectants: use of low toxic cryoprotectants **(Rall, 1987)** or mixtures of them **(Massip, 1986)**, addition of non-permeating cryoprotectants **(Fahy *et al*. 1984)**, short time of exposure to cryoprotectants **(Fahy *et al*. 1984; Arav *et al*. 1988)**, reduction of cryoprotectant concentration, and exposure at low temperature **(Rall,**