"EFFECT OF ASCORBIC ACID ON OXIDATIVE STRESS AND ITS THERMOPROTECTANT ROLE ON *IN VITRO* EMBRYONIC DEVELOPMENT OF BUFFALO *(BUBALUS BUBALIS)* EMBRYOS"

THESIS SUBMITTED TO
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF

MASTER OF TECHNOLOGY

IN

ANIMAL BIOTECHNOLOGY

BY

Mayank Roshan
B.Tech. (Biotechnology and Industrial Microbiology)

ANIMAL BIOTECHNOLOGY CENTRE
NATIONAL DAIRY RESEARCH INSTITUTE
(DEEMED UNIVERSITY)
KARNAL- 132001 (HARYANA), INDIA

2016

Reg. No. 14-M-BT-05

Effect of ascorbic acid on oxidative stress and its thermoprotectant role on *in vitro* embryonic development of buffalo *(Bubalus bubalis)* embryos

By

Mayank Roshan

THESIS SUBMITTED TO THE
NATIONAL DAIRY RESEARCH INSTITUTE
(DEEMED UNIVERSITY)
KARNAL (HARYANA)
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF

MASTER OF TECHNOLOGY
IN
ANIMAL BIOTECHNOLOGY

Approved by:

(27/07/16)
EXTERNAL EXAMINER

27.07.16
(Dr. M.S. Chauhan)
MAJOR ADVISOR

Members of Advisory Committee

1. **Dr. M.K. Singh**
   Scientist, Animal Biotech. Centre

2. **Dr. R.S. Kataria**
   Principal Scientist, NBAGR

3. **Dr. A. Kumaresan**
   Senior Scientist, ARGO Division

4. **Dr. Dheer Singh**
   Principal Scientist and Head, ABC

के.ब.अ.सं.
CIRG
1979
Mathura

भा.कृ.अ.प.- केन्द्रीय बकरी अनुसंधान संस्थान

मखदूम, पो० फरह - 281 122, मथुरा (उत्तर प्रदेश) भारत

ICAR- CENTRAL INSTITUTE FOR RESEARCH ON GOATS

Makhdoom, P.O. Farah - 281 122, Dist. Mathura (UP) INDIA
Tel.: +91-565-2763380 (O), +91-565-2763245 (R), Fax: +91-565-2763246
E-mail : director.cirg@icar.gov.in, chauhanabtc@gmail.com

भाकृअनुप
ICAR

डा. मनमोहन सिंह चौहान
निदेशक
Dr. M.S. Chauhan
DIRECTOR

Dated: 28th June, 2016

CERTIFICATE

This is to certify that the thesis entitled, **"Effect of ascorbic acid on oxidative stress and its thermoprotectant role on *in vitro* embryonic development of buffalo *(Bubalus bubalis)* embryos"** submitted by **Mayank Roshan** towards the partial fulfilment of the award of the degree of **Master of Technology in Animal Biotechnology** of the **National Dairy Research Institute (Deemed University)**, Karnal (Haryana), India, is a bonafide research work carried out by her under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

28.06.2016

Dated: 28.06.2016

(M.S.CHAUHAN)
MAJOR ADVISOR
(GUIDE)

Dedicated
To
My Dear Parents
Bhaiya & Bhabi
For their
Constant
Encouragement,
blessings & Love

ACKNOWLEDGEMENT

In the last one year I have had the pleasure to work in an excellent environment with dynamic people. It is a pleasant aspect that I have now the opportunity to express my gratitude for all those who helped me a lot. I want to thank all those who have been generous to me and helped me by any means during these years.

Words will never be enough to express admiration and gratitude to my guide ***Dr. M.S. Chauhan****, former Principal Scientist, Animal Biotechnology Centre, National Dairy Research Institute (NDRI), Karnal, India, currently, Director, Central Institute of Research on Goats, Makhdoom, Mathura, India for his tremendous help, sagacious guidance, constant encouragement, valuable suggestions, stimulating discussions, constructive criticism, everlasting affection and parental care which inspired me to bring this problem in hand to a successful end. His understanding, encouragement and personal guidance have provided a good basis for the present thesis. I learned from him-sincerity, punctuality and finally honesty. One line of him I will never forget that "You have to show your skill and capability in front of the people and kisi bhi field me jao koi bhi kam karo but apne 100% dedication ke sath or apna best dene ki koshish karo."*

I reverently and honestly acknowledge obligation to my advisory committee members, ***Dr. M.K. Singh, Dr. R.S. Kataria , Dr. A. Kumaresan*** *and* ***Dr. Dheer Singh*** *for their valuable suggestions and timely help in various phases of my study. My utmost respect to all teachers who made me sound in the subject, I will remain ever grateful to each one of them.*

The support and guidance received from ***Dr. R.S. Manik, Dr. P. Palta*** *and* ***Dr. S.K. Singla*** *is gratefully acknowledged. I am very grateful to* ***Dr. P. Palta*** *for his appreciation, suggestions, constructive criticism and providing me good concept during my scientific endeavour.*

I offer my good wishes and regards to ***Dr. M.K. Singh*** *for his advice, openhearted support, friendly gesture throughout my research programme and his cooperation at all stages of my work.I will never forget one line he used to say many times "Agar tum apni help khud nhi karogi to koi tumhari help nhi karega".I learned hard work and dedication towards the work from him.*

I thank ***Dr. A.K. Srivastava****, Director, NDRI for providing the necessary facilities for conducting this work. I am also thankful to all staff of Animal Biotechnology Division for their cooperation at all stages of my work.*

Special thanks to Deepu Mam and Karan Pratap sir for helping me during my research work. I wish to express my warm and sincere thanks to Narender sir, Tushar sir, Nagoor sir, Aditya sir, Anurag sir, Raghuvendra sir, Shrutika mam, Radha mam, Mehak mam, Sudha mam and Tanushree mam for their supporting behaviour.

Warm thanks to Neha mam for treating me like her brother, for helping me in my bad times, gifting take it easy attitude and whose encouragement, benevolent help, constant support and care and friendship will always remain in me forever.

I will never forget the beautiful moments spent with Parul mam, Diksha mam, Preeti mam, Nidhi mam and Ankur Sir we were just like a small family in NDRI. They were always with me in my bad as well as in good moment. Heartiest thanks to GOD for giving me such a nice people in my life.

I admit my inability to express my emotions through words for my friends Manish Tiwari ji, Disha, Amrita and Sonu for their extremely caring attitude, encouragement, moral support and showing confidence in me (each and every time). For that and for many other things I want to thank them once again. They are a

wonderful gift to me. Their friendship makes my life a wonderful experience. I remain ever their devoted friend.

I feel myself very lucky to have such a wonderful family Papa, Mummy, Bhaiya, Bhabhi and my most loving friend Himani without whose love and inspiration, I would never have achieved anything. Especially Papa, Mummy and Bhaiya for their unending support and faith. At those horrible moments when I have said I couldn't go on you have always said "you can do it".

Thanks to all those whose names I can't mention here for want of space, may the unknown eternal power bless all of them!

Finally, as though I am very small before him, still I would wish to acknowledge the Omnipotent, Omnipresent, and Omniscient 'GOD' without whose blessings, this small piece of work would have never been successful all through the way of truth and love and also creating such a beautiful world for us with all amenities (JAI BAJRANGBALI).

Dated: June 28, 2016 *(MAYANK ROSHAN)*

CONTENTS

Chapter		Title	Page No.
1.		**INTRODUCTION**	**....1-4**
2.		**REVIEW OF LITERATURE**	**....5-30**
	2.1	*In vitro* production of buffalo embryos	5-7
	2.1.1	*In vitro* maturation	6
	2.1.2	*In vitro* fertilization	6-7
	2.1.3	*In vitro* culture	7
	2.2	Oxidative stress	7-20
	2.2.1	Role of oxidative stress in embryo production	8-9
	2.2.2	Oxidative stress produces harmful reactive oxygen species	9-11
	2.2.3	Origin of ROS in Male Reproductive System/Sources of ROS	12-13
	2.2.4	Cryopreservation/ Freezing Thawing-Oxidative Stress	13
	2.2.5	Origin of ROS in Female Reproductive System/Sources of ROS	13-14
	2.2.6	Amelioration of oxidative stress by antioxidants	15
	2.2.7	Mechanism of radical scavenging activity of Ascorbic acid	15-16
	2.2.8	Supplementation of *in vitro* maturation and culture medium with Ascorbic Acid	16-20

2.2.8.1	Sheep	16-17
2.2.8.2	Pig	17
2.2.8.3	Goat	17-18
2.2.8.4	Buffalo	18-20
2.3	Heat Stress	20-25
2.3.1	Effect of heat stress on rat and mouse reproduction and embryo development	20-21
2.3.2	Effect of heat stress on bovine reproduction and embryo development	22-25
2.4	Genes Related To Apoptosis	25-27
2.4.1	*BAX*	26
2.4.2	*BID*	26
2.4.3	*BCL-XL*	27
2.4.4	*MCL-1*	27
2.5	Genes related to embryonic development	27-29
2.5.1	*BMP 15*	27-28
2.5.2	*GDF 9*	28-29
2.6	Genes responsible for thermo-tolerance responses	29-30
2.6.1	*HSP 70.1 and HSP 70.2*	29-30
3.	**MATERIALS AND METHODS**	**....31-42**

3.1	Materials	31-34
3.1.1	Glassware and plastic ware	31
3.1.2	Chemicals, cell culture media and supplements	31-32
3.1.3	Equipments	32-34
3.1.3.1	Microscopes	32
a	Zoom stereomicroscope	32
b	Inverted microscope	32
3.1.3.2	Laminar flow hood	32-33
3.1.3.3	CO_2 incubator	33
3.1.3.4	Centrifuge	33
3.1.3.5	Thermal cycler	33
3.1.3.6	Real time PCR	33
3.1.3.7	Electrophoresis unit and gel documentation	34
3.2	METHODS	34-44
3.2.1	Preparation of different media	34
3.2.2	Collection of buffalo follicular fluid	34
3.2.3	*In vitro* maturation and fertilization of oocytes	34-37
3.2.3.1	Collection and classification of oocytes	34-35
3.2.3.2	*In vitro* maturation of oocytes	35-36
3.2.3.3	Sperm preparation and *in vitro* fertilization	36

3.2.3.4	*In vitro* culture	36-37
3.2.4	Quantitative expression of genes	37-38
3.2.4.1	RNase-free plasticware	37
3.2.4.2	Preparation of RNase-free solutions	37
3.2.4.3	RNase free surface	38
3.2.4.4	RNA isolation Protocol	38
3.2.5	cDNA synthesis and real time quantification	38-39
3.2.6	Experimental design	41-42
3.2.7	Statistical analysis	42
4.	**RESULTS AND DISCUSSION**	**....43-56**
4.1	Effect of Ascorbic acid supplementation	43-53
4.1.1	Supplementation of *in vitro* maturation medium with different concentrations of Ascorbic acid	43-44
4.1.2	Supplementation of 50µM Ascorbic acid in *in vitro* culture medium	45
4.1.3	Supplementation of 50µM Ascorbic acid both *in vitro* maturation and culture media	45-49
4.1.4	Supplementation of both in vitro maturation and culture media with 50µM Ascorbic acid at 38.5°C, 39.5°C and 40.5°C	50-53
4.2	Gene expression study after supplementation of Ascorbic acid in IVM media	53-54

	4.2.1	Relative mRNA abundance of heat stress related genes	54
	4.3	Relative mRNA abundance of apoptosis related genes	54-55
	4.4	Relative mRNA abundance of developmental competence genes	56
5.	**SUMMARY AND CONCLUSIONS**		**....57-60**
	BIBLIOGRAPHY		**....i-xx**
	ANNEXURE		**....xxi-xxiv**

LIST OF FIGURES

Figure No.	Title		After page No.
1.	Immature slaughterhouse derived buffalo oocytes of usable quality (grade A+B)	:	44
2.	Mature buffalo oocytes of usable quality	:	44
3.	IVF embryos at 2-cell stage.	:	44
4.	IVF embryos at 4-cell stage.	:	44
5.	IVF embryos at 8-16 cell stage	:	44
6.	IVF embryos at morula stage	:	44
7.	IVF embryos at blastocyst stage	:	44
8.	IVF produced hatched blastocyst	:	44
9.	Melt curves and melt peaks of heat stress related genes: *HSP 70.1* (A, A'), *HSP 70.2* (B, B').	:	54
10.	Melt curves and melt peaks of different developmental competence related genes: *GDF9* (A, A'), *BMP15* (B, B').	:	54
11.	Melt curves and melt peaks of different apoptosis related genes: *BAX* (A, A'), *BID* (B, B'), *BCL-XL* (C, C') and *MCL1* (D, D').	:	54
12.	Expression of heat stress related gene (*HSP 70.1*) in blastocysts produced from control oocytes (without Ascorbic acid supplementation) and oocytes supplemented with 50 µM Ascorbic acid after initial treatment at 39.5°C for 12 h during IVM	:	56

13.	Expression of heat stress related gene (*HSP 70.2*) in blastocysts produced from control oocytes (without Ascorbic acid supplementation) and oocytes supplemented with 50 µM Ascorbic acid after initial treatment at 39.5°C for 12 h during IVM	:	56
14.	Expression of Pro-apoptotic gene (*BAX*) in blastocysts produced from control oocytes (without Ascorbic acid supplementation) and oocytes supplemented with 50 µM and 100 µM Ascorbic acid in IVM medium	:	56
15.	Expression of Pro-apoptotic gene (*BID*) in blastocysts produced from control oocytes (without Ascorbic acid supplementation) and oocytes supplemented with 50 µM and 100 µM Ascorbic acid in IVM medium	:	56
16.	Expression of Anti-apoptotic gene (*BCL-XL*) in blastocysts produced from control oocytes (without Ascorbic acid supplementation) and oocytes supplemented with 50 µM and 100 µM Ascorbic acid in IVM medium	:	56
17.	Expression of Anti-apoptotic gene (*MCL1*) in blastocysts produced from control oocytes (without Ascorbic acid supplementation) and oocytes supplemented with 50 µM and 100 µM Ascorbic acid in IVM medium	:	56
18.	Expression of developmental competence related (*GDF9*) gene in blastocysts produced from control oocytes (without Ascorbic acid supplementation) and oocytes supplemented with 50 µM and 100 µM Ascorbic acid in IVM medium	:	56
19.	Expression of developmental competence related (*BMP15*) gene in blastocysts produced from control oocytes (without Ascorbic acid supplementation) and oocytes supplemented with 50 µM and 100 µM Ascorbic acid in IVM medium	:	56

LIST OF TABLES

Table No.	Title		Page No.
3.1	Primer sequences and conditions for Real Time PCR	:	40
4.1	Effect of Ascorbic acid supplementation of IVM media on the developmental competence of buffalo oocytes	:	44
4.2	Effect of 50 µM Ascorbic acid supplementation of IVC media on the developmental competence of buffalo oocytes	:	45
4.3	Effect of 50 µM Ascorbic acid supplementation of IVM and IVC media on the developmental competence of buffalo oocytes	:	46
4.4	Effect of heat stress on the developmental competence of oocytes with 50 µM ascorbic acid supplementation at 39.5°C	:	50
4.5	Effect of heat stress on the developmental competence of oocytes with 50 µM Ascorbic acid supplementation at 40.5°C	:	51

ABBREVIATIONS AND SYMBOLS

AI	:	Artificial insemination
ANOVA	:	Analysis of variance
ART	:	Assisted reproductive technologies
BL	:	Blastocysts
BO	:	Brackett and Oliphant
BSA (FAF)	:	Bovine serum albumin (Fatty acid free)
BSA	:	Bovine serum albumin
cm	:	Centimeter
CO_2	:	Carbon dioxide
COC	:	Cumulus-oocyte complex
CR1aa	:	Charles Rosenkrans 1 amino acid
DNA	:	Deoxy ribonucleic acid
DPBS	:	Dulbecco's phosphate buffered saline
DPEC	:	Diethylpyrocarbonate
EDTA	:	Ethylenediamine tetra-acetic acid
FBS	:	Fetal bovine serum
FM	:	Fertilization medium
GPX	:	Glutathione peroxidase
GV	:	Germinal vesicle
H_2O_2	:	Hydrogen peroxide
HB	:	Hatched blastocyst
HR	:	Hatched rate
HS	:	Heat shock
I.U.	:	International units
IM	:	Immature oocyte
IVC	:	*In vitro* culture
IVEP	:	*In vitro* embryo production
IVF	:	*In vitro* fertilization
IVM	:	*In vitro* maturation
LN_2	:	Liquid nitrogen
mCR2aa	:	modified Charles Rosenkrans 2 amino acid

mg	:	Milligram
ml	:	Millilitre
MO	:	Matured oocyte
O_2	:	Oxygen
O_2^-	:	Superoxide anion
^oC	:	Degree Celsius
OH^-	:	Hydroxyl radical
PCR	:	Polymerase chain reaction
qPCR	:	Quantitative Polymerase chain reaction
RO^-	:	Alkoxyl radical
ROO^-	:	Peroxyl radical
ROS	:	Reactive oxygen species
RT-PCR	:	Reverse transcriptase- Polymérase chain reaction
SE	:	Standard error
SOD	:	Superoxide dismutases
TCM-199	:	Tissue culture medium-199
TCN	:	Total count number
%	:	Percent
μg	:	Microgram
μl	:	Microlitre
μm	:	Micrometer

****

Abstract

The present study was conducted to i) investigate the effect of supplementation of IVM and/or IVC media with ascorbic acid on developmental competence of buffalo embryos ii) to evaluate the themoprotectant role of ascorbic acid on *in vitro* development of buffalo embryos. Immature oocytes collected from slaughterhouse buffalo ovaries were subjected to *in vitro* maturation (IVM), fertilization (IVF) and culture (IVC). In experiment 1, the IVM or IVC or both IVM and IVC media was supplemented with 50 and 100 µM ascorbic acid. It was found that the cleavage and blastocyst rate ($p< 0.05$) was improved at 50 µM ascorbic acid but no significant difference in result was observed at 100 µM ($p< 0.05$). In experiment 2, three groups were taken; for group 1, IVF and IVC were carried out 38.5°C, group 2 immature oocytes were incubated initially at 39.5°C at IVM followed by IVM, IVF and IVC with 50 µM ascorbic acid, group 3 immature oocytes were incubated initially at 40.5°C at IVM followed by IVM, IVF and IVC with 50 µM ascorbic acid. No significant difference in developmental rate was observed at elevated temperature of 39.5°C and 40.5°C respectively. Among the heat stress-related genes, the expression level of *HSP 70.1* and *HSP 70.2* was increased both in control and treatment. The expression level of *BAX* increased at blastocyst stage in control but was significantly lower in treatment at 50 µM ($p< 0.05$). No significant difference was seen in gene expression of *BID* between control and treatement. Regarding anti-apoptotic gene expression of *BCL-XL* and *MCL1,* significant difference was observed in *MCL-1* it was comparatively higher for 50 µM ascorbic acid compared with control but no significant difference was observed in expression of *BCL-XL* between control and treatment. Among developmental gene *GDF 9 and BMP 15,* no discrepancy was concluded in control and treatment. From above results, it is concluded that ascorbic acid has an anti-oxidant as well as thermoprotectant role in developmental competence.

सारांश

वर्तमान अध्ययन के निम्न उद्देश्य थे (i) एस्कॉर्बिक अम्ल का आई.वी.एम. तथा/अथवा आई.वी. सी. मीडिया में पूरक के रूप में प्रयोग करने पर भैंस के भ्रुणों की विकास दक्षता पर प्रभाव ज्ञात करना। (ii) एस्कॉर्बिक अम्ल का भैंस के भ्रुणों के विकास पर एक थर्मोप्रोटेक्टेंट के रूप में योगदान का मूल्याकंन करना। बूचड़खाने से प्राप्त भैंस अण्डाशयों से अपरिपक्व अण्डाणु एकत्र किये गये। तत्पश्चात इनका इनविट्रो परिपक्वन (आई.वी.एम.), इनविट्रो निषेचन (आई.वी.एफ) तथा इनविट्रो कल्चर (आई.वी.सी.) किया गया। परीक्षण क्रमांक मे आई.वी.एम. अथवा आई.वी.सी. अथवा आई.वी.एम. एवम् आई.वी.सी. मीडिया, दोनों को 50 तथा 100μM एस्कॉर्बिक अम्ल से पूरित किया गया। यह ज्ञात हुआ कि विरलन तथा भ्रुण बनने के दर ($P< 0.05$) एस्कॉर्बिक अम्ल की 50μM सांद्रता पर बेहतर पाई गयी किन्तु 100μM पर कोई सार्थक अंतर नही पाया गया ($P< 0.05$)। परीक्षण क्रमांक 2 में चार अलग–अलग समूह लिए गये जिनमें समूह 1 में आई.वी.एफ तथा आई. वी.सी. 38.5^{0}C किए गये। समूह 2 में अपरिपक्व अण्डाणुओं को 39.5^{0}C पर आई.वी.एम. में इन्क्यूबेट किया गया। तत्पश्चात् आई.वी.एफ तथा आई.वी.सी 50μM एस्कॉर्बिक अम्ल से पूरित किया गया। समूह 3 और 4 में अपरिपक्व अण्डाणुओं के प्रारम्भ में 40.5^{0}C पर आई.वी.एम. में इन्क्यूबेट किया गया जिसके बाद आई.वी.एम., आई.वी.एफ. तथा आई.वी.सी. 50μM एस्कॉर्बिक अम्ल के साथ किये गये। विकास दर में कोई भी सार्थक अन्तर बढ़े तापमान क्रमशः 39.5^{0}C तथा 40.5^{0}C पर ˙ परिलक्षित नही हुआ। तापीय तनाव से संबन्धित जीनों में *HSP 70.1* तथा *HSP 70.2* दोनों की अभिव्यक्ति कंट्रोल तथा ट्रीटमैन्ट दानों समूहों में बढ़ा हुआ पाया गया। *BAX* जीन को अभिव्यक्ति कंट्रोल में ब्लास्टोसिस्ट स्तर पर अधिक पाई गयी किन्तु ट्रीटमैंट ग्रुप 50μM में सार्थक रूप से ($P< 0.05$) कम पाई गयी। *BID* जीन की अभिव्यक्ति में कंट्रोल तथा ट्रीटमैन्ट दोनों में कोई सार्थक अंतर नही पाया गया। *BCL XL* तथा *MCL-1* जैसे एन्टी अपॉप्टोटिक जीनों की अभिव्यक्ति देखने पर *MCL-1* की अभिव्यक्ति कंट्रोल ग्रुप में ट्रीटमैन्ट ग्रुप की तुलना में सार्थक रूप से अधिक थी। जबकि *BCL XL* की अभिव्यक्ति में कोई सार्थक ($P< 0.05$) अंतर नहीं पाया गया। विकास से संबंधित जीनों *GDF9* तथा *BMP15* की अभिव्यक्ति में दोनों समूहों में कोई विसंगति नही पाई गई। उपर्युक्त नतीजों से यह निष्कर्ष निकलता है कि एस्कॉर्बिक अम्ल का विकास दक्षता में न केवल एन्टी ऑक्सिडेंट के रूप में अपितू थर्मोप्रोटेक्टेंट के रूप में भी योगदान पाया गया।

CHAPTER – 1

Introduction

1. INTRODUCTION

Reproductive performance of farm animals in tropical and subtropical environments is affected throughout the year by a number of factors such as the physical environment, nutrients availability, adaptability, genetic composition of cattle, intensive or extensive management systems, socio-economic status of farmers. One of the most important factors of physical environment that lead to stress in farm animals is ***oxidative stress***, an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates.

Oxidative stress is involved in aetiology of defective embryo development. Oxygen species may originate from embryo metabolism and embryo surroundings. Reactive oxygen species (ROS) is a term which encompasses all highly reactive, oxygen-containing molecules, including free radicals. Types of ROS include the hydroxyl radical, the superoxide anion radical, hydrogen peroxide, singlet oxygen, nitric oxide radical, hypochlorite radical, and various lipid peroxides. All are capable of reacting with membrane lipids, nucleic acids, proteins and enzymes, and other small molecules, resulting in cellular damage. Embryo metabolism generates ROS via several enzymatic mechanisms involved in different biochemical reaction. ROS generated during embryo metabolism are highly reactive and alters most of cellular development (Guerin *et al.*, 2001).

Pathogenesis and clinical symptoms of oxidative stress

ROS are a double-edged sword: Oxidative stress impairs the intracellular environment resulting in diseased cells and endangered cell survival. ROS can affect a variety of physiological functions in the reproductive tract, and excessive levels can result in pathologies affecting animal reproduction. The oxidant status can influence early embryo development by modifying the key transition factors and hence modifying gene expression. During gestation

oxidative stress plays a role in the initiation of preterm labor and during normal parturition, assuring ovulation, ovarian steroidogenesis, oocyte maturation, blastocyst formation, luteolysis and luteal maintenance in pregnancy. Concentrations of ROS may play a key role both in the fertilization and implantation of oocytes. Oxidative stress operates during follicular development and cyclical endometrial changes. On the other hand, the pathological effects are exerted by various mechanisms including lipid damage, inhibition of protein synthesis and depletion of ATP (Ray *et al.,* 2012).

Heat stress can also disrupt development and function of the oocytes. The best evidence for this statement comes from the lactating dairy cow. In this animal, which is particularly sensitive to heat stress because of the metabolic demands of lactation, oocyte competence for fertilization and subsequent development is reduced during times of the year associated with heat stress. High air temperatures 10 days before oestrus were associated with low fertility. Steroid production by cultured granulosa and thecal cells were low when cells obtained from cows were exposed to heat stress (Roth *et al.,* 2001a).

Strategies for combating oxidative stress

Oxidative damage can be minimized by antioxidant defence mechanisms that protect the cells against cellular oxidants and repair system that prevent the accumulation of oxdatively damaged molecules. Antioxidants, both enzymatic and non-enzymatic, provide necessary defence against oxidative stress. Antioxidant vitamins like vitamin C and vitamin E have have proved to protect the biological membranes against the damage of ROS .The role of vitamin E as an inhibitor –“chain blocker”-of lipid peroxidation has been well established . Like vitamin E, ascorbate is also a chain breaking antioxidant. It prevents lipid peroxidation due to peroxyl radicals. It also recycles vitamin E. It protects against DNA damage induced by H_2O_2 radical. Vitamin C was found to assist in absorption of folic acid by reducing it to tetrahydrofolate;

the latter again acts as an antioxidant. Use of folic acid is impaired when vitamin C is deficient. Administration of ascorbic acid restores the androgenic and gametogenic activity of ethanol treated rats. Vitamin C along with electrolyte supplementation was found to ameliorate the heat stress in buffaloes (Kumar *et al*.,2010). Zinc and other trace elements like Cu and Cr act as typical antioxidants as they work indirectly. Reports have shown the impact of Cu and Zn deficiency on the antioxidant defence system and oxidative damage to cellular components (Picco *et al.,* 2004). The activity SOD, catalase (CAT) and glutathione peroxidise (GPx) is decreased in cu deficient animals. It is also reported that normal cu levels are necessary to maintain the structural integrity of DNA during oxidative stress.

Ascorbic Acid as an antioxidant

Ascorbic acid (AA), commonly known as vitamin C, is 1, 2-Dihydroxyethyl-3, 4-dihydroxyfuran-2(5*H*)-one, is a water-soluble free radical scavenger. Ascorbic acid is the enolic form of one α-ketolactone. Ascorbic acid solution is easily oxidized to the diketo form referred to as dehydro ascorbic acid, which can easily be converted into oxalic acid, diketogulonic acid or threonic acid. Moreover, it regenerates vitamin E in cell membranes in combination with GSH or compounds capable of donating reducing equivalents. Vitamin C, changes to ascorbate radical by donating an electron to the lipid radical in order to terminate the lipid peroxidation chain reaction. The pairs of ascorbate radicals react rapidly to produce one molecule of ascorbate and one molecule of dehydroascorbate. The dehydroascorbate does not have any antioxidant capacity. Hence, dehydroascorbate is converted back into the ascorbate by the addition of two electrons. The last stage of the addition of two electrons to the dehydroascorbate has been proposed to be carried out by oxido reductase.

Buffalo are the mainstay of dairy industry in India. Therefore, it is important to look for the causes that affect embryo development in buffalo. Effects of ascorbic acid on oxidative stress in embryonic development and

thermoprotectant role of ascorbic acid on *in vitro* produced buffalo embryos have not been studied till date.

In case of farm animals like cattle (Ealy *et al*., 1994), sheep (Dutt 1963) and pig (Tompkins *et al.,* 1967), the magnitude of the depression in embryonic survival is less when heat stress is applied later in the preimplantation period than when applied earlier in development. In cattle, for example, exposure of superovulated females to heat stress at day 1 after breeding (i.e., when embryos were at the one- or two-cell stage) reduced the proportion of embryos at day 8 of pregnancy that were at the blastocyst stage of development (Ealy *et al*., 1994). However, heat stress had no effect on the proportion of embryos at day 8, classified as blastocysts, when heat stress was applied at day 3 (4- to 8 cell stage), day 5 (16-cell to morula stage), or day 7 (morula to blastocyst stage). Thus, it might be inferred that preimplantation embryos undergo apoptosis in a stage-specific manner.

Taking this into consideration and the importance of the subject, the project was designed with the following objectives:

1. **To study the effect of ascorbic acid on *in vitro* development of IVM and IVC in buffalo *(Bubalus bubalis)* embryos**
2. **To study the role of ascorbic acid as thermoprotectant on developmental competence of embryos.**
3. **To study the expression of some developmental genes during embryonic development.**

CHAPTER – 2

Review of Literature

2. REVIEW OF LITERATURE

In vitro production (IVP) of embryos is one of the major areas of focus for the reproductive technologies currently being developed for buffalo. It is important not only for faster multiplication of superior germplasm through production of embryos from animals of superior genetic merit but is also an integral part of a number of other reproductive technologies like production of embryonic stem (ES) cells, cloning, transgenesis, gene targeting etc.

2.1 *In vitro* production of buffalo embryos

Buffalo holds tremendous potential in the livestock sector in Asian and Mediterranean countries due to their diversified advantages. Faster multiplication of superior genotypes and the conservation of endangered buffalo breeds are urgent concern for the buffalo scientists. Recent advances in assisted reproductive biotechnologies, including male and female assisted technologies, offer enormous opportunities to not only improve productivity, but also to use buffaloes to produce novel products for applications to human health and nutrition.

IVP of embryos is carried out through a combination of the techniques of *in vitro* maturation (IVM), fertilization (IVF) and culture (IVC) of oocytes. Various aspects of *in vitro* production of buffalo embryos have been extensively reviewed (Palta and Chauhan, 1998; Gasparrini, 2002). A plethora of information is now available on the IVP of embryos from different breeds of buffalo like Murrah (Totey *et al.*, 1992; 1993; 1996; Madan *et al.,* 1994a,b; Chauhan *et al.,* 1996; 1997a,b,c; 1998a,b,c,d,e,f; 1999; Narula *et al.,* 1996; Nandi *et al*., 1998; 2000; 2001) and Nili-Ravi (Samad *et al.,* 1998) and Swamp buffaloes (Liang *et al*., 2007).

2.1.1 *In vitro* maturation

The slaughterhouse ovaries are the major source of immature oocytes since oocytes can be obtained in numbers large enough for standardization and optimization of various techniques like IVM, sperm processing, IVF, IVC etc. Oocytes are collected from ovaries by aspiration of follicles or slicing of ovaries. However, these oocytes cannot be used to produce progeny of known pedigree. For this, oocytes have to be obtained from live animals through ultrasound-guided Transvaginal Oocyte Retrieval (TVOR) also called Ovum Pick-Up (OPU). The oocytes were then examined under a zoom stereomicroscope and graded on the basis of the appearance of their ooplasm and the cumulus mass surrounding them. For IVM, groups of usable quality oocytes in groups of 10-20 each are generally cultured for 24 h in 50-100 µl droplets of IVM medium under sterile paraffin oil at 38.5°C in a 5% CO_2 in air with 90-95% relative humidity (Totey *et al.,* 1992; Madan *et al.,* 1994a,b). Following IVM, the oocytes may be fixed and stained with Giemsa (Das *et al.,* 1997; 1999) or aceto orcein (Totey *et al.,* 1992) for observing the presence of metaphase-II stage chromosomes if the occurrence of nuclear maturation is to be examined. However, since this process kills the oocytes, the occurrence of maturation can alternatively be evaluated by assessing the degree of cumulus expansion by a classification scheme described originally for cattle by Leibfried and First (1979), modified subsequently by Loos *et al.* (1991) and later adapted for buffalo by Chauhan *et al.* (1998b). The *in vitro* matured oocytes which show an acceptable degree of cumulus expansion are then subjected to IVF.

2.1.2 *In vitro* fertilization

The spermatozoa artificially capacitated prior to their incubation with the oocyte for performing IVF. The spermatozoa were treated with an appropriate concentration of heparin for this. Moreover, since the semen generally used for IVF comes from frozen-thawed semen, the spermatozoa need to be treated with a motility enhancing substance like caffeine or the ophylline for increasing their motility (Madan *et al.,* 1994 a,b; Chauhan *et al.,* 1997 a,b,c; 1998 a,b,c,d,e,f;

1999; Nandi *et al*., 1998). The *in vitro* matured oocytes are incubated with the processed spermatozoa for an appropriate period of time for carrying out IVF.

2.1.3 *In vitro* culture

Embryos at the blastocyst stage can be used either for transfer to suitably synchronized recipients or cryopreserved for future use or used as raw material for obtaining inner cell mass (ICM) cells for the production of ES cells.

Probably the most serious problem which limits the applicability of this technology is the very low blastocyst yield which, at only around 6-10% of the total oocytes subjected to IVM, IVF and IVC (Palta and Chauhan 1998; Gasparrini 2002), is far lower than that of around 30 to 40% in cattle (Yang *et al*., 1998a). Any refinements in the IVM protocols directed towards improving blastocyst yield could be very useful not only for the faster multiplication of superior germplasm but also for increasing yields of cloned or transgenic blastocysts, and for production of ES cells. One of the important reasons for low blastocyst production rates is the oxidative stress imposed on the oocytes and embryos during *in vitro* culture.

2.2 Oxidative stress

It reflects an imbalance between the systemic manifestation of reactive oxygen species (ROS) and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA. Oxidative stress from oxidative metabolism causes base damage, as well as strand breaks in DNA. Base damage is mostly indirect and caused by reactive oxygen species (ROS) generated, e.g. O_2^- (superoxide radical), OH^- (hydroxyl radical) and H_2O_2 (hydrogen peroxide). Further, some reactive oxidative species act as cellular messengers in redox signalling. Thus, oxidative stress can cause disruptions in normal mechanisms of cellular signalling (Kala *et al*., 2015).

2.2.1 Role of oxidative stress in embryo production

All aspects of life, including reproduction are affected by reactive oxygen species (ROS). ROS are free radicals (FR), which play a crucial role in the physiological process of spermatogenesis, sperm capacitation, hyperactivation, penetration, oocyte maturation and regression of *corpus luteum* during normal ovarian reproductive cycle (Aitken *et al*., 2004).

There are several factors that contribute to the developmental competence and health of mammalian preimplantation embryos produced *in vitro*. Among these, oxidative stress, which arises as a result of high oxygen tension, appears to be a major factor impairing *in vitro* embryo development. In this respect, there are several reports reflecting the fact that embryos develop well at lower oxygen concentrations. In hamsters and rabbits, intrauterine O_2 concentration is found to decrease to 37 mm Hg (5.3% O_2) and 24 mm Hg (3.5% O_2), respectively, at the time of blastocyst formation and implantation, indicating that embryos develop *in vivo* under low oxygen concentrations. Such a reducing atmosphere that persists *in vivo* is required for proper embryonic development and embryo implantation in the uterus. In terms of rate of embryo development, embryos from mice (Umaoka *et al*., 1991; 1992), sheep (Thompson *et al*., 1990) and cattle (Thompson *et al*., 1990) cultured *in vitro* under low (5%) O_2 concentrations have been reported to show higher developmental rates than those cultured under 20% O_2.

Liu *et al.* (1995) demonstrated that an atmosphere with 5% O_2 was superior to that with 20% O_2 for the *in vitro* development of bovine embryos in terms of their yields (37% vs. 18%; $P<0.01$).

Fujitani *et al.* (1997) concluded, that the respective percentages in 5% vs. 20% O_2 concentration were found to be day 8-early blastocysts: 49% vs. 17%; day 8-expanded blastocysts: 19% vs. 6% and day 10-hatched blastocysts: 16% vs. 0%. Similar kind of results were also reported by Takahashi *et al.* (1993) who observed that after 8 days of culture, the extent of blastocyst formation was significantly decreased ($P<0.001$) when bovine embryos were cultured under

20% oxygen concentration (5.8 ± 2.4%) compared to embryos cultured under 5% oxygen concentration (35.1 ± 6.7%). The decrease in the blastocyst yield was correlated with the observation that a high DNA damage occurred in the embryos cultured under 20% oxygen concentration due to oxidative stress.

Iwamoto *et al.* (2005) reported that low oxygen tension during IVM improved parthenogenetic activation (38.5 ± 3.9% in 5% O_2 tension vs 24.5 ± 3.9% in 20% O_2) and subsequent development to the blastocyst stage in pig. The blastocyst quality measured by total cell number was significantly higher when oocytes were matured under 5% O_2 (34.6 ± 2.0) tension than when they were matured under 20% O_2 (25.9 ± 1.8) tension. This indicates that the detrimental effects of oxidative stress can be reduced under low oxygen tension and that low oxygen tension is useful for the development of parthenotes.

Altogether, these results indicate that a high O_2 concentration during *in vitro* culture of oocytes and embryos reduces their developmental ability due to oxidative stress.

2.2.2 Oxidative stress produces harmful reactive oxygen species

An oxygen rich atmosphere imposes an oxidative stress on oocytes and developing embryos by generating harmful reactive oxygen species (ROS), which are the molecules having an unpaired electron desperately in need of an electron in order to achieve stability. They steal an electron from neighbouring molecules thus destroying them and, in the process, initiate a chain of devastating reactions which ultimately proves highly deleterious for the cell. The important ROS are: hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$), peroxyl radicals ($ROO^{\cdot}$) and alkoxyl radicals ($RO^{\cdot}$). Though these harmful compounds are also produced under normal conditions during embryo development *in vivo*, but the extent of ROS generation is much greater under *in vitro* conditions.

A number of endogenous and exogenous conditions can lead to production of ROS. Endogenously, various metabolic pathways such as oxidative phosphorylation, and enzymes like NADPH oxidase and xanthine oxidase produce ROS. Amongst the exogenous factors, important ones are: high oxygen tension, exposure to visible light, exposure to ROS-producing spermatozoa, amine oxidase present in serum etc. ROS production in both mouse (Goto *et al*., 1993) and bovine (Nagao *et al*., 1994) embryos has been reported to increase during the culture at atmospheric oxygen concentration.

ROS produced as a result of oxidative stress are highly damaging to the oocytes and embryos since they

i) cause a developmental block of embryos (Goto *et al.*, 1993; Nagao *et al.*, 1994),

ii) damage cell membranes (Aitken *et al.*, 1989),

iii) induce nuclear DNA strand breakage (Munne *et al.,* 1991),

iv) cause oxidation of sulphydryl groups in proteins and disulphide formation (Halliwell *et al.*, 1991).

v) cause ATP depletion (Hyslop *et al*., 1988),

vi) lead to mitochondrial alterations (Kowaltowski *et al*., 1999),

vii) inhibit sperm-oocyte fusion (Aitken *et al.,* 1993) and

viii) induce apoptosis (Yang *et al*., 1998b).

ROS modulates gamete quality and gamete interaction. ROS influence spermatozoa, oocytes, embryos and their environment. Role of ROS in oocyte development, maturation, follicular atresia, corpus luteum function, steroidogenesis and luteolysis are being established.

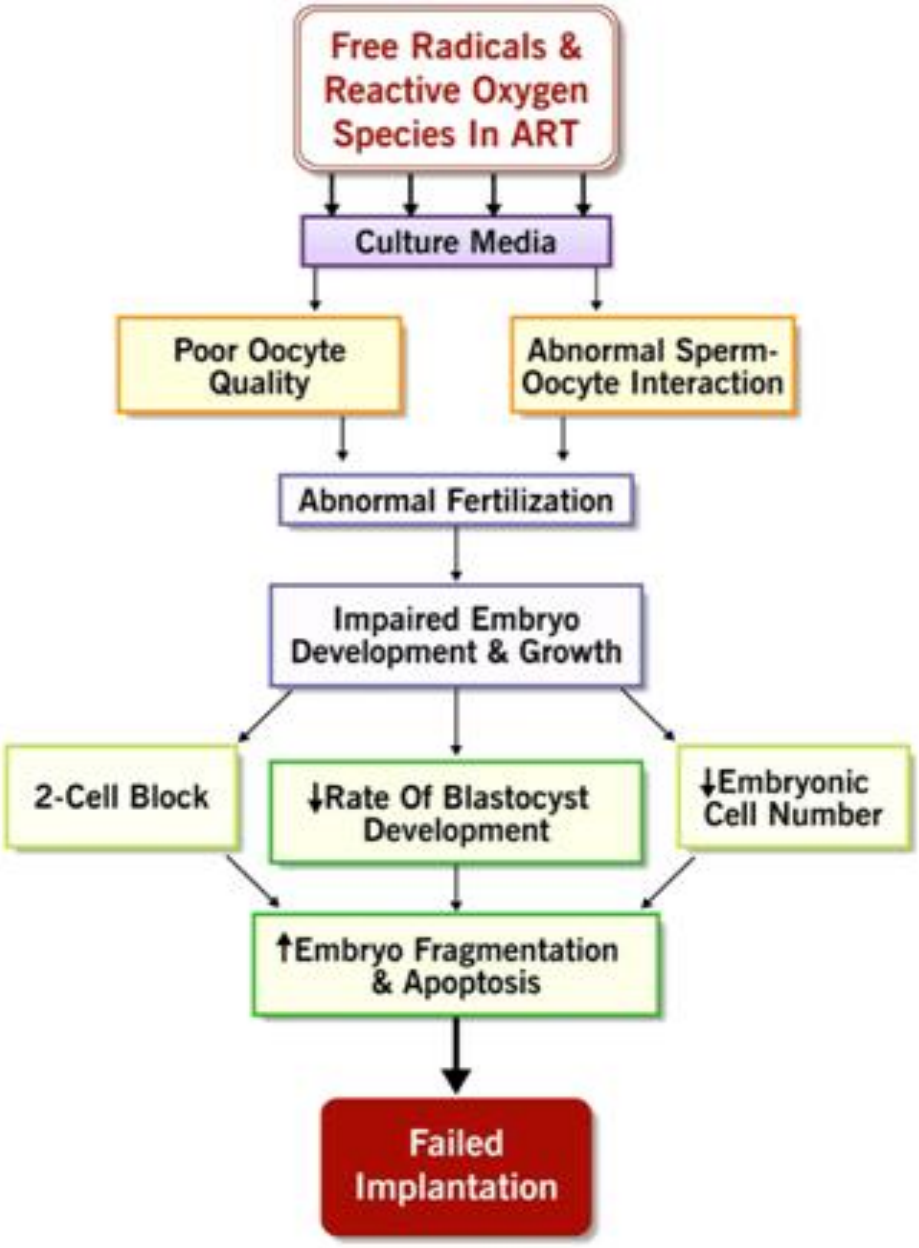

Fig. 1: Influence of the presence of free radicals and ROS in ART culture and subsquent effects on embryo development.

2.2.3 Origin of ROS in Male Reproductive System/Sources of ROS

ROS generated by spermatozoa play an important role in normal physiological processes such as, sperm capacitation, acrosome reaction, maintenance of fertilizing ability, and stabilization of the mitochondrial capsule in the mid-piece in bovine (Gocalves *et al*., 2010).

Controlled generation of ROS may function as signaling molecules (second messengers) in many different cell types; they are important mediators of sperm functions. Evidences have been reported that especially superoxide anion (O_2^-) is required for the late stage of embryo development such as, two germ cell layers and egg cylinder (Kodma *et al*., 1996).

Significant negative correlation between ROS and IVF fertilization rate has been found (Agarwal *et al*., 2005), yet, controlled generation of ROS has shown to be essential for the development of capacitation and hyper activation (Lamirande *et al*., 1993) the two processes of sperm that are necessary to ensure fertilization.

In vivo physiological concentrations of ROS are involved in providing membrane fluidity, maintaining the fertilizing ability and acrosome reaction of sperm (Bucak *et al*., 2010). The maintenance of a suitable ROS level is, therefore, essential for adequate sperm functionality. ROS cause adverse effects on the sperm plasma membrane, DNA, and physiological processes, thereby, affecting the quality of spermatozoa. The acrosome and associated dense fibers of the mid-piece in sperm are covered by mitochondria that generate energy from intracellular stores of ATP depletion (Bucak *et al*., 2008).

According to existing data, the cause of sterility in 30% - 80% of infertility cases is sperm that is disabled as a result of OS (Agarwal *et al*., 2006). OS causes sterility by several mechanisms:

1) ROS damage sperm membrane (containing a large number of polyunsaturated fatty acids, which are vulnerable to ROS attacks-the so-called lipid per-oxidation, leading to decreased motility and difficult fusion between sperm and oocyte (Zalata *et al*., 2004).

2) ROS mitochondrial damage, which reduces the energy available in the cell and thus impedes the movement of sperm. Impaired motility causes a smaller number of sperm reaching the egg, which in turn greatly reduces the likelihood of fertilization (De Lamirande *et al*., 1998).

3) ROS damage sperm DNA. They are capable of directly attacking both purine and pyrimidine bases and the sugar- phosphate backbone. Under normal conditions, sperm DNA is tightly packed with the participation of protamines, and this protects it from the attack by FR. During spermatogenesis histones are initially displaced by transition proteins and then by protamines. Protamines are significantly smaller than histones and contain many positively charged amino acid residues. This allows DNA strands which are highly negatively charged to wrap tightly around the protamine molecules. Moreover protamines contain cysteine residues, allowing formation of disulfide bonds between adjacent protamine molecules (Oliva, 2006).

2.2.4 Cryopreservation/ Freezing Thawing-Oxidative Stress

Freezing/thawing of sperm sample is routinely performed in cattle breeding industries in order to perform artificial insemination. These procedures are known to produce ROS in sperm samples. During cryopreservation, semen is exposed to cold shock and atmospheric oxygen, which in turn increases the susceptibility to lipid per-oxidation due to higher production of ROS. As the sperm plasma membrane is one of the key structures affected by cryopreservation, sperm cryopreservation and thawing is associated with increased ROS production and decreased antioxidant level (Cheema *et al*., 2009).

2.2.5 Origin of ROS in Female Reproductive System/Sources of ROS

Reactive oxygen species produced by the pre-ovulatory follicle are considered important inducers for ovulation (Ruder *et al*., 2009). Oxygen deprivation stimulates follicular angiogenesis, which is important for adequate growth and development of the ovarian follicle. Follicular ROS promotes

apoptosis, whereas GSH and follicular stimulating hormone (FSH) counterbalance this action in the growing follicle. Estrogen increases in response to FSH, triggering the generation of catalase in the dominant follicle, and thus avoiding apoptosis (Behrman *et al*., 2001).

Ovulation is essential for reproduction and commences by the LH surge, which promotes important physiological changes that result in the release of a mature ovum. An over abundance of post-LH surge inflammatory precursors generates ROS; on the other hand, depletion of these precursors impairs ovulation (Shkolnik *et al*., 2011).

Oxidative stress, iron stores, blood lipids, and body fat typically increase with age, especially after menopause. The cessation of menses leads to an increase in iron levels throughout the body. Elevated iron stores could induce oxidative imbalance, which may explain why the incidence of heart disease is higher in postmenopausal than premenopausal women (Crist *et al*., 2009).

Today, the task of maintaining some mammalian embryos in culture is challenging, particularly in species such as the canine and porcine. Recent studies have been reported with great improvement of *in vitro* culture systems by manipulating defined media via various combinatory supplements such as vitamins, growth factors, cytokines, hormones and other selective intracellular and extracellular modulators for biochemical processes in several species. Despite efforts to make improvements, the yield and quality of IVP porcine embryos are still low when compared with their *in vivo* counterpart. In a long-standing practice, ***the hydro soluble antioxidant vitamin* C** has often been used as a supplement in *in vitro* culture systems for oocytes and embryos. As a result of reduced O_2 tension, IVP embryos acquired improved development evident by a decreased reactive oxygen species (ROS) content and DNA fragmentation. Glutathione (GSH), a major intra cellular free thiol group, is involved in cellular proliferation and amino acid transport.

2.2.6 Amelioration of oxidative stress by antioxidants

The antioxidants can be broadly classified into two types, enzymatic and non-enzymatic. Amongst the enzymatic defense mechanisms, some important ones are superoxide dismutases (SODs) like the copper-zinc SOD-located in the cytosol and manganese-SOD located in mitochondria. These scavenge the superoxide radicals. Hydrogen peroxide-H_2O_2, the byproduct of SOD action, is eliminated by the action of catalase.

The important non-enzymatic antioxidants are vitamins A, C, E, pyruvate and sulphur compounds such as glutathione (GSH), hypotaurine, taurine, cysteine, cystine, cysteamine etc. Amongst these, GSH appears to be the main non-enzymatic defense system against ROS in embryos. Glutathione, a tripeptide (γ-glutamylcysteinylglycine) is a major non-protein sulphydryl compound in mammalian cells, which plays a critical role in protecting the cells from oxidative damages (Takahashi *et al*., 1993; Gardiner *et al*., 1998). It is both the most prevalent cellular thiol and the most abundant low molecular weight peptide. In many cells, GSH accounts for more than 90% of the total nonprotein sulfur. Inside the cell, synthesis of glutathione occurs in the cytoplasm via the sequential actions of γ–glutamyl cysteine synthetase and GSH synthetase (Meister, 1988).

2.2.7 Mechanism of radical scavenging activity of ascorbic acid

Vitamin C or ascorbic acid is a water-soluble free radical scavenger. Moreover, it regenerates vitamin E in cell membranes in combination with GSH or compounds capable of donating reducing equivalents. Vitamin C, changes to the ascorbate radical by donating an electron to the lipid radical in order to terminate the lipid per-oxidation chain reaction. The pairs of ascorbate radicals react rapidly to produce one molecule of ascorbate and one molecule of dehydroascorbate. The dehydroascorbate does not have any antioxidant capacity. Hence, dehydroascorbate is converted back into the ascorbate by the addition of two electrons. The last stage of the addition of two electrons to the dehydroascorbate has been proposed to be carried out by oxido-reductase (Oh *et al*., 2011).

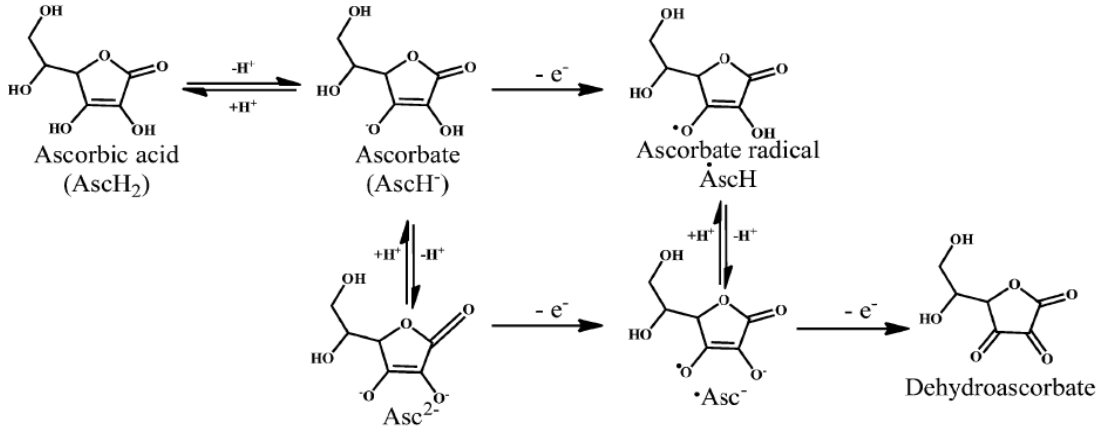

Fig. 2: Mechanism of radical scavenging activity of ascorbic acid

2.2.6 Supplementation of *in vitro* maturation and culture medium with ascorbic Acid

2.2.6.1 Sheep

In vitro embryo production procedures developed for sheep have tremendously been improved from decades, but many factors influencing their efficiency still need to be investigated. The overall of this study showed the production of the sheep embryos till blastocyst stage from the ovaries of the slaughtered ewes for IVM, IVF of the oocytes and then IVC in the complex culture media like TCM-199, TCM-199-, TCM-199+ and CR1aa. The morula yield was significantly higher in the wheat peptone and BSA supplemented group. The nuclear maturation rates of ovine oocytes matured in FBS (69.15 ± 1.07) group was more compared to BSA (56.47 ± 0.73) and wheat peptone (35.26 ± 0.79). The nuclear maturation of oocytes was significantly higher in the FBS group when compared to BSA and wheat peptone supplemented groups. The lowest maturation rate was observed in wheat peptone supplemented group. The development rates in 2, 4, 8, 16 and morula stages of ovine embryos produced from BSA group were high (74.22 ± 2.56, 60.93 ± 2.55, 42.97 ± 2.20 and 24.21 ± 1.28) compared to FBS group (73.58 ± 2.92, 58.77 ± 2.01,41.22 ± 1.68 and 20.17 ± 0.76) and wheat peptone (72.89 ± 1.09, 57.01 ± 0.76, 42.05 ± 0.36 and

24.29 ± 0.55). The cleavage, morula and blastocyst percent in CR1aa was found significantly high compared with TCM- 199, TCM-199- and TCM-199+. The cleavage, morula and blastocyst percent in L-Ascorbic acid at 100 µM with CR1aa is found more significant compared with other compositions used in the experimentation (Sreenivas *et al*., 2014).

2.2.6.2 Pig

Dose-response assessment performed to understand the relation between supplementation of media with L-ascorbic acid or vitamin C and porcine oocyte maturation and the *in vitro* development of parthenotes (PA) and handmade cloned (HMC) embryos. Various concentrations (0, 25, 50 and 100 µg/ml) of vitamin C supplemented in *in vitro* maturation (IVM) and culture (IVC) media were tested. When cultured in IVM- and/or IVC-supplemented media, the group supplemented with 50 µg/ml of vitamin C showed improved cleavage rates, blastocyst rates and total cell numbers per blastocyst ($P<0.05$) compared with other groups (control, 25 µg/ml and 100 µg/ml). In contrast, supplementation with 50 µg/ml vitamin C decreased ($P<0.05$) the apoptosis index as compared with the groups supplemented with 100 µg/ml. In addition, even with a lower blastocyst rate to start with (37.6 *vs.* 50.3%, $P<0.05$), supplementation of HMC embryos with vitamin C ameliorated their blastocyst quality to the extent of PA embryos as indicated by their total cell numbers (61.2 *vs.* 59.1). Taken together, an optimized concentration of vitamin C supplementation in the medium not only improves blastocyst rates and total cell numbers but also reduces apoptotic indices, whereas over dosages compromise various aspects of the development of parthenotes and cloned porcine embryos (Kere *et al*., 2014).

2.2.6.3 Goat

The effect of subcutaneous injections of vitamin C on the seminal characteristics of Markhoz bucks (2-4 year old) was studied. The bucks, trained to serve an artificial vagina, were randomly allotted into three equal groups (n = 4) and received daily either zero (1 mL normal saline; control group), or 20 (VitC

20 group) or 40 (VitC40 group) mg per kg body weight vitamin C from July 06, 2006 to Oct. 06, 2006. Blood samples were taken from the jugular vein at monthly intervals, and semen samples were collected at 15-day intervals. Testicular dimensions in the scrotum (circumference, width, and length) were also determined on the day before semen collection. The ejaculates were evaluated for volume, sperm concentration, pH, motility, and abnormal and live sperm. Testicular measurements were not affected by administration of vitamin C. The interaction between vitamin C and the sampling time was significant ($P<0.05$) for the concentration of vitamin C in the blood plasma and seminal fluid, sperm motility, sperm viability, sperm abnormality, and the number of live-normal sperm in the ejaculate. Vitamin C increased the levels of vitamin C in blood and seminal plasma. Both doses of vitamin C increased the percentage of progressively motile sperm showing forward motility. VitC40 injection for 90 days increased sperm motility and the effect was still evident up to 30 days after the cessation of injections (Fazeli *et al*., 2010).

2.2.6.4 Buffalo

Culture medium supplemented with either α-tocopherol or L-ascorbic acid at a concentration of 250 mM increased the quality of IVF-derived buffalo embryos, blastocyst rate and blastocyst cell number. Higher concentration of either α-tocopherol or L-ascorbic acid (500 mM) failed to improve *in vitro* embryo development. Vitamin C (ascorbic acid) is an important water-soluble antioxidant that reduces sulfhydryls, scavenges free radicals and protects against endogenous oxidative DNA damage. Vitamin C may become a pro-oxidant when free transition metals are present. Gasparrini *et al*. (2004) reported the effects of enriching the IVM medium with cystine, in the presence of cysteamine, on the *in vitro* embryo production efficiency. When the IVM was carried out in the absence or presence of 50 µM cysteamine, with or without 0.3 mM cystine, the intra cytoplasmic GSH concentration was found to be significantly higher than the control, with the highest GSH levels in oocytes matured in the presence of both thiol compounds (3.6, 4.7, 5.4 and 6.9 pM/oocyte in the control, cysteamine,

cystine and cystine + cysteamine groups, respectively; P<0.05). Cystine supplementation of IVM medium, both in the presence or absence of cysteamine, significantly increased the proportion of oocytes showing two normal synchronous pronuclei following fertilization. In all supplemented groups, cleavage rate was significantly improved compared to the control (55, 66.1, 73.5 and 78.4% in the control, cysteamine, cystine and cystine + cysteamine groups, respectively; P<0.05). Similarly, blastocyst yield was also increased in the three enriched groups compared to the control (17.1, 23.8, 29.3, 30.9% in the control, cysteamine, cystine and cystine + cysteamine groups, respectively; P<0.05). Overall, the addition of cystine to a cysteamine-enriched medium resulted in a significant increase of cleavage rate and transferable embryo yield compared to the medium supplemented with only cysteamine.

Supplementation of IVM medium with 50 µm cysteamine increased (*P*<0.01) the cleavage rate and blastocyst yield without affecting the HR and TCN whereas a higher concentration of 200 µm significantly (*P*<0.05) reduced the blastocyst yield but not TCN. Similar increases in blastocyst yield, without any effect on HR and TCN were observed after supplementation of the IVC medium with 100 (*P*<0.01) or 50 µm (*P*<0.05) cysteamine, whereas 200µm cysteamine was ineffective. Supplementation of both IVM medium with 50µm cysteamine and of IVC medium with 100µm cysteamine increased the yield of blastocysts and hatched blastocyst by over 100%(*P* <0.01) compared with the controls without any adverse effects on HR or TCN. The results of the present study suggest that supplementation of both IVM and IVC media improves the yield of blastocysts without compromising their health (Anand *et al*., 2008). Elamaran *et al*. (2012) found that cysteamine supplementation, the blastocyst rate and the relative mRNA abundance of *BCL-XL* and *MCL-1* was significantly higher (P<0.05) and that of *BAX* but not *BID* was lower (P<0.05) at many stages of embryonic development.

Buffalo oocytes cultured at 38.5°C (control) or exposed to 39.5°C or 40.5°C for 2 h once every day throughout *in vitro* maturation (IVM), fertilization (IVF) and culture (IVC). At both 8–16-cell and blastocyst stages, relative mRNA abundance of stress-related genes *HSP 70.1* and *HSP70.2* and pro-apoptotic genes *CASPASE-3*, *BID* and *BAX* was higher than that in controls with the exception of stress-related gene *HSF1*.The expression level of *ZAR-1* and *BMP-15* was not affected, whereas that of *GDF-9* was either increased by heat shock or the level of decrease during embryonic development was lower (Yadav *et al*., 2013).

2.3 Heat Stress

Heat stress is a type of abiotic stress. It is caused due to overexposure or overexertion in excess environmental temperature. Temperature determines metabolic rate, heart rate, hormone secretion, reproduction and other important factors within the bodies of animals, so an extreme temperature change can easily distress the animal's body. Temperature is a critical abiotic factor affecting organisms at ecological, organism, cellular and molecular levels (Somero *et al*., 2002). Thermal stress is a unique and complex phenomenon that brings about numerous challenges beyond the animal's baseline homeostatic mechanism causing alterations of the normal physiological mechanisms, thus, elicits a stressful response.

2.3.1 Effect of heat stress on rat and mouse reproduction and embryo development

Heat stress inhibits ovarian follicular development in mammalian species and suppresses follicular development. The ovaries and granulosa cells of follicles at different developmental stages were analyzed for gonadotropin receptor levels and aromatase activity; estradiol levels were measured in follicular fluid. Before injection, heat stress diminished only the amount of FSH receptor on granulosa cells of antral follicles. During PMSG- stimulated follicular development, heat stress strongly inhibited gonadotropin receptor levels and

aromatase activity in granulosa cells and estradiol levels in the follicular fluid of early antral, antral and preovulatory follicles. Heat-stressed granulosa cells showed a time-dependent increase in apoptosis. The *BCL-2* mRNA levels were similar in control and heat-stressed granulosa cells; *BAX* mRNA levels were increased in heat-stressed granulosa cells. The heat stress inhibits expression of gonadotropin receptors in granulosa cells and attenuates estrogenic activity of growing follicles, granulosa cells of heat-stressed follicles are susceptible to apoptosis and the BCL2/*BAX* system is not associated with heat stress-induced apoptosis of granulosa cells. This study suggests that decreased numbers and function of granulosa cells may cause ovarian dysfunction in domestic animals.

Paternal heat stress significantly reduced the proportion of embryos that developed normally during 24-120 h of *in vitro* culture, when zygotes were sired by males which had been heat stressed between 7 and 35 days prior to mating. Maximum impairment to development (including undeveloped, abnormal and dying/dead embryos) occurred in those embryos sired by males at days 14 and 21 after heating. Embryo development returned to control levels by day 42 after heat stress.

Furthermore, all stages of embryo development were affected by paternal heat stress, the proportion of embryos at the two-cell stage appeared to be most severely affected. Four-cell to morula stages and the morula to blastocyst stage also.demonstrated impairment at days 14, 21, 28 and 35 after heating. Single episode of paternal heat stress significantly reduces the development of preimplantation embryos, and this is not recovered until day 42 after heating (Zhu *et al.,* 2004). Development of mouse embryos was inhibited by exposure to 39.8°C beginning at the one-cell stage. Fiorenza and Mangia (1992) reported that mild hyperthermic conditions (38.5-40.8°C) during maturation disturbed the process of bivalent chromosome disjunction and blocked mouse oocytes at the metaphase I stage. Tseng *et al.* (2006) found that rates of blastocyst formation for pig oocytes were reduced post-maturation heat shock.

2.3.2 Effect of heat stress on bovine reproduction and embryo development

Heat stress can disrupt development and function of the oocytes. The best evidence for this statement comes from the lactating dairy cow. In this animal, which is particularly sensitive to heat stress because of the metabolic demands of lactation, oocyte competence for fertilization and subsequent development is reduced during times of the year associated with heat stress (Zeron *et al.,* 2001; Al-Katanani *et al.,* 2002; Sartori *et al.,* 2002). High air temperatures 10 days before oestrus were associated with low fertility (Al-Katanani *et al.,* 1999).

Steroid production by cultured granulosa and thecal cells was low when cells were obtained from cows exposed to heat stress 20-26 days previously (Roth *et al.,* 2001a), i.e. when follicles were 0.5-1 mm in diameter. Moreover, the resumption of fertility seen in lactating dairy cows in Israel in the autumn could be hastened by removing follicles formed in the summer (Roth *et al.,* 2001b).The mechanism by which heat stress during oogenesis compromises oocyte function is likely to involve alterations in follicular function.

Heat stress can alter follicular growth (Roth *et al.,* 2000), steroid secretion (Wolfenson *et al.,* 1997; Roth *et al.,* 2001a; Ozawa *et al.,* 2005) and gene expression (Argov *et al.,* 2005). Effects of heat stress on follicular function could involve changes at the level of the follicle or the secretion of the pituitary hormones that control development of the follicle.

Cultured follicular cells experience reduced steroid production at elevated temperature in cattle (Wolfenson *et al.,* 1997; Bridges *et al.,* 2005). One of the consequences of heat stress in lactating dairy cows is increased numbers of small and medium follicles; recruitment of these follicles into the growing pool seems to be due to a decrease in circulating concentrations of inhibin and increased FSH secretion (Roth *et al.,* 2000). The pre-implantation embryo is susceptible to maternal heat stress but the susceptibility declines as development proceeds.

Ealy *et al.* (1994) found that exposure of lactating cows to heat stress at day 1 after oestrus, when embryos were one to two cells, reduced the proportion of embryos that developed to the blastocyst stage at day 8 after oestrus. After *in vitro* fertilization, embryos were exposed to heat shock at 41°C for 6 h on days 0, 2, 4 and 6, respectively. On day 2, cleavage rate was not significantly different in all groups. However, the percentage of embryos developing to blastocyst stage after exposure to heat shock on day 0 (18.8 ± 4.3%) and day 2 (23.6 ± 3.7%) were significantly decreased compared with control (37.5 ± 4.0%), day 4 (40.0 ± 7.4%) and day 6 (38.1 ± 2.0%). In addition, the total cell number of blastocysts was significantly decreased by heat shock on day 0 (107.5 ± 6.6) and day 2 (112.8 ± 5.7) compared with the control (143.2 ± 9.4).

Sakatani *et al.* (2004) reported that heat stress also induces the oxidative stress by production of reactive oxygen species (ROS), when embryos were exposed to heat shock on days 0 and 2. However, heat shock on day 4 and day 6 did not increase the ROS production. This study indicates that heat shock to earlier stage embryos causes a decrease in development to blastocysts and cell proliferation and the decrease in development by heat shock could be involved in an increase of intracellular oxidative stress. Oocytes collected from slaughter house during summer produced lower number of blastocysts after *in vitro* maturation, fertilization and culture as compared with oocytes collected from slaughter house at the time of winter seasons. Oocytes obtained from winter animals by aspiration formed more blastocysts as compared with the heat exposed animals. Summer depression in oocyte quality in Holstein cows was evident, but cooling cows for 42 d did not alleviate that seasonal effect (Al-Katanani *et al.,* 2002).

Krininger *et al.* (2002) observed that *in vitro* heat stress during the critical stage of early embryo development significantly increases the incidence of early embryonic mortality. Heat treatment during the first 48 h of IVC significantly impaired embryo development, the percentage of heat treatment zygotes that developed to ≥8-cell stage embryos after 72 h IVC was 2.0% (n=459) compared with 28.4% (n=458) for the control zygotes (P<0.01). The subsequent yield of morulae or blastocysts after 144 h IVC for the heat treatment and control groups

was 0.9% (n=457) and 12.3% (n=456) (P<0.01), respectively. The percentage of zygotes that developed into morulae and blastocysts following heat treatment during the fourth day of IVC was 4.5% (n=468) compared to 10.5% (n=456) for the control group (P<0.01). Heat shock, conversely, reduced development to the blastocyst stage when applied at the two-cell stage but not when applied to embryos ≥ 16-cells at day 5 after insemination. Some actions of elevated temperature on the preimplantation embryo probably involve increased production of reactive oxygen species.

Maternal heat stress in mouse has resulted in increased reactive oxygen species activity in oviducts and embryos (Ozawa *et al.,* 2002; Matsuzuka *et al.,* 2005) and reduced glutathione content in recovered embryos (Ozawa *et al.,* 2002; Matsuzuka *et al.,* 2005). Treatment of female mice with either melatonin (Matsuzuka *et al.,* 2005) or vitamin E (Sakamoto *et al.,* 2008) reduced the effects of heat stress on embryonic development. Female embryos are better able to survive effects of elevated temperature than male mice and this gender difference has been demonstrated to be caused by reduced reactive oxygen species production in females (Perez-Crespo *et al.,* 2005).

Increased reactive oxygen species production in response to elevated culture temperature has also been reported for cattle (Sakatani *et al.,* 2004, 2008) and treatment with the antioxidant 2-mercaptoethanol has been reported to alleviate the negative effects of heat shock on development in one study (Sakatani *et al.,* 2008), although not in another (Castroe Paula and Hansen, 2008). Recent studies indicate that rate for blastocyst formation decreased significantly as maturation temperature increased from 38.5°C to 39°C.

Roth and Hansen (2004) found that while bovine oocytes matured at 38.5°C were mostly at metaphase II stage, the majority of heat-shocked oocytes were blocked at the first metaphase, first anaphase or first telophase stages of *in vitro* produced embryo. In addition, (Roth and Hansen, 2004) showed that exposure of bovine oocytes to thermal stress during the first 12 h of maturation reduced cleavage rate and the number of oocytes developed during the blastocyst stage.

Edwards *et al*. (1997) found that exposure of bovine cumulus oocyte complexes to 41°C did not alter the number of embryos that cleaved but reduced the number that developed to the blastocyst stage. In contrast, exposure to 42°C reduced both cleavage and developmental rates.

Oocytes harvested from cows during the summer showed reduced ability to develop into blastocysts after fertilization *in vitro* (Al-Katanani *et al.,* 2002). Exposure of heifers to heat stress between the onset of estrus and insemination increased the proportion of abnormal and retarded embryos (Putney *et al.,* 1989). This suggests that the process of oocyte maturation is susceptible to heat stress. In fact, it has been shown that exposure of bovine oocytes to elevated temperature during *in vitro* maturation decreased their subsequent cleavage and blastocyst rates (Edwards *et al.,* 1997; Roth and Hansen, 2004).

2.4 Genes Related To Apoptosis

Apoptosis is an evolutionary highly conserved mechanism that allows the organism to tightly control cell numbers, tissue size and protect itself from dangerous cells that threaten homeostasis. There are at least two major protein families involved in the regulation of apoptosis, namely***, BCL-2 and BAX***. The *BCL-2* family members include ***BCL-2, BCL-XL, BCL-X***, and ***BCL-W*** has been shown to protect cells from apoptosis whereas the *BAX* family members Bad, *BAX* and *BAK* induce apoptosis in somatic cells. These patterns also appear to be true for embryonic development as poor quality bovine embryos that are undergoing more apoptosis express more *BAX* and less *BCL* as compared to good quality embryos (Yang and Rajamahendran, 2002). It is difficult to obtain pig embryos of homogeneous quality due to the relatively high incidence of polyspermy during *in vitro* fertilization. Therefore, diploid parthenotes have frequently been used to study early development in the mammals.

2.4.1 *BAX*

BAX stimulates the release of cytochrome c from mitochondria to induce downstream caspases (proteases) that can dismantle the cell. Mice with targeted disruption in the *BAX* gene show abnormalities in granulosa cell apoptosis (Knudson *et al.,* 1995) and increased resistance to follicular atresia resulting in a dramatic extension of ovarian life span. In addition, mice carrying a null of *BAX* are more resistant to apoptosis induced by chemotherapy agent (Perez-crespo *et al.,* 2005).

Yang and Rajamahendran (2002) reported that expression of *BCL-2* was high in good quality oocytes and embryos, low in fragmented embryos, and hardly detectable in denuded oocytes. In contrast, the expression of *BAX* was found in all types of oocytes and embryos with the highest expression in the denuded oocytes. This implies that the ratio of *BCL-2* to *BAX* may be used to gauge the tendency of oocytes and embryos towards either survival or apoptosis. Transcripts of the anti-apoptotic gene, *BCL-XL*, were detected in all stages of bovine embryo development and displayed a maternal/embryonic expression profile with a dramatic increase at the blastocyst stage. Augustin *et al.,* (2003) reported the expression of this anti-apoptotic gene in bovine blastocysts.

2.4.2 *BID*

BID, a pro-apoptotic member of the *BCL-2* family, was initially discovered through binding to both pro-apoptotic *BAX* and anti-apoptotic *BCL-2*. *BCL-2* family of proteins that regulate the permeabilization of the outer mitochondrial membrane (OMM), a critical event during apoptosis. *BCL-2* family proteins, including *BID*, *BIM* and *BAD*, contain sequence homology only in the BH3 region and hence are referred to as BH3-only proteins (Kim *et al*., 2006).

2.4.3 *BCL-XL*

BCL-XL (*BCL-2* like 1) is a member of the group of anti-apoptotic proteins. It inhibits the association of apoptotic peptidase activating factor (*APAF-1*) with procaspase-9 and thereby prevents caspase-9 activation and subsequent loss of mitochondrial function (Chinnaiyan *et al.,* 1997). According to Shimizu *et al.* (1999), survival factors such as *BCL-2* and *BCL-XL* prevent cytochrome c release from the mitochondria by binding to *BAX*.

2.4.4 *MCL1*

MCL1 is a member of the *BCL-2* family that acts as an apical molecule in apoptosis control, promoting cell survival by interfering at an early stage in a cascade of events leading to release of cytochrome c from mitochondria. *MCL1* is required for embryonic development and the function of the immune system (Michels *et al*., 2005). *MCL1* can also readily be cleaved by caspases during apoptosis to produce a cell death promoting molecule. *MCL1* plays a critical role in controlling life and death decisions in response to rapidly changing environmental stimuli as suggested by the multiple levels of control of *MCL1* expression.

2.5 Genes related to embryonic development

2.5.1 *BMP15*

Bone morphogenetic protein (*BMP15*), a member of the transforming growth factor β (*TGF-β*) superfamily is closely related to growth and differentiation factor (*GDF9*), structurally and functionally. In mammals, *BMP15* is predominantly produced by oocytes and exerts important regulatory functions within the ovary, such as promoting early folliculogenesis, preventing premature luteinization and enhancing cumulus cell expansion. Besides ovary, expression of this gene in other tissues has also been reported (Dube *et al.,* 1998).

In mammals, *BMP15* is the product of an X-linked gene and is predominantly expressed in oocytes within the mammalian ovary (Silva *et al.,* 2005) which further suggest a role for BMPs in modulating oocyte development and maturation. Defects in folliculogenesis have been shown to be caused by mutations in *BMP15* in ewes and women.

Di Pasquale *et al.* (2004) demonstrated that an inherited mutation in *BMP15* caused hypergonadotropic ovarian failure in humans. *BMP15* knockout mice have normal follicle development but show only sub fertility with defects in ovulation and early embryonic development (Yan *et al.,* 2001). Difference in *BMP15* processing and secretion cause it to have different roles in different species. While mature human *BMP15* is processed and secreted throughout folliculogenesis in mouse, mature *BMP15* is not detectable until after exposure to the LH surge (Hashimoto *et al.,* 2005; Yoshino *et al.,* 2006).

BMP15 induces granulosa cell proliferation and the expression of kit ligand, a factor known to be essential for early folliculogenesis (Otsuka and Shimasaki, 2002). *BMP15* induces cumulus cell expansion *in vitro* (Yoshino *et al.,* 2006). *BMP15* is known to increase oocyte developmental competence in bovines (Gilchrist *et al.,* 2008). Similarly, human *BMP15* in the follicular fluid is positively correlated with the developmental potential of the oocytes (Wu *et al.,* 2007).

2.5.2 *GDF9*

Growth differentiation factor 9 (*GDF9*) is a well-characterized oocyte-derived growth factor that plays crucial roles in follicle growth and ovulation in all mammalian species studied, including rodents (Yan *et al.,* 2001), domestic ruminants (Bodin *et al.,* 2007; Galloway *et al.,* 2000) and humans (Chand *et al.,* 2006; Di Pasquale *et al.,* 2006; Palmer *et al.,* 2006). Growth differentiation factor-9 (*GDF9*) is a distant member of the *TGFβ* superfamily with highest homology to *BMP15* (Dube *et al.,* 1998). Culturing oocytes from wild-type and mutant mice demonstrated that *GDF9*-deficient oocytes have defects in meiosis. Follicle development beyond the primary stage occurs neither in *GDF9* null mice (Dong

et al., 1996) nor in ewes homozygous for naturally occurring inactivating mutations in the *GDF9* gene which indicates an obligatory role for this oocyte-derived growth factor.

In vitro, *GDF9* has been shown to stimulate progesterone production (Vitt *et al.,* 2000) and has been proposed to be the oocyte-derived factor responsible for maintaining cumulus cell function Thus, *GDF9* plays a critical function as a growth and differentiation factor during early folliculogenesis and as a key regulator of several granulosa cell proteins involved in cumulus expansion and maintenance of an optimal oocyte microenvironment.

2.7 Genes responsible for thermo-tolerance responses

2.7.1 *HSP 70.1 and HSP 70.2*

Elisabeth *et al.* (1995) reported that *HSP 70.1* gene is highly transcribed at the onset of zygotic genome activation and transcription of this gene began as early as the 1-cell stage. Expression of the gene continued through the early 2-cell stage but was repressed before the completion of the second round of DNA replication. Retinal photic injury was prevented by hyperthermia-induced *HSP70*. *HSP*70 from *HSP70.3* may be a rapid and short-lived responder, and that from *HSP70.1* is a slower and more sustained responder. *HSP*70 from *HSP70.3* may be an initial retinal chaperone while *HSP*70 from *HSP70.1* may be a sustained chaperone (Jin *et al.,* 2007).

The activities of both SOD-1 and SOD-2 were significantly decreased in *HSP 70.1* KO mice than in the wild type (WT) littermates. SOD-1 protein level in the *HSP 70.1* KO mice was lower than that of WT. Camargo *et al.* (2007) studied developmental competence and expression of the *HSP 70.1* gene in immature oocytes from *B. taurus* (Holstein) and *B. indicus* (Gyr) dairy cows raised in a tropical region by taking cumulus–oocyte complexes in spring and early autumn, and subjected to *in vitro* maturation and fertilization. Cleavage and blastocyst rates were greater ($P < 0.05$) for Gyr (n= 390 oocytes) than Holstein (n= 505) breed (66.7% versus 53.1% of cleavage and 19.6% versus 10.8% of blastocysts,

respectively) but pregnancy rates were not significantly different following transfer to recipients (44.5% for 36 Gyr embryos; 60% for 10 Holstein embryos). Holstein immature oocytes had a higher level ($P < 0.05$) of *HSP 70.1* relative expression (1.82 ± 0.22; mean ± S.E.M.) than Gyr oocytes (1.12 ± 0.11). In conclusion, Gyr oocytes obtained in a tropical region were less subjected to stress and more likely to develop (after IVF) than Holstein oocytes. Sharma *et al.* (2012) showed quantitative expression of *HSPA-1A* mRNA in immature oocytes (IMO), matured oocytes (MO), *in vitro* produced (IVP) and *in vivo*-derived (IVD) buffalo embryos to assess the level of stress to which embryos are exposed under *in vivo* and *in vitro* culture conditions. The *HSPA-1* expression analysis was studied in 72 oocytes, 76 IVP and 55 IVD buffalo embryos. Expression of *HSPA-1A* was found in oocytes and throughout the developmental stages of embryos examined irrespective of the embryo source; however, higher ($P< 0.05$) expression was observed in 8- to 16-cell, morula and blastocyst stages of IVP embryos as compared to IVD embryos. Sharma *et al.* (2012) concluded that higher level of *HSPA-1A* mRNA in IVP embryos in comparison with *in vivo*-derived embryos is an indicator of cellular stress in IVP system.

CHAPTER – 3

Material and Methods

3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Glassware and plastic ware

All the glassware used in the present investigation was made of high-grade Pyrex glass. The glassware, wherever used, were thoroughly cleaned and rinsed with triple distilled water and then heat sterilized at 250°C for 4h. Pasteur pipettes were from Labco, Ambala, India. The plastic ware, which included disposable 100 mm x 100 mm square Petri dishes with 13 mm grid (searching dishes), 35 mm x 10 mm and 60 mm x 15 mm cell culture Petri dishes, 15 and 50 ml Falcon tubes and tissue culture flasks were purchased either from Becton, Dickinson and Co., Lincoln Park, NJ, USA or from Nunc, Rosklide, Denmark. The 0.22 µm filters were from Millipore Ireland Ltd, IRL or from Nalgene, Nalgene Company, Rochester, New York, USA. Disposable, non-toxic and non-pyrogenic plastic syringes of assorted sizes were from Norm-Ject, Henke-Sass Wolf GmbH, Germany. Sterile disposable 18 gauge hypodermic needles of Dispovan were from Hindustan Syringes & Medical Devices Ltd., Faridabad, India, whereas autoclavable disposable tips for micropipettes were from Tarsons Products Pvt. Ltd, Kolkata, India. Plasticware like microtips, tip boxes, eppendorf tubes etc., were made RNase free by soaking them in 0.1% diethylpyrocarbonate (DEPC) over night, followed by autoclaving.

3.1.2 Chemicals, cell culture media and supplements

The different media used in the present study for the culture of oocytes/embryos, which included tissue culture medium-199 (TCM-199), and Dulbecco's phosphate buffered saline (DPBS) were from Sigma Chemical Company, St. Louis, MO, USA. The media were in the ready-to-use liquid form. Various supplements which included bovine serum albumin (BSA), antibiotics (gentamicin and streptomycin), porcine follicle stimulating hormone (pFSH), sodium pyruvate, L-glutamine, fatty acid-free BSA, heparin, caffeine, mineral oil

and all the chemicals used in various experiments were also purchased from Sigma Chemical Company, St. Louis, MO, USA unless otherwise indicated. Most of the chemicals used in the present study were of embryo tested or cell culture tested grade. Fetal bovine serum (FBS) was from Hyclone (Logan, Utah, US).

3.1.3 Equipments

3.1.3.1 Microscopes

a) Zoom stereomicroscope

Low magnification zoom stereo microscopes (Nikon, Japan, Model SMZ-745T or Olympus SZX 7) were used for searching the aspirated oocytes, for evaluating the quality of the oocytes and for collection of various embryonic stages.

b) Inverted microscope

An inverted microscope (Nikon, Japan, Model TMD) was used for the examination of embryos for monitoring health, morphological characteristics and growth of the various embryonic stages. The microscope with the light source at the top and a long working distance allowed embryos in culture dishes to be viewed and photographed whenever needed. The microscope was equipped with an incubator attachment to enable maintenance of optimum temperature during working. The inverted microscope was also equipped with UV fluorescence and differential interference contrast (DIC) attachment, which helped in capturing the images of *in vitro* produced embryos. The microscope was equipped with programmable still photography and video recording facilities.

3.1.3.2 Laminar flow hood

Experiments including searching, grading and, *in vitro* embryo production (IVM, IVF, IVC) procedures were carried out in Laminar flow cabinet (CLEANAIR Laminar Flow Systems, India), which served the purpose of minimizing the incidences of microbial contamination and ensuring the safety of the operator. UV irradiation and thorough cleaning of working places with ethanol

(70% v/v) was used to maintain hygienic and sterile environment throughout the experiments.

3.1.3.3 CO_2 incubator

For studying the effects of heat stress the oocytes and embryos were cultured in a Thermo Fisher Scientific (Marietta, Ohio, USA, Model 3131) CO_2 incubator, which provided a 5% CO_2 in air (90-95% relative humidity) environment. For studying the effects of heat stress on oocytes and embryos three CO_2 incubators were used at three different temperatures 38.5°C, 39.5°C and 40.5°C.

3.1.3.4 Centrifuge

Refrigerated centrifuge (Sigma 3K30, Germany) with facilities to adjust centrifugation speed, time and temperature was used for centrifugation of chemicals, and washing of oocytes and embryonic stages etc. as and when needed.

3.1.3.5 Thermal cycler

A thermal cycler (My Cycler, BIO-RAD, Hercules, CA, USA) was used for synthesizing cDNA from mRNA of buffalo embryos through reverse transcription in the presence of reverse transcriptase enzyme for amplification of genes of interest with gene specific primers and heat stable taq polymerase. It gave 2^n number of DNA strands, where n= number of cycles.

3.1.3.6 Real time PCR

Real time PCR (CFX96 Real time system, BIO-RAD, Hercules, USA) was used for quantitative expression of genes related to heat shock, embryonic development and apoptosis in normal and experimental embryos by using SsoFast EvaGreen (double stranded DNA specific fluorescence dye) qPCR Super Mix (BIO RAD, USA.).

3.1.3.7 Electrophoresis unit and gel documentation

Agarose gel electrophoresis was performed for the resolution of PCR products. The electrophoretic unit (Power Pac Basic, BIORAD, USA) included the buffer chamber, safety lid with cables, UV transparent tray, casting trays, comb set and power supply. For analyzing the PCR products and for capturing the images gel documentation system, (Gel doc, BIO-RAD, Hercules, CA, USA) was used.

3.2 METHODS

3.2.1 Preparation of different media

For details regarding the composition of various media used in the present study, please see ANNEXURE-I

3.2.2 Collection of buffalo follicular fluid

For the collection of buffalo follicular fluid (buFF), buffalo ovaries were obtained from Delhi slaughterhouse and were transported to the laboratory at 4°C within 6h of collection. Follicular fluid was aspirated from all visible surface follicles (4 to 10 mm in diameter) with a 23-gauge needle. The cellular debris was removed by centrifugation at 10,000 rpm for 30 min at 4°C. The supernatant was carefully collected and sterilized by filtration through a 0.45 µm filter. The follicular fluid was divided into 1 ml aliquots in micro centrifuge tubes and was stored at -20°C until further use. The same pool of buFF was used throughout the study.

3.2.3 *In vitro* maturation and fertilization of oocytes

3.2.3.1 Collection and classification of oocytes

Buffalo ovaries were collected from Delhi slaughterhouse immediately after slaughter. These were washed 3-4 times with isotonic saline (32-37°C) containing a 100 µg/ml streptomycin. The washed ovaries were then put in a

thermos flask containing warm saline and antibiotics. The collected ovaries were transported to the laboratory within 6 h of slaughter. In the laboratory, the ovaries were rinsed twice, trimmed to remove the extra tissue and washed properly with warm saline containing antibiotics.

Oocytes were collected by aspiration of surface follicles (2-8 mm diameter) with an 18 gauge needle attached to a 10 ml syringe containing the aspiration medium (TCM-199+ 0.3% BSA+ 0.68 mM L-glutamine + 50 μg/ml gentamicin sulfate). The contents of the syringe, which included the aspirated oocytes, follicular fluid, granulosa cells and other debris, were poured in 100 mm x 100 mm square Petri dishes with 13 mm grid. The oocytes were searched under a zoom stereomicroscope at around 20X magnification. The oocytes were then shifted to 35 mm Petri dishes containing the washing medium (TCM-199 + 10% FBS + 0.81 mM sodium pyruvate + 0.68 mM L-glutamine + 50 μg/ml gentamicin sulfate). The aspirated oocytes were graded according to the criteria already in use in the laboratory:

Usable quality: Compact cumulus-oocyte complexes (COCs) with an unexpanded cumulus mass having ≥2 layers of cumulus cells, and with homogenous, evenly granular ooplasm.

Unusable quality: Oocytes partially or wholly denuded or with expanded or scattered cumulus cells or with an irregular ooplasm.

Oocytes of only usable quality were used for *in vitro* maturation.

3.2.3.2 *In vitro* maturation of oocytes

The oocytes were washed six times with the washing medium (TCM-199 + 10% FBS + 0.81 mM sodium pyruvate + 50 μg/ml gentamicin sulfate), then twice with the IVM medium (TCM-199 + 10% FBS + 10% follicular fluid + 1 μg/ml estradiol-17β + 5 μg/ml pFSH + 0.81 mM sodium pyruvate + 0.68 mM glutamine + 50 μg/ml gentamicin sulfate). For IVM, groups of 18-20 COCs were placed in

100 µl droplets of the IVM medium, overlaid with sterile mineral oil in 35 mm Petri dishes and cultured for 24 h in a humidified CO_2 incubator at 38.5°C.

3.2.3.3 Sperm preparation and *in vitro* fertilization

The spermatozoa used for IVF throughout the study were from the same donor tested for IVF earlier. The spermatozoa were prepared for fertilization as described earlier (Chauhan *et al*., 1998a). Briefly, two straws of frozen-thawed buffalo semen were washed twice with the washing Brackett and Oliphant (BO) medium (BO medium containing 10 µg/ml heparin, 137.0 µg/ml sodium pyruvate and 1.942 mg/ml caffeine sodium benzoate). The pellet was re-suspended in around 0.5 ml of the washing BO medium. The *in vitro* matured oocytes were washed thrice with the fertilization BO medium and transferred to 50 µl droplets (18-20 oocytes/droplet) of the capacitation and fertilization BO medium (washing BO medium containing 10 mg/ml fatty acid-free BSA). The spermatozoa in 50 µl of the capacitation and fertilization BO medium (2-4 million spermatozoa/ ml) were then added to the droplets containing the oocytes, covered with sterile mineral oil and placed in a CO_2 incubator at 38.5°C for 16-18 h for IVF.

3.2.3.4 *In vitro* culture

After the end of sperm-oocyte incubation, the cumulus cells were washed off the oocytes by gentle pipetting. The oocytes were then washed several times with modified Charles Rosenkrans medium with amino acids (mCR2aa) containing 0.8% BSA and cultured in this medium for 48 h post insemination. After this, the embryos were shifted to the IVC medium (mCR2aa + 0.6% BSA + 10% FBS) and cultured in 100 µl droplets of this medium on original beds of granulosa cells for up to 9 days post insemination in a humidified CO_2 incubator at 38.5°C. The medium was replaced with 50% of fresh IVC medium every 48 h. The cleavage rate was recorded on Day 2 post insemination and the percentage of oocytes that developed to 4-cell, 8- to 16-cell, morula and blastocyst stages was recorded on days 3, 4, 5 and 8 post insemination,

respectively, in Experiment 1. Embryos at 8- to 16-cell and blastocyst stage of development were collected on days 4 and 8 post insemination, respectively, for Experiments 2 and 3.

3.2.4 Quantitative expression of genes

3.2.4.1 RNase-free plasticware

RNase-free plastic ware was prepared by immersing the tubes and tips in 0.1% DEPC (Diethyl pyrocarbonate) overnight at 37˚C over a magnetic stirrer. The solution was prepared by adding 1.5 ml of DEPC to 1.5 liters of distilled water and then mixing vigorously over a magnetic stirrer. The solution was prepared fresh every time. DEPC has been reported to destroy the enzymatic activity of ribonuclease by modifying -NH, -SH, -OH group in RNase (Ambion, USA). DEPC is also a known carcinogen, therefore, extra precaution was taken and gloves were worn every time it was handled. The DEPC solution with immersed materials was stirred 3-4 times so that all tips and tubes were soaked completely in the solution. Next day, the solution was drained off and the treated material was dried after wrapping in aluminium foil in hot air oven. Tips and tubes were not touched with anything and gloves were used while handling. After drying, tips were carefully filled in the tip boxes and tubes were filled in jars and then the materials were double autoclaved to remove the remaining traces of DEPC which can otherwise interfere with the RT reaction during cDNA synthesis.

3.2.4.2 Preparation of RNase-free solutions

Buffers and solutions are a common source of RNase contamination. DEPC treatment is the most commonly used method for eliminating RNase contamination from water, buffer and other solutions. However, DEPC cannot be used with certain reagents containing primary amine groups (e.g. TAE buffer), secondary or tertiary amines (e.g. HEPES). The amino groups tend to react with and ‘sop up’ the DEPC, making it unavailable for inactivating RNase. To avoid the RNase contamination all solutions were prepared using baked glassware and

DEPC treated pyrogen-free MilliQ water. RNase-free chemicals and glassware were handled with baked and autoclaved spatula.

3.2.4.3 RNase-free surface

Prior to using the laboratory surfaces e.g., work table, bench tops, laminar hoods centrifuge and electrophoresis tanks, the surfaces were decontaminated by wiping it down with RNase inhibitor solution, RNAZap (Ambion). For total RNA isolation from oocytes and embryos the RNAqueous- Micro Kit (Ambion) was used.

3.2.4.4 RNA isolation Protocol

Total RNA was isolated from embryos by using the RNAqueous-Micro Kit (Ambion, USA) according to the manufacturer's instructions, with some modifications. Briefly, the embryos at different stages of development were lysed with the lysis buffer after which RNA was eluted with column after several washings with the wash solutions (wash Solution 1 and wash solution-2/3). The genomic DNA contamination was removed by DNase treatment at 37°C for 20 min. Before cDNA synthesis, the concentration of RNA was measured, and was set at 20 ng/ µl for oocytes and embryos.

3.2.5 cDNA synthesis and real time quantification

The cDNA was synthesised by sensiscript cDNA synthesis kit (QIAGEN, Hilden) according to the manufacturer's instructions. For cDNA synthesis, 1µL of total RNA (20 ng), 2 µl dNTP mix (5 mM), 2 µl oligo dT (10 µM), 2 µl 10x RT-buffer, 1µl RNAse inhibitor, 1µl superscript III RT and nuclease-free water was added to make volume 20 µl in 200 µl tube. The reaction mixture was mixed and incubated at 37°C for 60 min in a thermal cycler. The synthesized cDNA was stored at -80˚C until use for Real-Time PCR.

The relative quantification of mRNA of various genes was done by using CFX96 Real time system (Bio-Rad, Hercules, USA). For this, cDNA of different stages (8-to16- cell stage and blastocyst stage) of embryos from all three groups was randomly diluted in 1:3 ratio. GAPDH was used as reference gene for all experiments. The qRT-PCR reactions were performed using the SYBR green(double stranded DNA-specific flouroscent dye) master mix from BIORAD, each run was performed in duplicate in a 10 µl reaction volume which contained 5 µl fluorescence dye, 2 µl of gene specific primers (forward and reverse) from 10 µM stock and 1 µl template. The final volume was made up with nuclease-free water. The PCR condition used for all genes was as follows: Initial denaturation at 95°C for 3 min, 40 cycles (denaturation 95°C for 10 sec., annealing 60°C for 10 sec., and extension at 72°C for 10 sec), melting cycle starting from 65°C up to 95°C with a 0.5°C/sec transition rate. The annealing temperature of all genes is mentioned in Table 1. The qRT-PCR specificity was confirmed by the analysis of the melting curve shown by machine by CFX Manager Software. During data analysis, the C_t value of housekeeping gene was subtracted from the C_t value of target gene to obtain change in C_t (ΔC_t). The Δ C_t value of target gene sample was subtracted from the calibrator (control) to get $\Delta\Delta$ C_t values. Difference in the transcript abundance for the target genes was calculated using the equation $2^{-\Delta\Delta C_t}$.

To detect the expression of genes, specific primers are essential for amplification of target gene of interest. The gene specific primers were designed with a target to amplify a fragment of around 150-200 bp preferably from the end of cDNA. The primers were designed with highly conserved region of either Bovine or Buffalo sequences using Primer3 Software. (http://www-genome.wi.mit.edu/cgi-bin/prime/primer3-www.cgi.

Table 3.1: Primer sequence and conditions for Real Time PCR.

Gene	Primer sequence	Size (bp)	Annealing temp.(ºC)	Accession no.
HSP70.1	F-GACAAGTGCCAGGAGGTGAT R-ACATGAGCAATCCAGGGAAG	238	58	AJ812563
HSP70.2	F-AAGCACAAGAAGGACATTGCACCC R-AAGTGTAGAAATCCACGCCCTCGT	103	60	NM_174344.1
BMP15	F- CATCCCTTACGGTATATGCTG R- GTTTGGTCTCAGAGGAAAGTC	179	56	DQ463368.1
GDF9	F- CCCTAAATCCAACAGAAGCC R- GTTCCACAACAGTAACACGA	148	60	NM_174681.2
BCL-XL	F- TTGTGGCCTTTTTCTCCTTC R- GATCCAAGGCTCTAGGTGGT	128	60	ENSBTAT00000008572
MCL1	F- TCGGAAACTGGACATCAAAA R- CCACAAAGGCACCAAAAGAA	128	58	ENSBTAT00000020159
BID	F- CTGTCGGAGGAGGACAGGAG R- GTGGTCGGCTATCTTTTTGG	135	60	NM_001075446.1
BAX	F- CCTTTTGCTTCAGGGTTTCA R- CGCTTCAGACACTCGCTCA	123	60	NM_001191220.1
GAPDH	F-TCAAGAAGGTGGTGAAGCAG R-CCCAGCATCGAAGGTAGAAG	122	57	GU324291.1

3.2.6 Experimental design

Effect of Ascorbic acid on oxidative stress

Experiment 1: Ascorbic acid supplementation of IVM medium.

A total of 1270 oocytes, spread over 3 replicates were randomly divided into 3 groups. The IVM medium was supplemented with control, 50 µM and 100 µM Ascorbic acid.

Experiment 2: Ascorbic acid supplementation of IVC medium.

Immature buffalo oocytes were subjected to IVM and IVF. A total of 418 cleaved buffalo embryos obtained on Day 2 post insemination from 659 oocytes which were randomly distributed into two groups and cultured in IVC medium supplemented with control and 50 µM Ascorbic acid. The experiment was repeated 4 times.

Experiment 3: Ascorbic acid supplementation of both IVM and IVC media.

In this experiment, both the IVM and IVC media were supplemented with Ascorbic acid. Based on the results of Experiments 1 and 2, both the IVM and IVC media was supplemented with 50 µM Ascorbic acid. A total of 782 oocytes were randomly allocated into two groups.

Effect of Ascorbic acid on heat stress

Effect of Ascorbic acid on development of buffalo embryos exposed to heat shock (39.5 °C for 12 h).

During the course of culture; the oocytes were initially matured at 39.5°C for 12 h, supplemented with and without 50 µM Ascorbic acid.

Effect of Ascorbic acid on development of buffalo embryos exposed to heat shock (40.5 °C for 12 h).

During the course of culture; the oocytes were initially matured at 40.5°C for 12 h, supplemented with and without 50 µM Ascorbic acid.

3.2.7 Statistical analysis

The data were analyzed using SPSS 17.0 (IBM, USA) after Arcsine transformation of percentage values. The cleavage and blastocyst rate were analyzed on Day 8 post insemination The differences were analyzed by one way analysis of variance (ANOVA) followed by Fisher's LSD test or Student's t-test.

CHAPTER – 4

Results and Discussion

4. RESULTS AND DISCUSSION

The present study was conducted to investigate the effect of supplementation of antioxidant Ascorbic acid in IVM or IVC or both IVM and IVC media and to find out if it could improve developmental competence of buffalo embryos *in vitro* and to assess the thermoprotectant role of Ascorbic acid in embryonic development.

For *in vitro* production of embryos, immature oocytes (Figure 1) were collected by aspiration of follicles from slaughtered buffalo ovaries. Oocytes with homogeneous cytoplasmic granulation and ≥2 layers of unexpanded cumulus layers were considered to be of usable grade whereas oocytes with partially or wholly denuded or with expanded or scattered cumulus cells or with an irregular ooplasm were considered to be unusable. Oocytes of only usable grade were subjected to IVM (Figure 2), followed by IVF after 24h of maturation and then subjected to IVC. The cleavage rate was recorded on Day 2 post-insemination, followed by 4-cell, 8-to16- cell, morula, blastocyst and hatched blastocyst on days 3, 4, 5, 8 and 9 of culture (Figures 3-8), respectively.

4.1 Effect of Ascorbic acid supplementation

4.1.1 Supplementation of *in vitro* maturation medium with different concentrations of Ascorbic acid

A total of 1270 oocytes were used in this experiment in which IVM media was supplemented with 50 µM and 100 µM Ascorbic acid for 24 h. In the control group, a total of 403 oocytes were subjected to IVC after IVM and IVF. The cleavage rate was 62.77 ± 2.71 percent. These cleaved oocytes were further cultured and 72.72 ± 1.77, 53.35 ± 0.70, 24.50 ± 1.07 and 10.67 ± 0.24 percent developed to 4- cell, 8-to 16- cell, morula and blastocyst stages (Table 4.1), respectively. When 50 µM Ascorbic acid was supplemented, the cleavage rate, 4-cell, 8-to 16-cell, morula and blastocyst formation rates were 66.67 ± 2.27,

78.85 ± 2.77, 59.06 ± 1.65, 32.55 ± 1.32 and 16.67 ± 1.26 percent, respectively. While there was a significant increase in the blastocyst production rate, increase in all other stages of development was non-significant. When higher concentration of 100 µM Ascorbic acid was supplemented to IVM media, 54.04 ± 2.20, 62.11 ± 2.56, 33.03 ± 2.59, 15.85 ± 0.37 and 6.16 ± 0.37 percent of the cultured oocytes (n=420) developed to 2- cell, 4- cell, 8-to 16- cell, morula and blastocyst, respectively (Table 4.1). These rates were lower as compared to control group as well as 50 µM Ascorbic acid supplemented group but the decrease was non-significant for all stages of embryonic development. Hence, it can be concluded from the experiment that supplementation of 50 µM Ascorbic acid in IVM media leads to increase in development rate of buffalo oocytes while supplementation of 100 µM Ascorbic acid led to decrease in blastocyst production rate due to increased toxicity.

Table 4.1: Effect of Ascorbic acid supplementation of IVM media on the developmental competence of buffalo oocytes.

Group	Oocytes cultured (n)	Percentage development n (%)				
		2-cell stage[1]	4-cell stage[2]	8-to 16-cell[2]	Morula[2]	Blastocyst[2]
Control	403	253 (62.77±2.71)	184 (72.72±1.77)	135 (53.35±0.70)	62 (24.50±1.07)	27 (10.67±0.24)[a]
50 µM Ascorbic acid	447	298 (66.67±2.27)	235 (78.85±2.77)	176 (59.06±1.65)	97 (32.55±1.32)	50 (16.67±1.26)[b]
100 µM Ascorbic acid	420	227 (54.04±2.20)	141 (62.11±2.56)	75 (33.03±2.59)	36 (15.85±0.37)	14 (6.16±0.37)[a]

Given values are percentage (Mean ± SEM) as well as absolute values (n) from total number of respective oocytes taken (n).

[a,b] Values within a column having different superscripts differ significantly (P <0.05).

[1]2-cell%: number of embryos cleaved/number of embryos cultured.

[2]4-cell%, 8 to 16 cell%, morula%, blastocyst%: number of embryos of respective stage/number of embryos cleave.

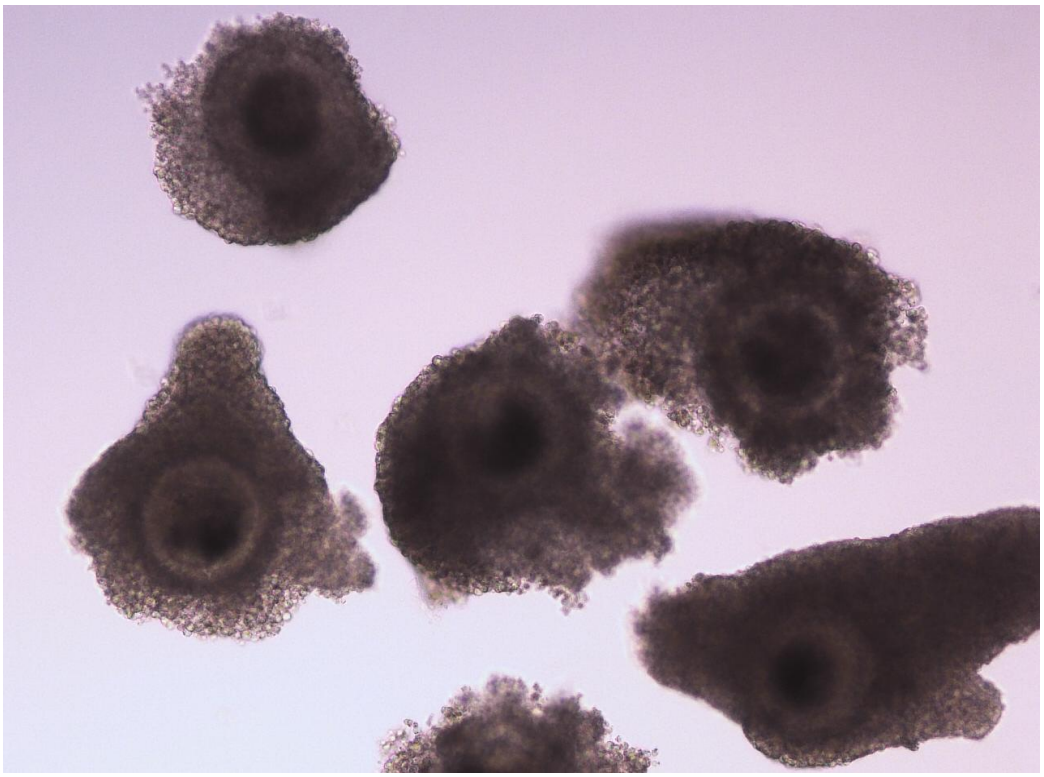

Fig. 1: Immature slaughterhouse derived buffalo oocytes of usable quality (grade A+B).

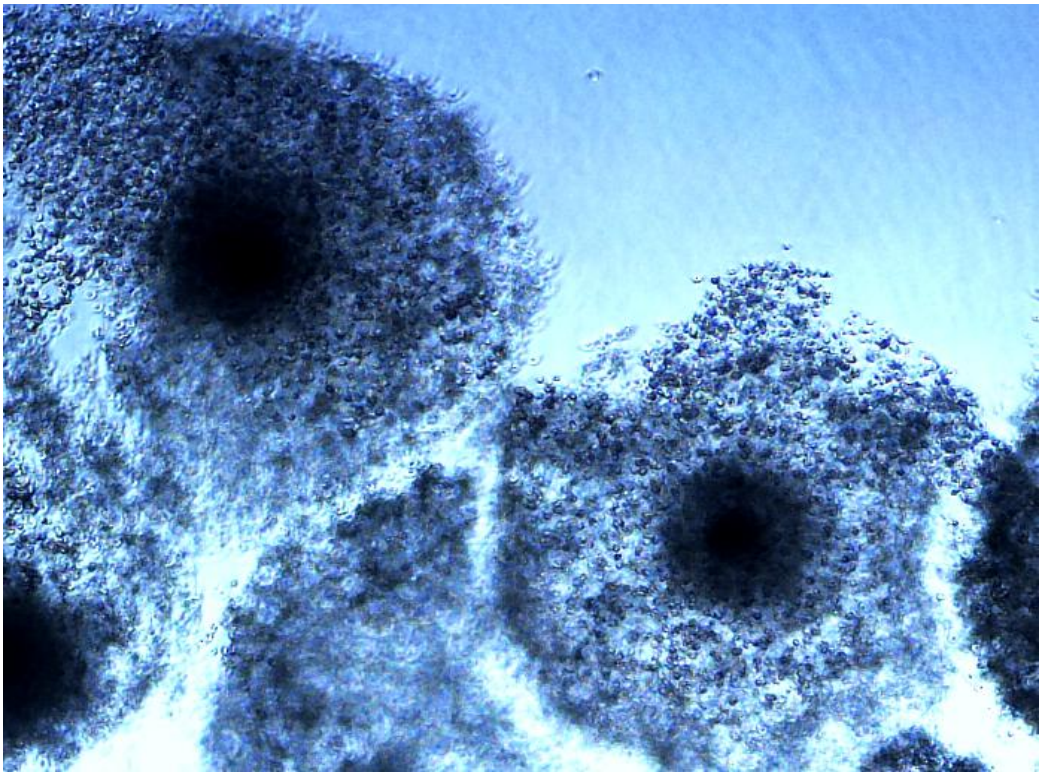

Fig. 2: Mature buffalo oocytes of usable quality.

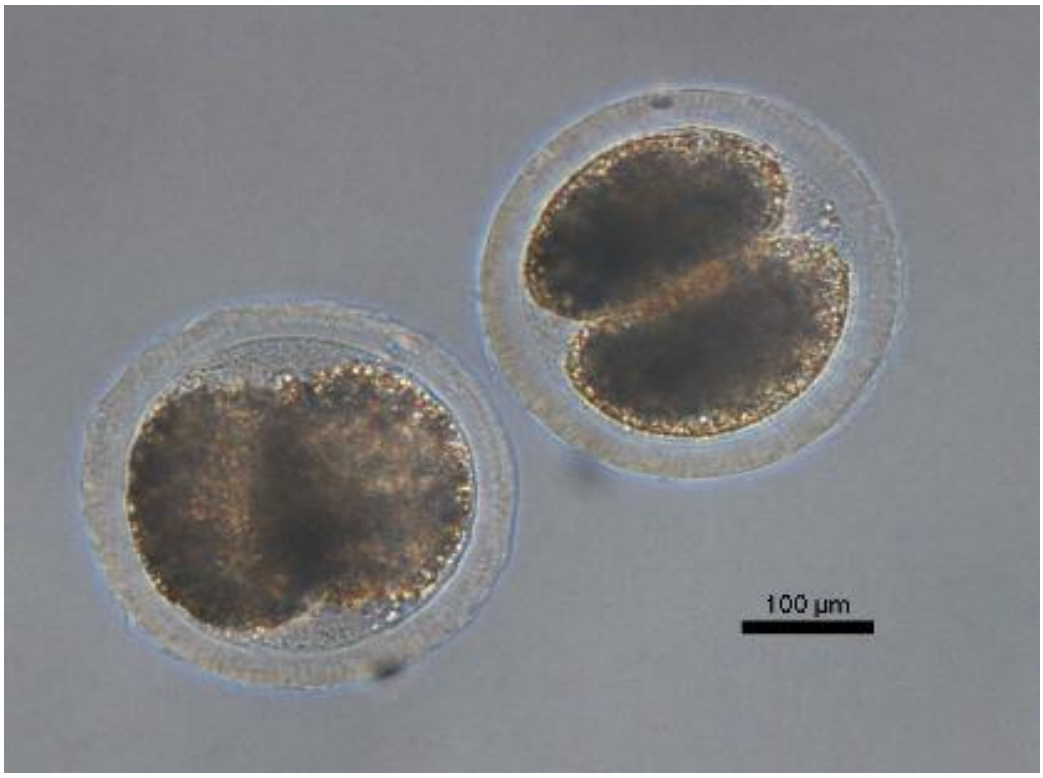

Fig. 3: IVF embryos at 2-cell stage.

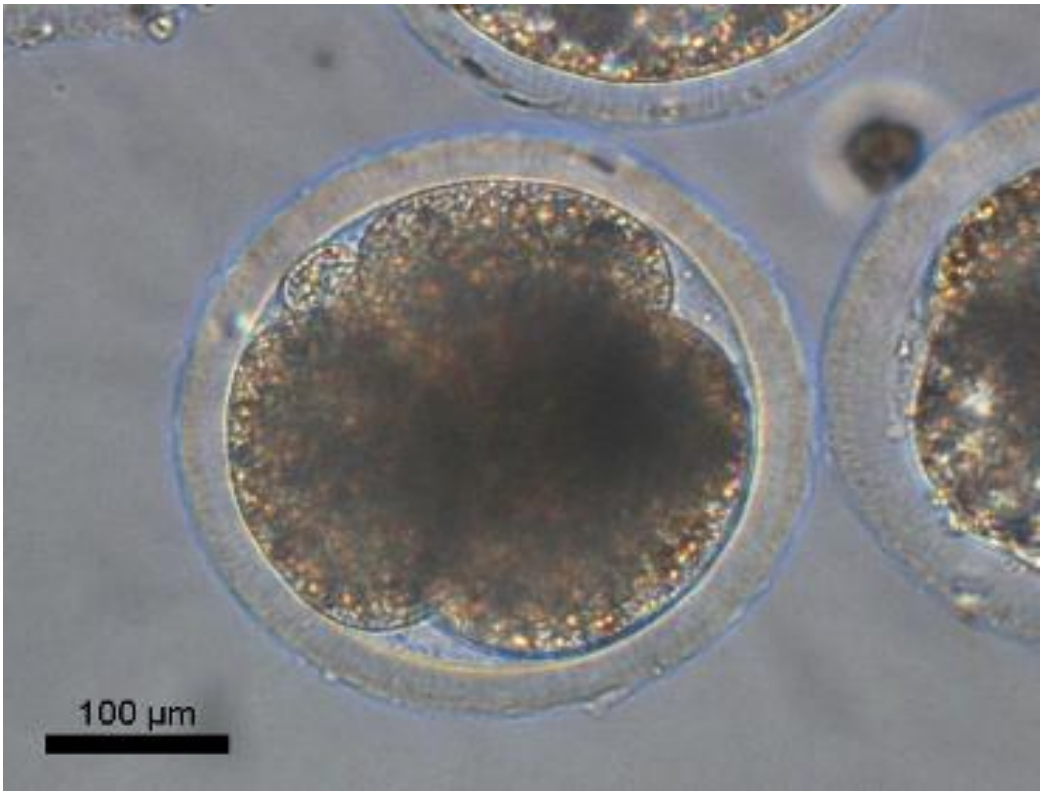

Fig. 4: IVF embryos at 4-cell stage.

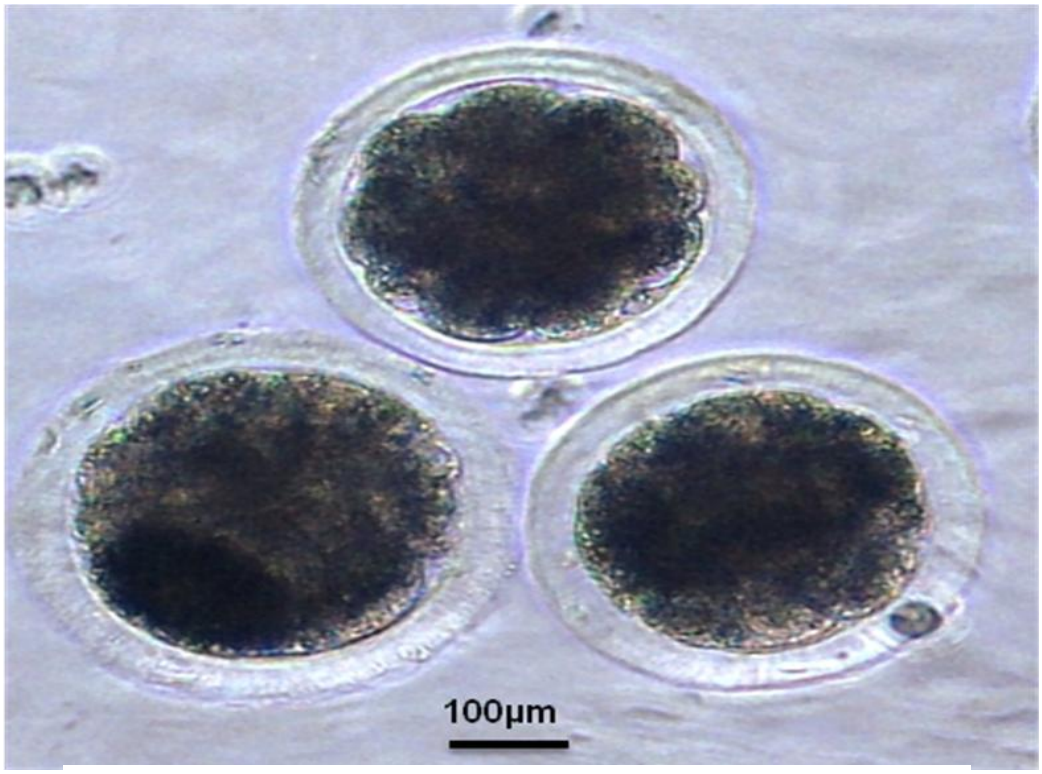

Fig. 5: IVF embryos at 8-16 cell stage

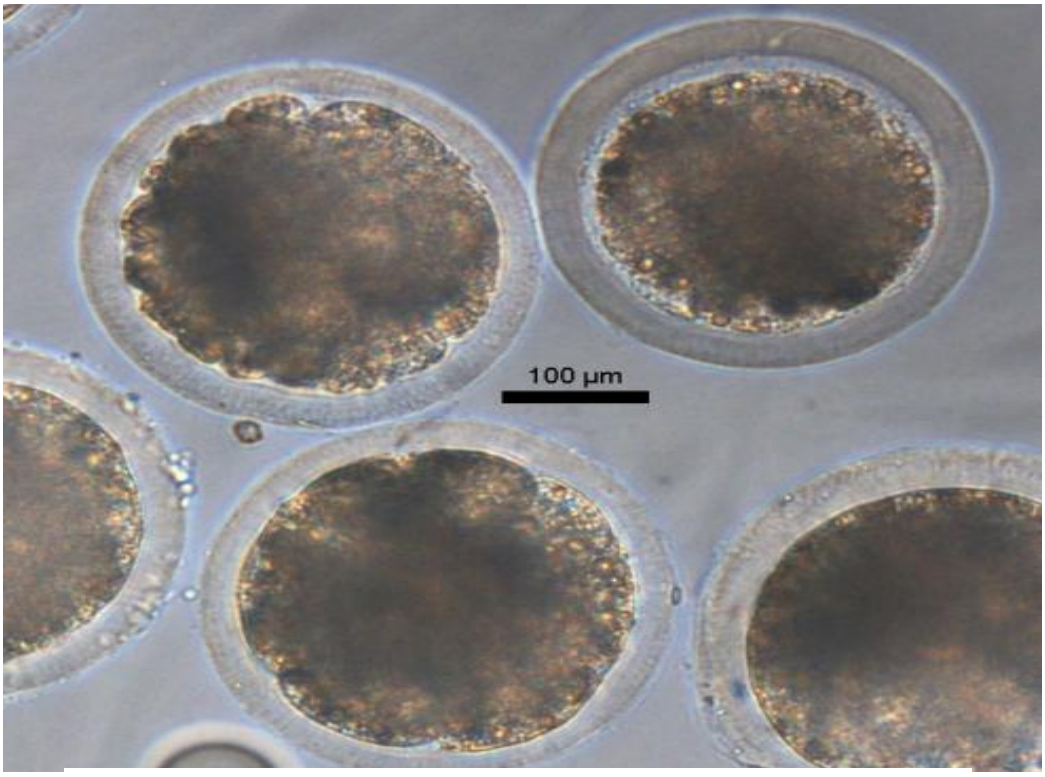

Fig. 6: IVF embryos at morula stage

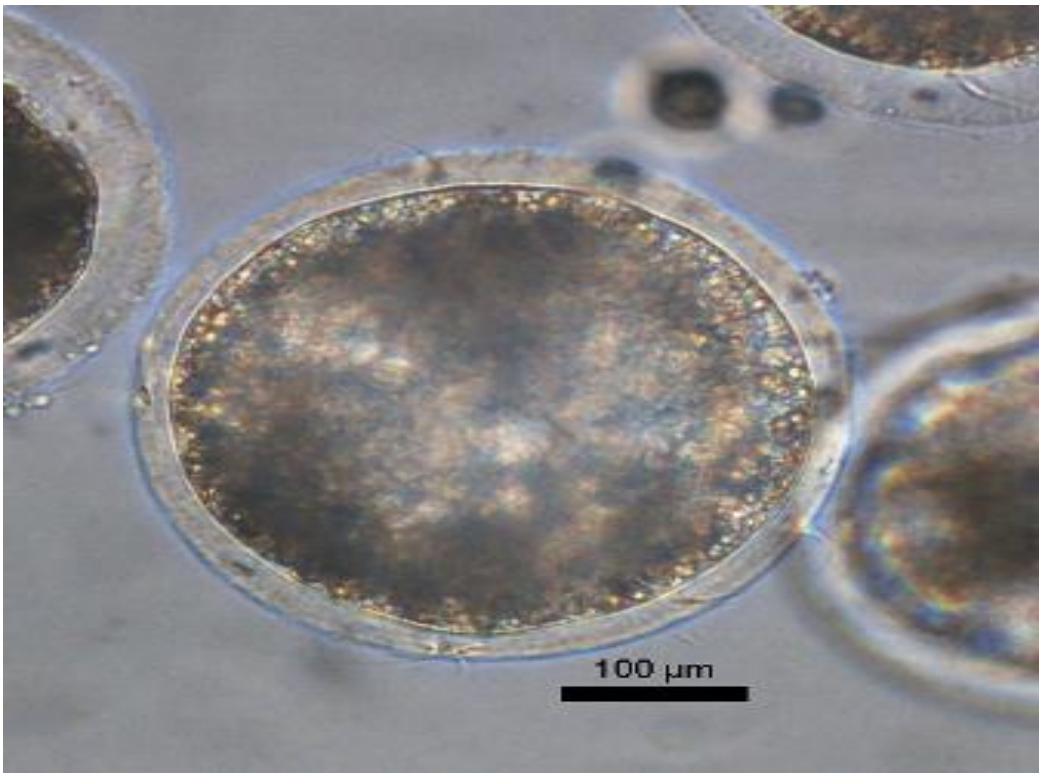

Fig. 7: IVF embryos at blastocyst stage

Fig. 8: IVF produced hatched blastocyst

4.1.2 Supplementation of 50 µM Ascorbic acid in *in vitro* culture medium

When 50 µM Ascorbic acid was supplemented in IVC media for 8 Days of culture period, in the control group, out of 330 oocytes cultured 57.57 ± 1.55, 65.78 ± 2.01, 46.84 ± 1.25, 23.15 ± 1.08 and 9.47 ± 0.86 percent developed to 2-cell, 4- cell, 8-to 16- cell, morula and blastocyst, respectively. In the treatment group (n=329), cleavage rate, 4- cell, 8-to 16- cell, morula and blastocyst formation rates were , 69.09 ± 3.22, 80.26 ± 2.05, 60.96 ± 2.05, 34.21 ± 1.04 and 20.18 ± 0.86 percent, respectively (Table 4.2). Conclusively, on supplementation of 50 µM Ascorbic acid in IVC media, developmental rates increased significantly ($P<0.05$) for all the stages including blastocyst stage except cleavage rate.

Table 4.2: Effect of 50 µM Ascorbic acid supplementation of IVC media on the developmental competence of buffalo oocytes.

Group	Oocytes cultured (n)	Percentage development n (%)				
		2-cell stage[1]	4-cell stage[2]	8-to 16-cell[2]	Morula[2]	Blastocyst[2]
Control	330	190 (57.57±1.55)	125 (65.78±2.01)[a]	89 (46.84±1.25)[a]	44 (23.15±1.08)[a]	18 (9.47±0.86)[a]
50µM Ascorbic acid	329	228 (69.09±3.22)	183 (80.26±2.05)[b]	139 (60.96±2.05)[b]	78 (34.21±1.04)[b]	46 (20.18±0.86)[b]

Given values are percentage (Mean ± SEM) as well as absolute values (n) from total number of respective oocytes taken (n).

[a,b] Values within a column having different superscripts differ significantly ($P<0.05$).

[1]2-cell%: number of embryos cleaved/number of embryos cultured.

[2]4-cell%, 8 to 16 cell%, morula%, blastocyst%: number of embryos of respective stage/number of embryos cleave.

4.1.3 Supplementation of 50 µM Ascorbic acid both in *in vitro* maturation and culture media

Based on the results of above experiments, both, IVM and IVC media were supplemented with 50 µM Ascorbic acid. A total of 782 oocytes were used in this experiment. A non-significant increase in cleavage rate (66.67 ± 2.23% vs

54.83 ± 1.94%) was observed for the supplemented group as compared to control.The percentage development rate was significantly higher at 4- cell, 8-to 16- cell, morula and blastocyst stages in 50 µM Ascorbic acid treated group (81.57 ± 2.27%, 62.40 ± 1.24%, 38.72 ± 1.72% and 28.57 ± 0.37%) as compared to control (66.19 ± 27.8%, 46.67 ± 3.58%, 25.71 ± 1.64% and 11.42 ± 1.45%) respectively (Table 4.3).

The above results led to the conclusion that the supplementation of both IVM and IVC media with 50 µM Ascorbic acid led to better developmental rate as compared to supplementation of Ascorbic acid alone either in IVM or IVC.

Table 4.3: Effect of 50 µM Ascorbic acid supplementation of IVM and IVC media on the developmental competence of buffalo oocytes.

Group	Oocytes cultured (n)	Percentage development n (%)				
		2-cell stage[1]	4-cell stage[2]	8-to 16-cell[2]	Morula[2]	Blastocyst[2]
Control	383	210 (54.83±1.94)	139 (66.19±27.8)[a]	98 (46.67±3.58)[a]	54 (25.71±1.64)[a]	24 (11.42±1.45)[a]
50µM Ascorbic acid	399	266 (66.67±2.23)	217 (81.57±2.27)[b]	166 (62.40±1.24)[b]	103 (38.72±1.72)[b]	76 (28.57±0.37)[b]

Given values are percentage (Mean ± SEM) as well as absolute values (n) from total number of respective oocytes taken (n).

[a,b] Values within a column having different superscripts differ significantly (P <0.05).

[1]2-cell%: number of embryos cleaved/number of embryos cultured.

[2]4-cell%, 8 to 16 cell%, morula%, blastocyst%: number of embryos of respective stage/number of embryos cleave.

In vitro embryo production (IVP) comprises of three major processes: *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) (Palta and Chauhan, 1998). Maturation of mammalian oocytes is defined as the sequence of events occurring from the germinal vesicle stage to completion of the second meiotic division with formation of the second polar body (Khatun *et al.,* 2011).

Selection of good quality gametes (oocytes and spermatozoa) is the first bottleneck of *in vivo* and inculpate a tremendous loss of unfit oocytes mostly by atresia in the ovarian and of sperm cells in the male and in the female reproductive tract before and after mating, respectively (Van Soom *et al.,* 2007). To enhance reproductive efficiency, several protocols of oestrus and ovulation synchronization have been adopted from their use in commercial cattle production. These protocols yield encouraging pregnancy rates of 30% to 50%, which are comparable to those achieved in buffaloes bred at natural oestrus (Warriach *et al.,* 2015).

The efficiency of *in vitro* maturation, fertilization of oocytes and development of embryos is not sufficiently high as compared to *in vivo*. The number of pregnancies obtained is lesser when the transferred embryos are produced *in vitro* than when they are produced *in vivo*. Though the efforts have been made to improve bovine *in vitro* embryo production (IVP), but still the efficiency of blastocyst is only 30% to 40% obtained from oocytes after *in vitro* maturation, fertilization and embryo culture (Sirad *et al.,* 2006). Oocytes can be protected from oxidant-induced apoptosis by supplementing media with vitamin E and C (Gupta *et al.,* 2010). The major constraint that has limited the felicity of this technology is very low blastocyst yields which, at only 5-10% of the oocytes subjected to IVM (Palta and Chauhan, 1998), are much lower in comparison to that of around 30% to 40% in cattle (Yang *et al*., 1998, Kumar *et al*., 2012). The primary objective to use these protocols was to improve yields of IVP blastocysts which can be expected not only to lead to faster multiplication of superior germplasm in buffalo, but these would also contribute towards development of other advanced reproductive technologies like cloning, transgenesis, production of embryonic stem cells etc.

The current trend in bovine assisted reproduction in recent years is that *in vitro* production (IVP) is increasingly overtaking multi-ovulation embryo transfer

as the technology of choice in several countries, including Brazil (Taylor-Robinson *et al*., 2014). Parrish (2014) concluded that bovine IVP has become a competitive alternative to multiple ovulation embryo transfer (MOET) and plays an important role in commercial settings. Besides highly transferable IVP embryos correlate with cattle possessing considerable numbers of ovarian follicles (Dos Santos *et al*., 2016). In addition, Pontes *et al.* (2009) Indicated that the number of IVP embryos was greater than those attained by *in vivo* production over the same period of time. Moreover, Taylor *et al*. (2014) contended that IVP may increase genetic gain in a breeding herd. Further, there is no doubt that bovine *in vitro* fertilization (IVF) technology is a vital research tool which may be used in order to understand early embryo development (Besenfelder *et al*., 2010).

IVM and IVC of buffalo oocytes is carried out in the presence of serum (Palta and Chauhan, 1998) or follicular fluid (Chauhan *et al.,* 1997c), both of these biological fluids contain a number of growth factors, hormones and other unknown factors, their incorporation in a culture system introduces a high variability among different batches. Undoubtedly specific effects of any substance on IVP of embryos cannot be studied in the presence of serum or follicular fluid. Development of serum-free IVM and IVC culture systems, tailor-made for buffalo is, therefore, necessary. As the blastocyst yields are remarkably low in the absence of serum, substances which could be used to improve the blastocyst yield need to be identified.

The results of the present study demonstrated that Ascorbic acid had a significant effect on the oocyte maturation and embryonic development compared with the other groups, and this positive effect was observed till morula stage. It also suggested that the Ascorbic acid concentration used for supplementation of media is an important factor influencing the cleavage rate and blastocyst yield. For IVM, 50 µM concentrations gave the better results.

Supplementation of 50 µM Ascorbic acid in IVC alone also showed positive results with developmental competence rate of blastocyst increased from 16.67 ± 1.26% to 20.18 ± 0.86% in control and treatment groups, respectively (Tao *et al*., 2010).

These results confirm and extend those of previous studies in which supplementation of Ascorbic acid to an embryo culture significantly improved the blastocyst development rate (Wang *et al*., 2002). IVC medium supplemented with either α-tocopherol or L-Ascorbic acid at a concentration of 250 mM enhanced the rate of embryonic developmental competence of buffalo embryos to the blastocyst stage when compared to medium alone (Saikhun *et al*., 2008). Sreenivas *et al*. (2014) concluded that in sheep, the cleavage, morula and blastocyst percent in L-Ascorbic acid at 100 µM with CR1aa is found more significant compared with other compositions used in the experimentation.

Better developmental competence rate was obtained when both IVM and IVC were supplemented with 50 µM Ascorbic acid. In accordance to our result, Kere *et al*. (2014) also indicated that among Ascorbic acid concentrations (0, 25, 50 and 100 µg/ml) supplemented in porcine *in vitro* maturation (IVM) and culture (IVC) media, 50 µg/ml of vitamin C showed improved cleavage rates, blastocyst rates and total cell numbers per blastocyst ($P<0.05$) compared with other groups (control, 25 µg/ml and 100 µg/ml). Ascorbic acid also improves the developmental competence of porcine oocytes (Tatemoto *et al*., 2001) and overcome apoptosis in granulosa cells (Murray *et al*., 2001) and ovarian follicular cells (Eppig *et al*., 2000). Ascorbic acid addition to the culture medium has been shown to inhibit follicular apoptosis in rat and mouse follicles, and improved mouse blastocyst production (Tatemoto *et al*., 2001). The efficiency of teratoma formation by human embryonic stem cells was improved with Ascorbic acid treatment, leading to enhancement of potential differentiation ability (Yu *et al*., 2015).

4.1.4 Supplementation of both *in vitro* maturation and culture media with 50 µM Ascorbic acid at 39.5°C and 40.5°C

For this experiment, the immature oocytes were divided into the following 4 groups:

Group 1: Without Ascorbic acid supplementation, oocytes were given heat treatment at 39.5°C initially for 12 h during IVM.

Group 2: With 50 µM Ascorbic acid supplementation, oocytes were given heat treatment at 39.5°C initially for 12 h during IVM.

Group 3: Without Ascorbic acid supplementation, oocytes were given heat treatment at 40.5°C initially for 12 h during IVM.

Group 4: With 50 µM Ascorbic acid supplementation, oocytes were given heat treatment at 40.5°C initially for 12 h during IVM.

In this experiment, oocytes of all 4 groups were subjected to IVM, IVF and IVC. The developmental competence was compared among the 4 groups by examining the cleavage rate on Day 2 post insemination and development of 4-cell, 8-to 16-cell, morula and blastocyst stages at Day 3, 4, 5 and 8, respectively.

Table 4.4: Effect of heat stress on the developmental competence of oocytes with 50µM Ascorbic acid supplementation at 39.5°C

Group	Oocytes cultured (n)	Percentage development n (%)				
		2-cell stage[1]	4-cell stage[2]	8-to 16-cell[2]	Morula[2]	Blastocyst[2]
Control	354	55 (15.53±0.50)	45 (81.80±1.21)	30 (54.54±3.71)	22 (40.00±2.20)	2 (3.60±2.30)
50µM Ascorbic acid	381	62 (16.27±1.70)	46 (74.19±4.47)	34 (54.83±2.28)	24 (38.70±3.62)	2 (3.23±2.34)

Given values are percentage (Mean ± SEM) as well as absolute values (n) from total number of respective oocytes taken (n).

[1]2-cell%: number of embryos cleaved/number of embryos cultured.

[2]4-cell%, 8 to 16 cell%, morula%, blastocyst%: number of embryos of respective stage/number of embryos cleave.

* Initially all the groups were treated at 39.5°C for 12 h in IVM

Table: 4.5 Effect of heat stress on the developmental competence of oocytes with 50µM Ascorbic acid supplementation at 40.5°C

Group	Oocytes cultured (n)	Percentage development n (%)				
		2-cell stage[1]	4-cell stage[2]	8-to 16-cell[2]	Morula[2]	Blastocyst[2]
Control	360	50 (13.88±0.50)	41 (82.00±1.21)	23 (46.00±3.71)	09 (18.00±2.20)	0
50µM Ascorbic acid	398	53 (13.31±3.77)	38 (71.69±2.65)	26 (49.05±2.79)	08 (15.09±0.90)	0

Given values are percentage (Mean ± SEM) as well as absolute values (n) from total number of respective oocytes taken (n).

[1]2-cell%: number of embryos cleaved/number of embryos cultured.

[2]4-cell%, 8 to 16 cell%, morula%, blastocyst%: number of embryos of respective stage/number of embryos cleave.

* Initially all the groups were treated at 40.5°C for 12 h in IVM

To study the thermoprotectant role of Ascorbic acid, oocytes were divided into 4 groups as mentioned above. When control oocytes (n=354) were subjected to heat treatment at 39.5°C along with treatment group (n=381) with 50 µM Ascorbic acid supplementation, it was observed that embryonic development rates did not differ significantly between two groups as rates of production of 2-cell stage (15.53 ± 0.50% vs 16.27 ± 1.70%), 4- cell (81.80 ± 1.21% vs 74.19 ± 4.47%), 8-to 16- cell (54.54 ± 3.71% vs 54.83 ± 2.28%), morula (40.00 ± 2.20% vs 38.70 ± 3.62%) and blastocyst (3.60 ± 2.30% vs 3.23 ± 2.34%) were almost similar (Table 4.4). Earlier, Yadav *et al*. (2013) has reported significant decrease in all stages of embryonic development when oocytes were treated at 39.5°C for 2 h only during IVM. Supplementation of Ascorbic acid combated the effect of heat shock which resulted in almost similar developmental rates in heat treated group as that of control.

When the corresponding treatment was given to group 3 and 4 at 40.5°C, it was observed that heat treatment had profound effect on embryonic development as no blastocyst was formed in both control as well as treatment

group. Supplementation of Ascorbic acid helped to combat the heat shock as evident from the non-significant decrease for all stages of embryonic development (13.88 ± 0.50% vs 13.31 ± 3.77% for 2- cell, 82.00 ± 1.21% vs 71.69 ± 2.65% for 4- cell, 18.00 ± 2.20% vs 15.09 ± 0.90% for morula) except 8- to 16- cell stage (46.00 ± 3.71% vs 49.05 ± 2.79%) (Table 4.5). Recently, Ashraf *et al*. (2014) also reported severely compromised development of oocytes to blastocysts when given heat treatment at 40.5°C for 12 h during IVM.

From the above results, it was concluded that increased temperature disrupt embryonic development in buffalo embryos and that embryos become more labile to heat shock as they proceed through development. Further, it was concluded that the Ascorbic acid does play a significant role in mitigating heat stress at elevated temperatures.

Several stages of embryo development are susceptible to elevated ambient temperature such as oocyte germinal vesicle-stage (Al-Katanani *et al*., 2002), the stage near or after fertilization and the early cleavage-stage embryos (Ju *et al*., 2004). The majority of early embryonic losses in dairy cattle with environmental heat stress occur before Day 20 of gestation (Hansen *et al*., 1999). The losses are more pronounced during temperature induced stressful conditions and may continue up to Day 40 to 50 of gestation (Cartmill *et al*., 2001). Effects of elevated temperature manifested as heat stress are particularly prominent during estrus (Day 1) when the oocyte is undergoing meiotic maturation in preparation for fertilization (Gwazdauskas *et al*., 1973; Cavestany *et al*., 1985). It has already been established that maternal hyperthermia affected the meiotic maturation of an oocyte (Roth *et al*., 2004; Rensis and Scaramuzzi, 2003) in addition to a reduction in embryonic development with direct or *in vitro* exposure of bovine oocytes to elevated temperature (Edwards *et al*., 2005; Schrock *et al*., 2007) to a similar degree as seen *in vivo* (Putney *et al*., 1989). Yadav *et al*. (2013) have found that physiologically relevant heat shock (40.5°C) to buffalo oocytes or embryos for 2 h once every Day throughout IVM, IVF and IVC resulted in a decreased ($p<0.05$) percentage of buffalo oocytes that developed to 8-to 16- cell or blastocyst stage. The effect of 2 h heat shock

throughout IVM, IVF and IVC was so profound that not a single blastocyst was formed at 40.5°C. Recently, Ashraf *et al*. (2014) reported that development of oocytes to blastocyst was severely compromised (P<0.001) when matured at 40.5°C and 41.5°C for both exposure periods (12 h and 24 h). It was found that the cleavage rates, blastocyst yield and mean cell number decreased remarkably (p<0.001) in the treatment groups compared to control. These results are in accordance with our findings.

4.2 Gene expression study after supplementation of Ascorbic acid in IVM media

The relative quantification of genes related to heat stress (*HSP 70.1* and *HSP 70.2*), apoptosis (pro-apoptotic: *BAX* and *BID* as well as anti-apoptotic: *BCL-XL* and *MCL1*) and developmental competence (*GDF-9* and *BMP-15*) was done by Real Time PCR. The melt curves and melt peaks of different genes under study are shown in Figures 9-11

The gene expression for apoptosis related and developmental competence related genes was studied in following groups:

Control: Blastocysts produced from oocytes cultured without Ascorbic acid supplementation.

Group 1 (50 µM): Blastocysts produced from oocytes cultured with 50 µM Ascorbic acid supplementation during IVM.

Group 2 (100 µM): Blastocysts produced from oocytes cultured with 100 µM Ascorbic acid supplementation during IVM.

The gene expression for heat stress related genes was studied in following groups:

Control: Without Ascorbic acid supplementation, oocytes were given heat treatment at 39.5°C initially for 12 h during IVM.

Group 1 (50 µM): With 50 µM Ascorbic acid supplementation, oocytes were given heat treatment at 39.5°C initially for 12 h during IVM.

4.2.1 Relative mRNA abundance of heat stress-related genes

The relative mRNA abundance of *HSP 70.1* and *HSP 70.2*, was almost similar (non-significantly higher) in oocytes matured at 39.5°C or 40.5°C after supplementation with 50 µM Ascorbic acid as compared to their corresponding controls in which Ascorbic acid was not supplemented (Figures 12-13). *Hsp 70* family (*70.1, 70.2, 70.8*) and other small *Hsps* (*10, 60*) form the most important group of stress responsive elements in embryonic cells (Kawarsky *et al*., 2001; Salvetti *et al*., 2010). The relative importance of these proteins in oocyte/embryo growth and development could be potentially used as biomarkers for *in vitro* as well as *in vivo* studies concerning stressful conditions (Oliveria *et al*., 2005). The rapid induction of *Hsp70* and other small *Hsps* represents a unique feature of the physiological function of these molecules. It is well known that *Hsp* expression is accomplished by mechanisms of transcriptional activation and translation involving heat shock transcription factors (*HSFs*). These results are also in accordance with the previous findings carried out in cattle, pig, horse and mouse. Earlier studies conducted in our lab (Yadav *et al*., 2013 and Ashraf *et al*., 2014) also reported that heat treated embryos show significantly high expression of *HSP 70.1* and *HSP 70.2* as compared to control embryos which were cultured at 38.5°C throughout the procedure of IVEP. Correlating the gene expression of *HSP 70.1* and *HSP 70.2* to embryonic development (Tables 4.4 and 4.5), it can be concluded that Ascorbic acid does play role as thermoprotectant. Had it not been playing a role in mitigating heat stress at elevated temperatures, a significant increase in heat shock protein expression would have been observed.

4.3 Relative mRNA abundance of Apoptosis related genes

While the relative mRNA abundance of pro-apoptotic genes, *BAX* and *BID* was lower in blastocysts produced after supplementation of oocytes with 50 µM Ascorbic acid during IVM, it was higher in blastocysts produced with supplementation of 100 µM Ascorbic acid. The expression pattern was significantly different for *BAX* but for *BID*, the expression pattern did not differ significantly (Figures 14-15).

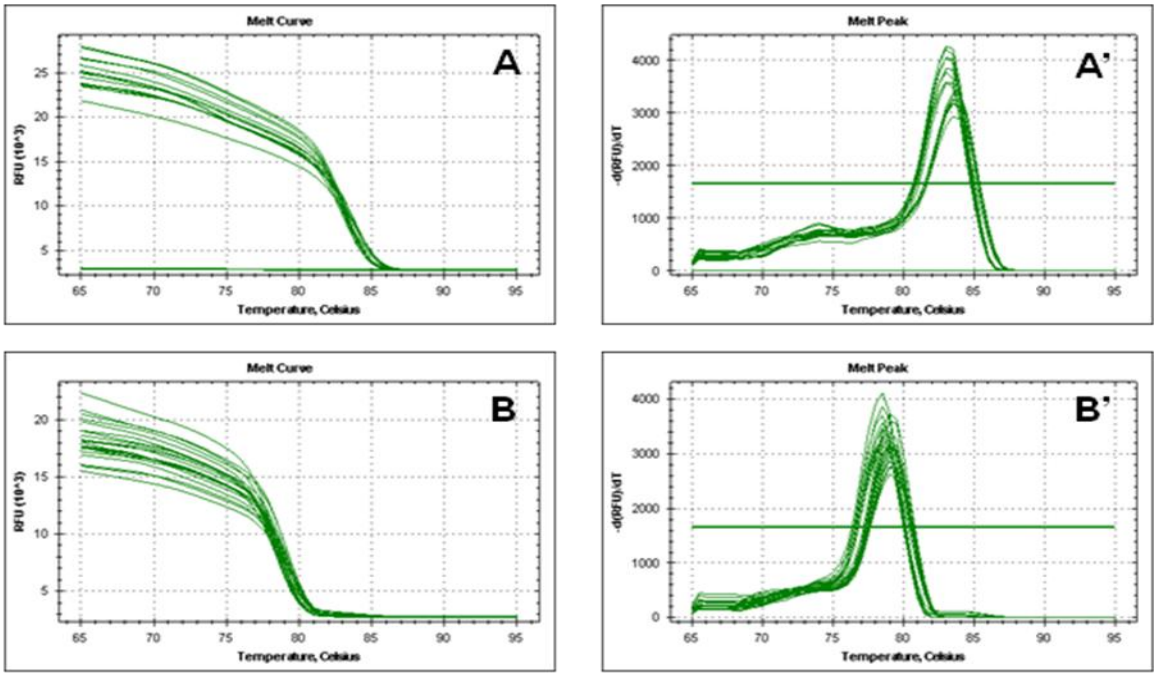

Fig. 9: **Melt curves and melt peaks of heat stress related genes: *HSP 70.1* (A, A'), *HSP 70.2* (B, B').**

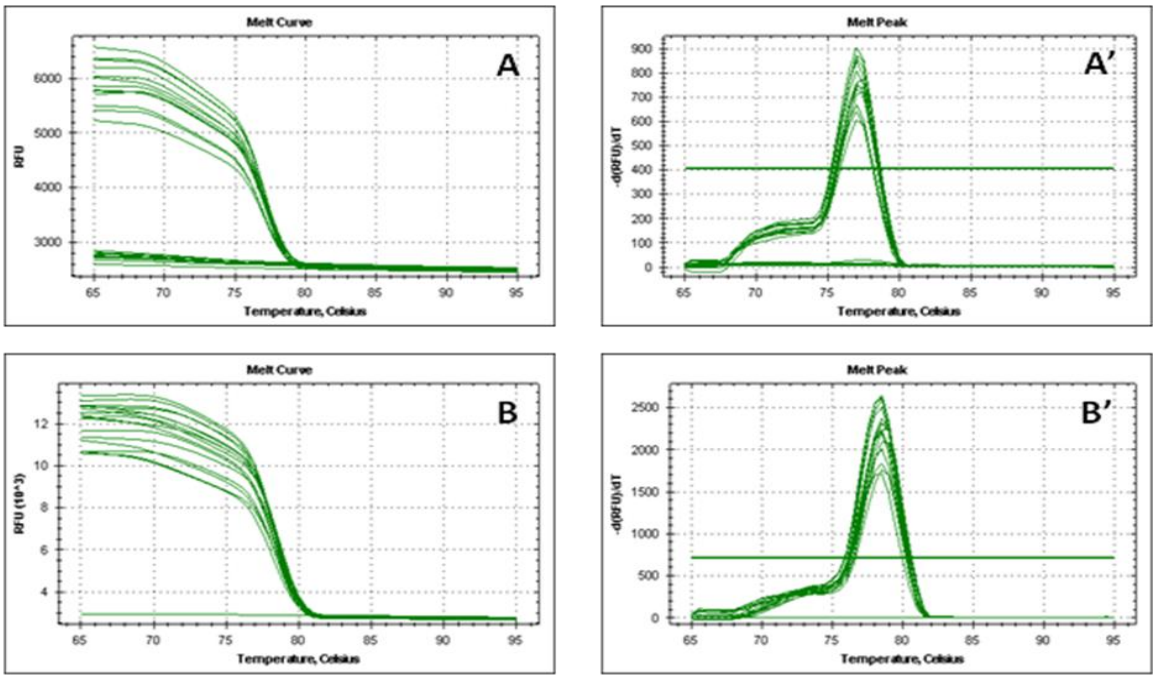

Fig. 10: **Melt curves and melt peaks of different developmental competence related genes: *GDF9* (A, A'), *BMP15* (B, B').**

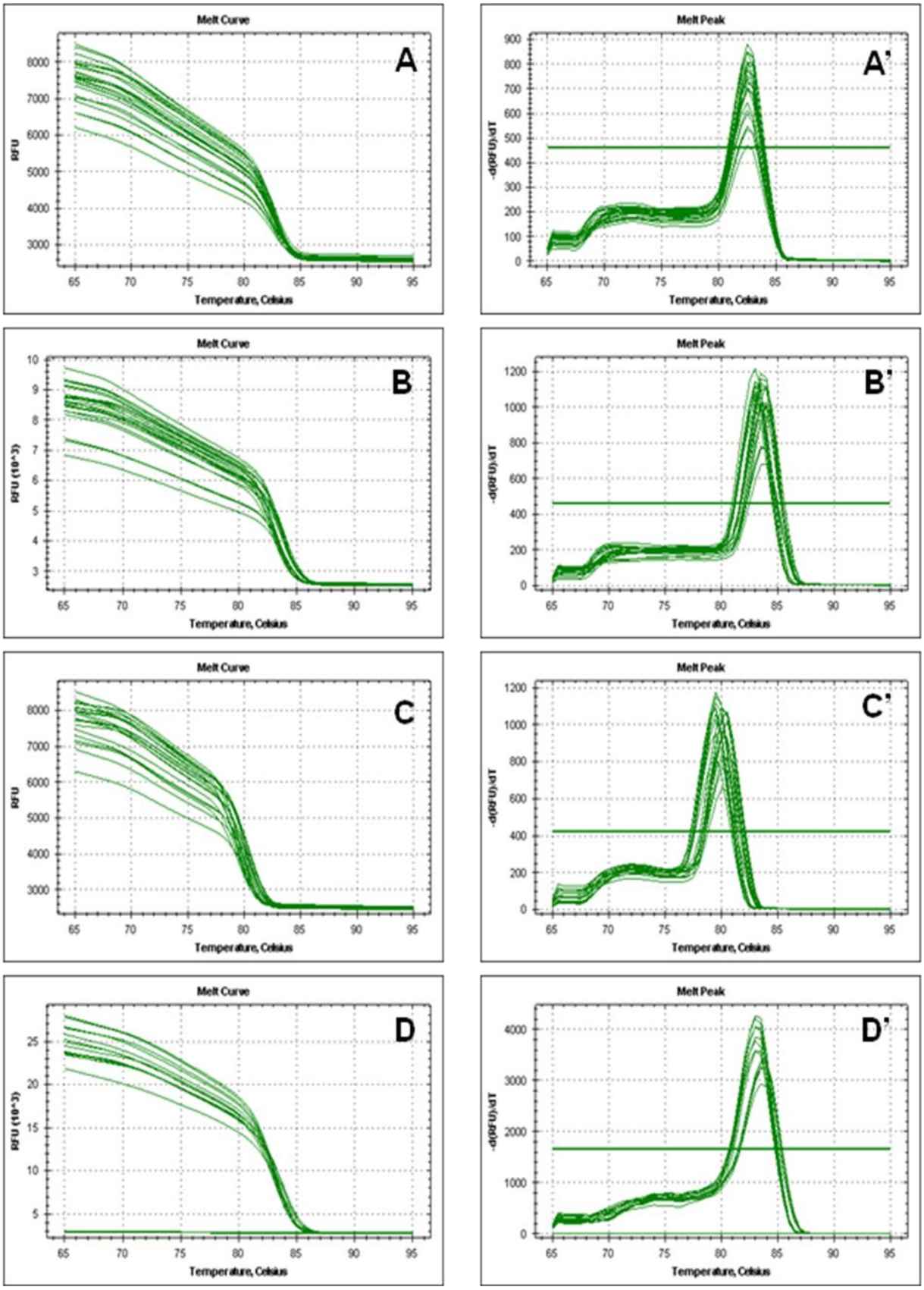

Fig. 11: Melt curves and melt peaks of different apoptosis related genes: *BAX* (A, A'), *BID* (B, B'), *BCL-XL* (C, C') and *MCL1* (D, D').

For the anti-apoptotic genes, i.e., *BCL-XL* and *MCL1* studied, the relative mRNA abundance in case of *BCL-XL* was non-significantly higher for group of oocytes supplemented with 50 µM Ascorbic acid and non-significantly lower for group supplemented with 100 µM Ascorbic acid in IVM media as compared to control group. The trend observed for *MCL1* was similar to that of *BCL-XL* except the fact that increase and decrease in mRNA abundance was significant ($P<0.05$) (Figure 16-17).

Apoptosis, also called 'programmed cell death', is a highly conserved and regulated suicidal form of cell death that occurs in single cells. It has received an increasing amount of attention because of its potential role in embryonic loss (Li *et al*., 2009; Antunes *et al*., 2010). Induction of apoptosis has been reported to be one of the important manners through which ROS manifest their deleterious effects (Yang *et al*. 1998). One of the approaches to ameliorate the oxidative stress is to supplement the culture media with low molecular weight thiol compounds like Cysteamine, β-mercaptoethanol etc., which can scavenge the ROS leading to improved blastocyst production as observed in cattle (de Matos *et al*., 2002; Oyamada and Fukui, 2004) and buffalo (Gasparrini *et al*., 2003; Anand *et al*., 2008). Earlier in our lab, supplementation of the IVM and IVC media with Cysteamine increased ($P< 0.05$) the relative mRNA abundance of anti-apoptotic genes *BCL-XL* at 4- cell, morula and blastocyst stages and that of *MCL1* at 2-, 4-, 8-to 16-cell and morula stages. Also, Cysteamine supplementation decreased ($P< 0.05$) the transcriptional level of *BAX* (Elamaran *et al*., 2012).

Our results indicate that supplementation of 50 µM Ascorbic acid in IVM media increased the blastocyst rate as well as relative mRNA abundance of anti-apoptotic genes, i.e., *BCL-XL* and *MCL1* while decreasing the expression of pro-apoptotic genes i.e., *BAX* and *BID*. However, Ascorbic acid seemed to have toxic effects at higher concentration of 100 µM as it reduced ($P<0.05$) the rate of formation of blastocysts.

4.4 Relative mRNA abundance of Developmental Competence genes

When gene expression related to developmental competence, i.e., *GDF9* and *BMP15* was compared amongst 3 groups, it was observed that supplementation of 50 µM Ascorbic acid in IVM media led to a non-significant increase in relative mRNA abundance of both the genes while a non-significant decrease was observed on supplementation of 100 µM Ascorbic acid to IVM media (Figures 18-19).

To the best of our knowledge, for the first time we report the effects of supplementation of culture media with Ascorbic acid on the gene expression of heat stress, apoptosis and developmental competence related genes. The present findings related to embryonic development and gene expression after supplementation of IVM or IVC or both IVM and IVC media with Ascorbic acid improved blastocyst production rate in buffalo due to its anti-oxidant as well as thermoprotectant role.

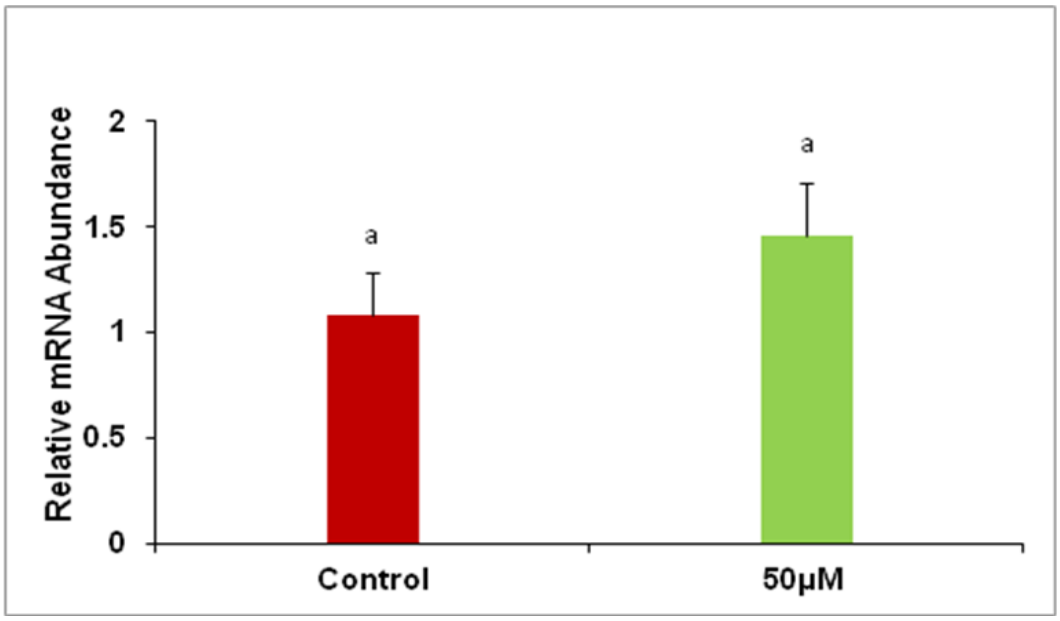

Fig. 12: Expression of heat stress related gene (*HSP 70.1*) in blastocysts produced from control oocytes (without Ascorbic acid supplementation) and oocytes supplemented with 50 µM Ascorbic acid after initial treatment at 39.5°C for 12 h during IVM

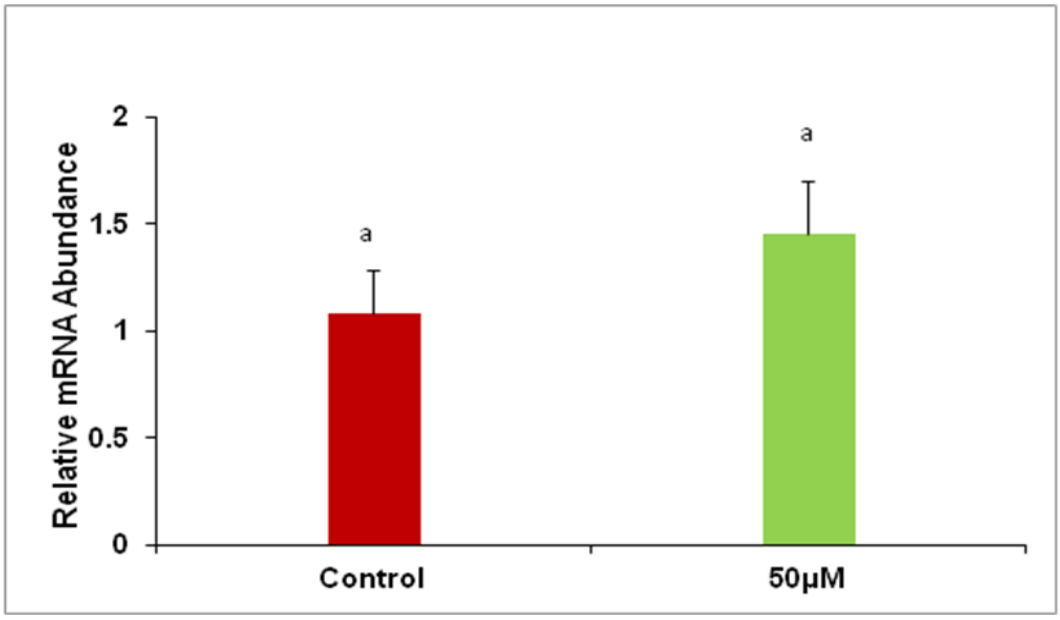

Fig. 13: Expression of heat stress related gene (*HSP 70.2*) in blastocysts produced from control oocytes (without Ascorbic acid supplementation) and oocytes supplemented with 50 µM Ascorbic acid after initial treatment at 39.5°C for 12 h during IVM

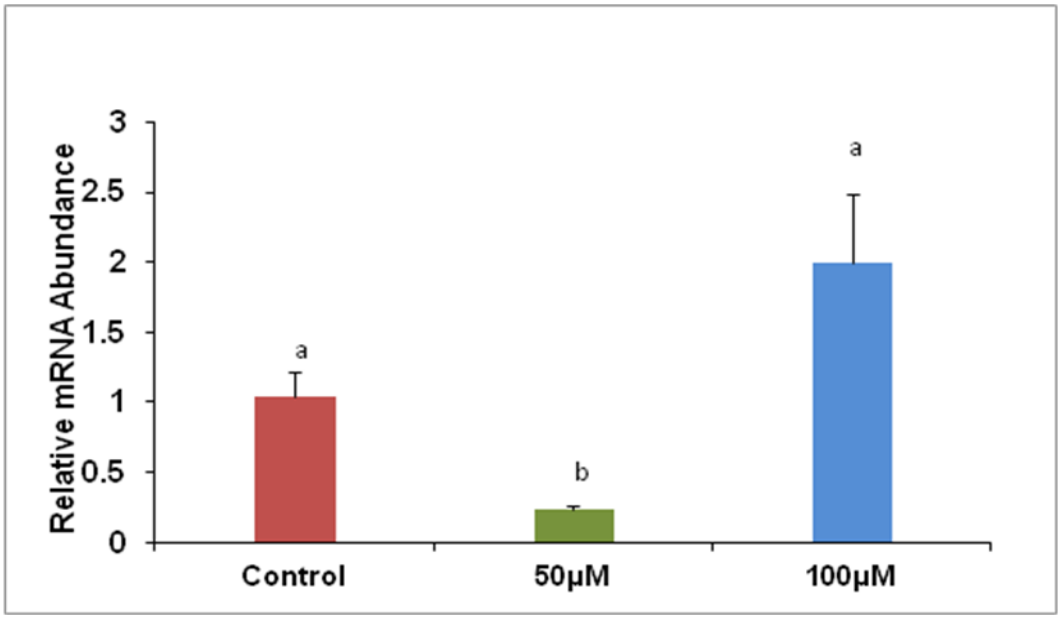

Fig. 14: Expression of Pro-apoptotic gene (*BAX*) in blastocysts produced from control oocytes (without Ascorbic acid supplementation) and oocytes supplemented with 50 µM and 100 µM Ascorbic acid in IVM medium

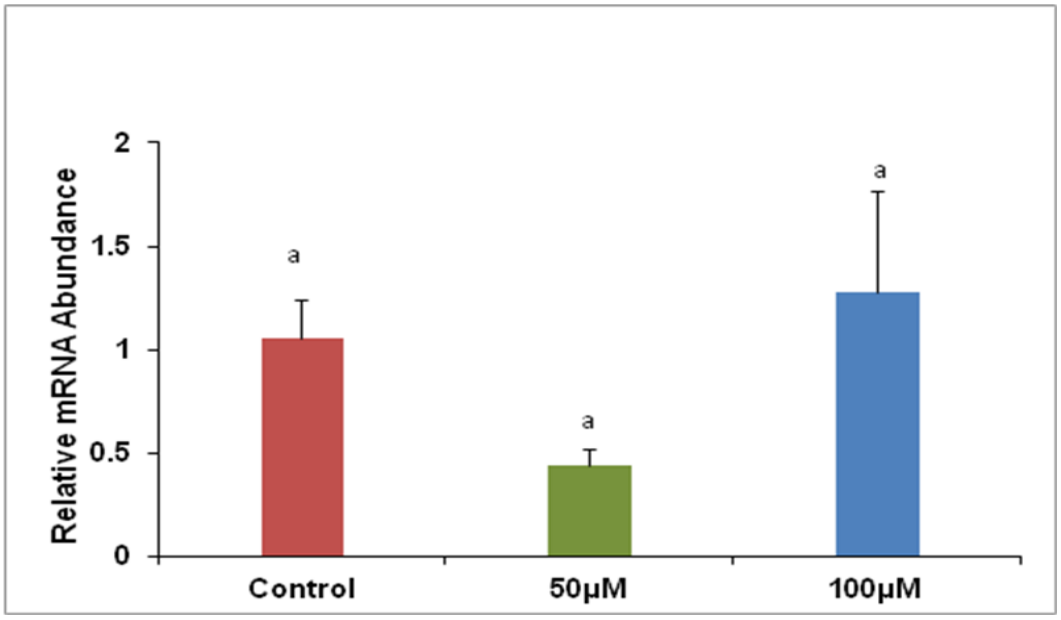

Fig. 15: Expression of Pro-apoptotic gene (*BID*) in blastocysts produced from control oocytes (without Ascorbic acid supplementation) and oocytes supplemented with 50 µM and 100 µM Ascorbic acid in IVM medium

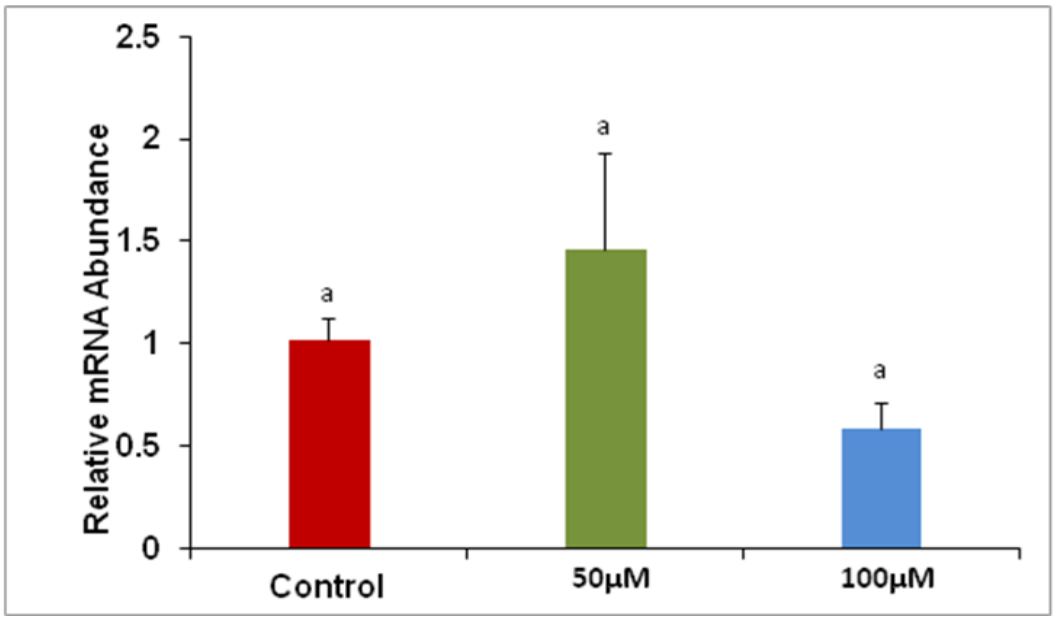

Fig. 16: Expression of Anti-apoptotic gene (*BCL-XL*) in blastocysts produced from control oocytes (without Ascorbic acid supplementation) and oocytes supplemented with 50 µM and 100 µM Ascorbic acid in IVM medium

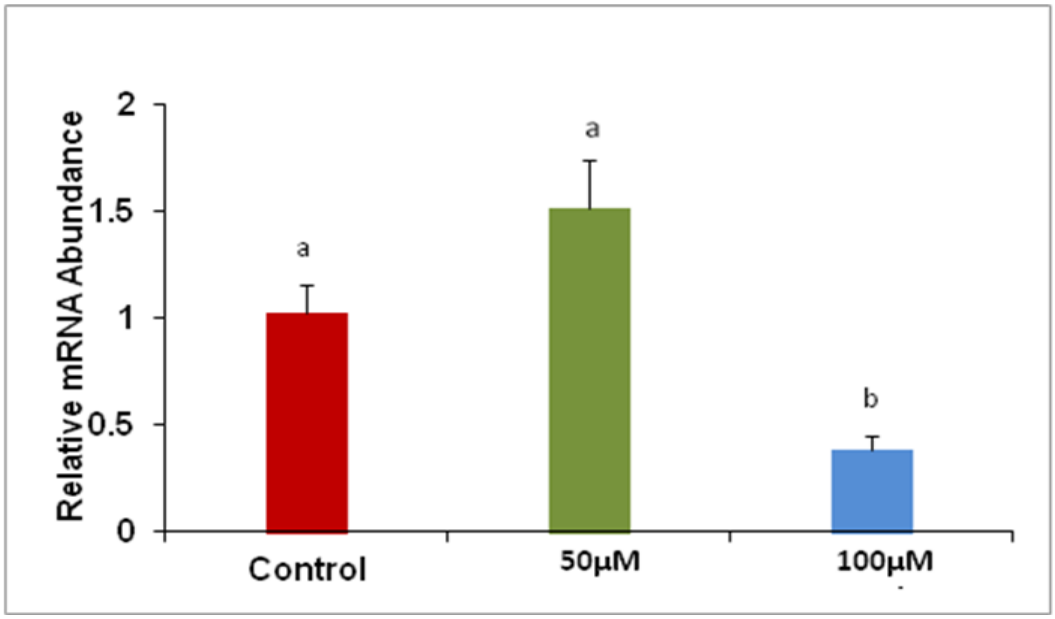

Fig. 17: Expression of Anti-apoptotic gene (*MCL1*) in blastocysts produced from control oocytes (without Ascorbic acid supplementation) and oocytes supplemented with 50 µM and 100 µM Ascorbic acid in IVM medium

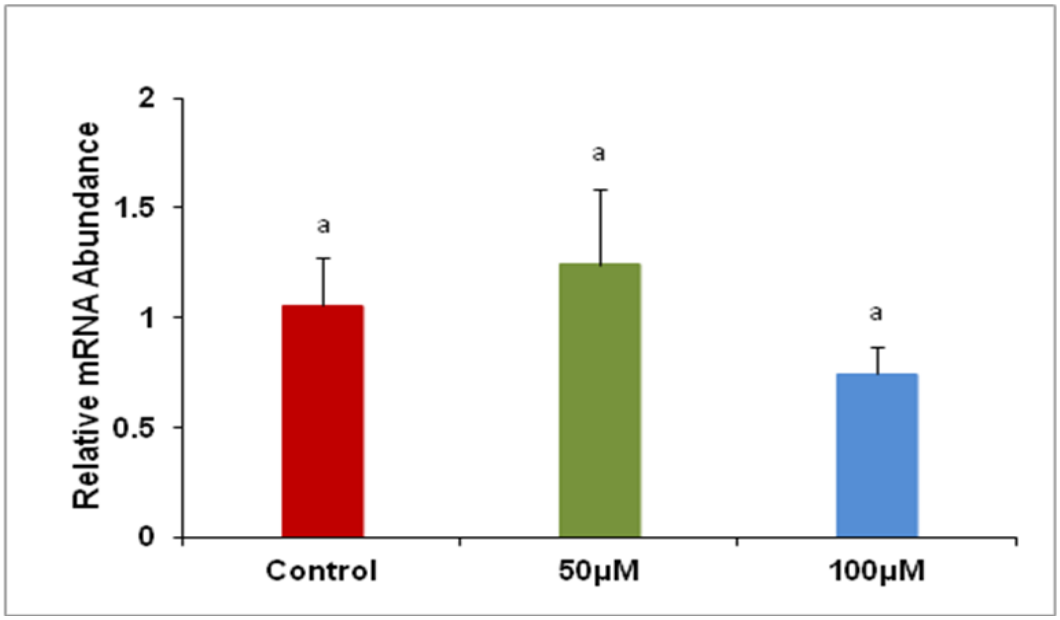

Fig. 18: Expression of developmental competence related (*GDF9*) gene in blastocysts produced from control oocytes (without Ascorbic acid supplementation) and oocytes supplemented with 50 µM and 100 µM Ascorbic acid in IVM medium

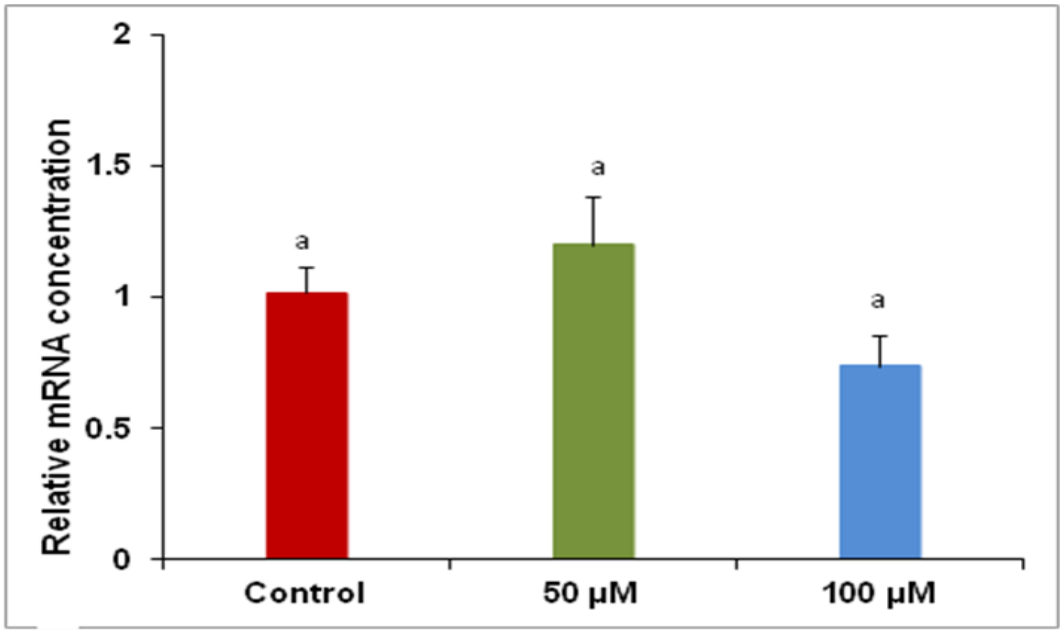

Fig. 19: Expression of developmental competence related (*BMP15*) gene in blastocysts produced from control oocytes (without Ascorbic acid supplementation) and oocytes supplemented with 50 µM and 100 µM Ascorbic acid in IVM medium

CHAPTER – 5

Summary and Conclusions

5. SUMMARY AND CONCLUSIONS

Reproductive performance of farm animals in tropical and subtropical environments is affected throughout the year by a number of factors such as the physical environment, nutrients availability, adaptability, genetic composition of cattle, intensive or extensive management systems, socio-economic status of farmers. One of the most important factors of physical environment that lead to stress in farm animals is ***oxidative stress***, an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates. Antioxidant vitamins like vitamin C and vitamin E have proved to protect the biological membranes against the damage of ROS. The role of vitamin E as an inhibitor –"chain blocker"-of lipid per-oxidation has been well established. Like vitamin E, ascorbate is also a chain breaking antioxidant. It prevents lipid peroxidation due to peroxyl radicals. It also recycles vitamin E. It protects against DNA damage induced by H_2O_2 radical. Vitamin C was found to assist in absorption of folic acid by reducing it to tetrahydrofolate; the latter again acts as an antioxidant. Use of folic acid is impaired when vitamin C is deficient. It has been reported that administration of ascorbic acid restores the androgenic and gametogenic activity of ethanol treated rats. Vitamin C along with electrolyte supplementation was found to ameliorate the heat stress in buffaloes. The present study was, therefore, carried out to i) To study the effect of ascorbic acid on *in vitro* development of IVM and IVC in buffalo *(Bubalus bubalis)* embryos. ii) To study the role of ascorbic acid as thermoprotectant on developmental competence of embryos. iii) To study the expression of some developmental genes during embryonic development.

For experiment 1, Ascorbic acid was supplemented at 50 µM and 100 µM in IVM medium only and IVF and IVC were carried out. The developmental competence was compared by recording the cleavage and different stages that the cloned embryos developed to. The cleavage rate was 62.77 ± 2.71 percent. These cleaved oocytes were further cultured and 72.72 ± 1.77, 53.35 ± 0.70, 24.50 ± 1.07 and 10.67 ± 0.24 percent developed to 4-

cell, 8-to 16- cell, morula and blastocyst stages respectively. When 50 µM Ascorbic acid was supplemented the cleavage rate, 4- cell, 8-to 16- cell, morula and blastocyst formation rates were 66.67 ± 2.27, 78.85 ± 2.77, 59.06 ± 1.65, 32.55 ± 1.32 and 16.67 ± 1.26 percent, respectively. When higher concentration of 100 µM Ascorbic acid was supplemented to IVM media, 54.04 ± 2.20, 62.11 ± 2.56, 33.03 ± 2.59, 15.85 ± 0.37 and 6.16 ± 0.37 percent of the cultured oocytes (n=420) developed to 2- cell, 4- cell, 8-to 16-cell, morula and blastocyst, respectively. Thus, supplementation of 50 µM Ascorbic acid in IVM media leads to increase in development rate of buffalo oocytes while supplementation of 100 µM Ascorbic acid led to decrease in blastocyst production rate due to increased toxicity.

For experiment 2, Ascorbic acid was supplemented at 50 µM in IVC medium only, in the control group, 57.57 ± 1.55, 65.78 ± 2.01, 46.84 ± 1.25, 23.15 ± 1.08 and 9.47 ± 0.86 percent developed to 2-cell, 4- cell, 8-to 16- cell, morula and blastocyst, respectively. In the treatment group (n=329), cleavage rate, 4- cell, 8-to 16- cell, morula and blastocyst formation rates were , 69.09 ± 3.22, 80.26 ± 2.05, 60.96 ± 2.05, 34.21 ± 1.04 and 20.18 ± 0.86 percent, respectively. Hence, on supplementation of 50 µM Ascorbic acid in IVC media increased significantly ($P<0.05$) for all the stages including blastocyst stage except cleavage rate.

In experiment 3, 50 µM Ascorbic acid was added to both IVM and IVC media. A non-significant increase in cleavage rate (66.67 ± 2.23% vs 54.83 ± 1.94%) was observed for the supplemented group as compared to control. While the percentage development rate was significantly higher at 4- cell, 8-to 16- cell, morula and blastocyst stages in 50 µM Ascorbic acid treated group (81.57 ± 2.27%, 62.40 ± 1.24%, 38.72 ± 1.72% and 28.57 ± 0.37%) as compared to control (66.19 ± 27.8%, 46.67 ± 3.58%, 25.71 ± 1.64% and 11.42 ± 1.45%) respectively. Thus, supplementation of both IVM and IVC media with 50 µM Ascorbic acid led to better developmental rate as compared to supplementation of Ascorbic acid alone either in IVM or IVC

To study the effect of Ascorbic acid on development of buffalo embryos exposed to heat shock, the oocytes were initially matured at 39.5°C or 40.5°C for 12 h, supplemented with 50 µM Ascorbic acid. When control oocytes

(n=354) were given heat treatment at 39.5°C along with treatment group (n=381) with 50 µM Ascorbic acid supplementation, it was observed that embryonic development rates did not differ significantly between two groups as rates of production of 2- cell stage (15.53 ± 0.50% vs 16.27 ± 1.70%), 4-cell (81.80 ± 1.21% vs 74.19 ± 4.47%), 8-to 16- cell (54.54 ± 3.71% vs 54.83 ± 2.28%), morula (40.00 ± 2.20% vs 38.70 ± 3.62%) and blastocyst (3.60 ± 2.30% vs 3.23 ± 2.34%) were almost similar. When the corresponding treatment was given to group 3 and 4 at 40.5°C, it was observed that heat treatment had profound effect on embryonic development as no blastocyst was formed in both control as well as treatment group. Supplementation of Ascorbic acid helped to combat the heat shock as evident from the non-significant decrease for all stages of embryonic development (13.88 ± 0.50% vs 13.31 ± 3.77% for 2- cell, 82.00 ± 1.21% vs 71.69 ± 2.65% for 4- cell, 18.00 ± 2.20% vs 15.09 ± 0.90% for morula) except 8-to 16- cell stage (46.00 ± 3.71% vs 49.05 ± 2.79%). Supplementation of Ascorbic acid combated the effect of heat shock which resulted in almost similar developmental rates in heat treated group as that of control.

The relative quantification of mRNA for genes related to heat stress (*HSP 70.1*, *HSP 70.2*), apoptosis (Pro-apoptotic: *BAX, BID*; Anti-apoptotic: *BCL-XL, MCL1*) and developmental competence (*GDF9, BMP15*) related genes was done by real time PCR. The relative mRNA abundance of *HSP 70.1* and *HSP 70.2*, was almost similar (non-significantly higher) in oocytes matured at 39.5°C or 40.5°C after supplementation with 50 µM Ascorbic acid as compared to their corresponding controls in which Ascorbic acid was not supplemented. When the relative mRNA abundance of pro-apoptotic genes, *BAX* and *BID* was lower in blastocysts produced after supplementation of oocytes with 50 µM Ascorbic acid during IVM, it was higher in blastocysts produced with supplementation of 100 µM Ascorbic acid. The expression pattern was significantly different for *BAX* but for *BID*, the expression pattern did not differ significantly. For the anti-apoptotic genes, i.e., *BCL-XL* and *MCL1* studied the relative mRNA abundance in case of *BCL-XL* was non-significantly higher for group of oocytes supplemented with 50 µM Ascorbic acid and non-significantly lower for group supplemented with 100 µM Ascorbic

acid in IVM media as compared to control group. The trend observed for *MCL1* was similar to that of *BCL-XL* except the fact that increase and decrease in mRNA abundance was significant ($P<0.05$). When gene expression related to developmental competence, i.e., *GDF9* and *BMP15* was compared among 3 groups, it was observed that supplementation of 50 µM Ascorbic acid in IVM media led to a non-significant increase in relative mRNA abundance of both the genes while a non-significant decrease was observed on supplementation of 100 µM Ascorbic acid to IVM media

In conclusion, the results of the present study suggest that:

i) Supplementation of IVM or IVC or both IVM and IVC media with 50 µM Ascorbic acid improves the blastocyst production rates in buffalo.

ii) Ascorbic acid plays a thermoprotectant role and helps buffalo embryos combating the effects of heat stress.

Bibliography

BIBLIOGRAPHY

Agarwal, A., Gupta, S. and Sharma, R.K., 2005. Role of oxidative stress in female reproduction. *Reproductive Biology and Endocrinology*, **3(28):** 1-21.

Agarwal A., Sharma, R.K., Nallella, K.P., Thomas Jr., A.J., Alvarez, J.G. and Sikka, S.C. 2006 Reactive oxy- gen species as an independent marker of male factor in- fertility. *Fertility and Sterility*, **86:** 878-885.

Aitken, R.J., Clarkson, J.S., Fishel, S. 1989. Generation of reactive oxygen species, lipid peroxidation, and human sperm function. *Biology of Reproduction*, **41:** 183-197.

Aitken, R.J., Harkiss, D., Buckingham, D. 1993. Relationship between iron-catalyzed lipid peroxidation potential and human sperm function. *Journal of Reproduction & Infertility*, **98:** 257-265.

Aitken, R.J., Ryan, A.L., Baker, M.A. and McLaughlin, E.A. 2004. Redox activity associated with the matura- tion and capacitation of mammalian spermatozoa. *Free Radical Biology and Medicine*, **36:** 994-1010.

Al-Katanani, Y.M., Webb, D.W. and Hansen, P.J. 1999. Factors affecting seasonal variation in 90 day non-return rate to first service in lactating Holstein cows in a hot climate. *Journal of Reproduction & Infertility*, **82:** 2611-2615.

Al-Katanani, Y. M., Paula-Lopes, F.F. and Hansen, P.J. 2002. Effect of season and exposure to heat stress on oocyte competence in Holstein cows. *Journal of Reproduction and Infertility*, **85:** 390-396.

Anand, T., Kumar, D., Chauhan, M. S., Manik, R. S and Palta, P. 2008. Cysteamine supplementation of *in vitro* maturation medium, *in vitro* culture medium or both media promotes *in vitro* development of buffalo (*Bubalus bubalis*) embryos. *Reproduction, Fertility and Development*, **20:** 253-257.

Antunes, G., Chaveiro, A., Santos, P., Marques, A., Jin, H.S. and Moreira da Silva, F. 2010. Influence of apoptosis in bovine embryo's development. *Reproduction in Domestic Animals*, **45(1):** 26-32.

Argov, N., Moallem, U. and Sklan, D. 2005. Summer heat stress alters the mRNA expression of selective-uptake and endocytotic receptors in bovine ovarian cells. *Theriogenology*, **64:** 1475-1489.

Ashraf, S., Shah, S.M., Saini, N., Dhanda, S., Kumar, A., Goud, T.S., Singh, M.K., Chauhan, M.S. and Upadhyay, R.C. 2014. Developmental competence and expression pattern of bubaline (*Bubalus bubalis*) oocytes subjected to elevated temperatures during meiotic maturation *in vitro*. *Journal of Assisted Reproduction and Genetics*, **31(10):** 1349-1360.

Augustin, R., Pocar, P., Wrenzycki, C., Niemann, H. and Fischer, B. 2003. Mitogenic and anti-apoptotic activity of insulin on bovine embryos produced *in vitro. Reproduction,* **126:** 91-99.

Behrman, H.R., Kodaman, P.H., Preston, S.L. and Gao, S. 2001. Oxidative stress and the ovary. *Journal of the Society for Gynecologic Investigation*, **8:** S40-S42.

Besenfelder, U., Havlicek, V., Kuzmany, A. and Brem, G., 2010. Endoscopic approaches to manage in vitro and in vivo embryo development**:** use of the bovine oviduct. *Theriogenology*, **73(6):** 768-77.

Bodin, L., Di Pasquale, E., Fabre, S., Bontoux, M., Monget, P., Persani, L. and Mulsant, P. 2007. A novel mutation in the bone morphogenetic protein 15 gene causing defective protein secretion is associated with both increased ovulation rate and sterility in Lacaune sheep. *Endocrinology,* **148:** 393-400.

Bridges, P.J., Brusie, M.A. and Fortune, J.E. 2005. Elevated temperature (heat stress) *in vitro* reduces androstenedione and estradiol and increases progesterone secretion by follicular cells from bovine dominant follicles. *Domestic Animal Endocrinology*, **29:** 508-522.

Bucak M.N., Ateahin A. and Yuce A. 2008. Effect of antioxidants and oxidative stress parameters on ram semen afterthe freeze-thawing process. *Small Ruminant Research*, **75:** 128-134.

Bucak, M.N., Sariozkan, S. and Tuncer, P. B. 2010. The effect of antioxidants on post-thawed Angora goat (Capra hircusancryrensis) sperm parameters, lipid peroxidation and antioxidant activities. *Small Ruminant Research*, **89:** 24-30.

Camargo, L.S., Viana, J., Ramos, A. and Vale, F. 2007. Developmental competence and expression of the Hsp 70.1 gene in oocytes of *Bos indicus* and *Bos Taurus* dairy cows in tropical environment. *Theriogenology,* **68:** 626-632.

Cartmill, J.A., El-Zarkouny, S.Z., Hensley, B.A., Rozell, T.G., Smith, J.F. and Stevenson, J.S. 2001. An alternative AI breeding protocol for dairy cows exposed to elevated ambient temperatures before or after calving or both. *Journal of Dairy Science*, **84(4):** 799-806.

Castro Paula L.A. and Hansen P.J. 2008. Modification of actions of heat shock on development and apoptosis of cultured preimplantation bovine embryos by oxygen concentration and dithiothreitol. *Molecular Reproduction and Development,* **75:** 1338-1350.

Cavestany, D., El-Wishy, A.B. and Foote, R.H. 1985. Effect of season and high environmental temperature on fertility of Holstein cattle. *Journal of Dairy Science*, **68(6):** 1471-1478.

Chand, A.L., Ponnampalam, A.P., Harris, S.E., Winship, I.M. and Shelling, A.N. 2006. Mutational analysis of BMP15 and GDF9 as candidate genes for premature ovarian failure. *Fertility and Sterility,* **86:** 1009-1012.

Chauhan, M.S., Katiyar, P.K., Madan, M.L., Singla, S.K. Manik, R.S. 1996. Influence of follicle stimulating hormone on *in vitro* maturation and cleavage of buffalo (*Bubalus bubalis*) oocytes after *in vitro* fertilization. Theriogenology, **45:** 243.

Chauhan, M.S., Katiyar, P.K., Singla, S.K., Manik, R.S., Madan, M.L. 1997a. Production of buffalo calves through *in vitro* fertilization. *The Indian Journal of Animal Sciences,* **67:** 306-308.

Chauhan, M.S., Manik, R.S., Singla, S.K., Katiyar, P.K., Madan, M.L. 1997b. Effect of insulin on *in vitro* development of *in vitro* produced embryos in co-culture system. *The Indian Journal of Animal Production and Management*, **13:** 19-24.

Chauhan, M.S., Palta, P., Das, S.K., Katiyar, P.K. Madan, M.L. 1997c. Replacement of serum and hormone additives with follicular fluid in the IVM medium**:** effects on maturation, fertilization and subsequent development of buffalo oocytes *in vitro*. *Theriogenology,* **48:** 461-469.

Chauhan, M.S., Palta, P., Das, S.K., Tomer, O.S. 1998a. Effect of culture conditions on the hatching ability of *in vitro* produced buffalo *(Bubalus bubalis)* embryos. *Veterinary Record***, 142:** 169-171.

Chauhan, M.S., Singla, S.K., Palta, P., Manik, R.S., Madan, M.L. 1998b. *In vitro* maturation and fertilization, and subsequent development of buffalo *(Bubalus bubalis)* embryos**:** Effects of oocyte quality and type of serum. *Reproduction, Fertility and Development*, **10:** 173-177.

Chauhan, M.S., Singla, S.K., Palta, P., Manik, R.S., Madan, M.L. 1998c. Individual variation among buffalo bulls in fertilization and subsequent embryonic development *in vitro*. *Indian Journal of Animal Science,* **68:** 454-456.

Chauhan, M.S., Singla, S.K., Palta, P., Manik, R.S., Madan, M.L. 1998d. Influence of theophylline on cleavage rate and embryonic development following *in vitro* fertilization of buffalo oocytes. *Indian Journal of Animal Science,* **68:** 920-922.

Chauhan, M.S., Singla, S.K., Palta, P., Manik, R.S., Tomer, O.S. 1998e. IGF-II stimulation of *in vitro* maturation, *in vitro* fertilization and subsequent development of buffalo (*Bubalus bubalis)* oocytes *in vitro*. *Veterinary Record***, 142:** 727-728.

Chauhan, M.S., Singla, S.K., Palta, P., Manik, R.S., Tomer, O.S. 1998f. Development of *in vitro* produced buffalo (*Bubalus bubalis*) embryos in relation to time. Asian-Austral. *Journal of Animal Science,* **11:** 398-403.

Chauhan, M.S., Singla, S.K., Palta, P., Manik, R.S., Madan, M.L. 1999. Effect of epidermal growth factor on cumulus expansion, meiotic maturation and development of buffalo oocytes *in vitro*. *Veterinary Record***, 144:** 266-267.

Cheema, R.S., Bansal, A.K. and Bilaspuri, G.S. 2009. Manganese provides antioxidant protection for sperm cryopreservation that may offer new consideration for clinical fertility. *Oxidative medicine and cellular longevity*, ***2*(3):** 152-159.

Chinnaiyan, A.M., O'Rourke, K., Lane, B.R. and Dixit, V.M. 1997. Interaction of CED-4 with CED-3 and CED-9**:** a molecular framework for cell death. *Science,* **275:** 1122-1126.

Crist, B.L., Alekel, D.L., Ritland, L.M., Hanson, L.N., Genschel, U. and Reddy, M.B. 2009. Association of oxidative stress, iron, and centralized fat mass in healthy postmenopausal women. *Journal of Women's Health*, **18(6):** 795-801.

Das, S.K., Chauhan, M.S., Palta, P. and Tomer, O.S. 1997. Influence of cumulus cells on *in vitro* maturation of denuded buffalo oocytes. *Veterinary Record*, **141:** 522-523.

Das, S.K., Chauhan, M.S., Palta, P. and Tomer, O.S. 1999. Chromosome configurations of buffalo oocytes matured *in vitro*. *Indian Journal of Animal Sciences*, **69 (10):** 303-305.

De Lamirande, E., Tsai, C.O.N.Y.E.E., Harakat, A.Z.I.Z. and Gagnon, C.L.A.U.D.E. 1998. Involvement of reactive oxygen species in human sperm acrosome reaction induced by A23187, lysophosphatidylcholine, and biological fluid ultrafiltrates. *Journal of andrology*, **19:**.585-594.

de Matos, D.G., Herrera, C., Cortvrindt, R., Smitz, J., Van Soom, A., Nogueira, D. and Pasqualini, R.S. 2002. Cysteamine supplementation during in vitro maturation and embryo culture**:** a useful tool for increasing the efficiency of bovine in vitro embryo production. *Molecular reproduction and development*, **62(2):** 203-209.

Di Pasquale, E., Beck-Peccoz, P. and Persani, L. 2004. Hypergonadotropic ovarian failure associated with an inherited mutation of human bone morphogenetic protein-15 (BMP15) gene. *The American Journal of Human Genetics*, **75:** 106-111.

Di Pasquale, E., Rossetti, R., Marozzi, A., Bodega, B., Borgato, S., Cavallo, L., Einaudi, S., Radetti, G., Russo, G. and Sacco, M. 2006. Identification of new variants of human BMP15 gene in a large cohort of women with premature ovarian failure. *The Journal of Clinical Endocrinology & Metabolism,* **91:** 1976-1979.

Dong, J., Albertini, D.F., Nishimori, K., Kumar, T.R., Lu, N. and Matzuk, M.M. 1996. Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature*, **383:** 531- 535.

Dos Santos, G.M.G., Silva-Santos, K.C., Barreiros, T.R.R., Morotti, F., Sanches, B.V., de Moraes, F.L.Z., Blaschi, W. and Seneda, M.M., 2016. High numbers of antral follicles are positively associated with in vitro embryo production but not the conception rate for FTAI in Nelore cattle. *Animal reproduction science*, **165:** 17-21.

Dube, J.L., Wang, P., Elvin, J., Lyons, K.M., Celeste, A.J. and Matzuk, M.M. 1998. The bone morphogenetic protein 15 gene is X-linked and expressed in oocytes. *Molecular Endocrinology***, 12:** 1809-1817.

Dutt, R.H. 1963. Critical period for early embryo mortality in ewes exposed to high ambient temperature. *Journal of Animal Science*, **22:** 713-719.

Ealy, A.D. and Hansen, P.J. 1994. Induced thermotolerance during early development of murine and bovine embryos. *Journal of Cellular Physiology,* **160:** 463-468.

Edwards, J.L., Ealy, A.D., Monterroso, V.H. and Hansen, P.J. 1997. Ontogeny of temperature-regulated heat shock protein 70 synthesis in preimplantation bovine embryos. *Molecular Reproduction and Development,* **48:** 25-33.

Edwards, J.L., Saxton, A.M., Lawrence, J.L., Payton, R.R. and Dunlap, J.R. 2005. Exposure to a physiologically relevant elevated temperature hastens in vitro maturation in bovine oocytes. *Journal of Dairy Science*, **88(12):** 4326-4333.

Elamaran, G., Singh, K.P., Singh, M.K., Singla, S.K., Chauhan, M.S., Manik, R.S. and Palta, P. 2012. Oxygen Concentration and Cysteamine Supplementation During In vitro Production of Buffalo (*Bubalus bubalis*) Embryos Affect mRNA Expression of *BCL-2, BCL-XL, MCL-1, BAX* and *BID*. *Reproduction in Domestic Animals*, ***47*(6):** 1027-1036.

Elisabeth, C., Campion, E. and Thompson, E. 1995. Expression of Hsp 70.1 gene, a landmark of early zygotic activity in mouse embryo, is restricted to the first burst of transcription. *Development,* **121:** 113-122.

Eppig, J.J., Hosoe, M., O'Brien, M.J., Pendola, F.M., Requena, A. and Watanabe, S., 2000. Conditions that affect acquisition of developmental competence by mouse oocytes in vitro**:** FSH, insulin, glucose and ascorbic acid. *Molecular and Cellular Endocrinology*, **163(1):**.109-116.

Fazeli, P., Zamiri, M.J., Farshad, A. and Khalili, B., 2010. Effects of vitamin C on testicular and seminal characteristics of Markhoz goats. *Iranian Journal of Veterinary Research*, **11(3):** 267-272.

Fiorenza, M.T. and Mangia, F. 1992. Hyperthermia specifically inhibits bivalent chromosome disjunction in maturing mouse oocytes. *Biology and Reproduction*, **46:** 658–664.

Fujitani, Y., Kasai, K., Ohtani, S., Nishimura, K., Yamada, M., Utsumi, K. 1997. Effect of oxygen concentration and free radicals on *in vitro* development of in-vitro produced bovine embryos. *Journal of Animal Science*, **75 (2):** 438-439.

Galloway, S.M., McNatty, K.P., Cambridge, L.M., Laitinen, M.P., Juengel, J.L., Jokiranta, T.S., McLaren, R.J., Luiro, K., Dodds, K.G., Montgomery, G.W., Beattie, A.E., Davis, G.H. and Ritvos, O. 2000. Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. *Nature Genetics*, **25:** 279-283.

Gardiner, C.S., Salmen, J.J. and Brandt, C.J. 1998. Glutathione is present in reproductive tract secretions and improves development of mouse embryos after chemically-induced glutathione depletion. *Biology of Reproduction*, **59:** 431-436.

Gasparrini, B. 2002. *In vitro* embryo production in buffalo species**:** state of the art. *Theriogenology,* **57(1):** 237-56.

Gasparrini, B., Sayoud, H., Neglia, G., de Matos, D.G., Donnay, I. and Zicarelli, L., 2003. Glutathione synthesis during in vitro maturation of buffalo (*Bubalus bubalis*) oocytes**:** effects of cysteamine on embryo development. *Theriogenology*, **60(5):** 943-952.

Gilchrist, R.B., Lane, M. and Thompson, J.G. 2008. Oocyte-secreted factors**:** regulators of cumulus cell function and oocyte quality. *Human Reproduction Update,* **14:** 159-177.

Gonçalves, F.S., Barretto, L.S.S., Arruda, R.P.D., Perri, S.H.V. and Mingoti, G.Z., 2010. Effect of antioxidants during bovine *in vitro* fertilization procedures on spermatozoa and embryo development. *Reproduction in Domestic Animals*, ***45*(1):** 129-135.

Goto, Y., Noda, Y., Mori, T. and Nakano, M. 1993. Increased generation of reactive oxygen species in embryos cultured *in vitro*. *Free Radical Biology and Medicine*, **15(1):** 69-75.

Guerin, P., Mouatassium, S.El. and Menezo, Y. 2001. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Human Reproduction Update*, **7(2):** 175-189.

Gupta, S., Sekhon, L., Kim, Y. and Agarwal, A. 2010. The role of oxidative stress and antioxidants in assisted reproduction. *Current Women's Health Reviews*, ***6*(3):** 227-238.

Gwazdauskas, F.C., Thatcher, W.W. and Wilcox, C.J., 1973. Physiological, Environmental, and Hormonal Factors at Insemination Which May Affect Conception 1, 2. *Journal of dairy science*, **56(7):** 873-877.

Halliwell, B. and Aruoma, O.I. 1991. DNA damage by oxygen derived species**:**Its mechanism and measurement in mammalian systems. *FEBS Letters*, **281:** 9-91.

Hansen, P.J. and Arechiga, C.F. 1999. Strategies for managing reproduction in heat stressed dairy Cows. *Journal of Dairy Science*, **82(2):** 36–50.

Hashimoto, O., Moore, R.K. and Shimasaki, S. 2005. Posttranslational processing of mouse and human BMP-15**:** potential implication in the determination of ovulation quota. *Proceedings of the National Academy of Sciences of the United States of America,* **102:** 5426-5431.

Hyslop, P.A., Hinshaw, D.B. and Hasley, W.A. 1988. The glycolytic and mitochondrial pathways of ADP phosphorylation are major intracellular targets inactivated by hydrogen peroxide. *Journal of Biological Chemistry*, **263:** 1665-1675.

Iwamoto. M., Onishi. A., Fuchimoto. D., Somfai. T., Takeda. K., Tagami. T., Hanada. H., Noguchi. J., Kaneko. H., Nagai. T. and Kikuchi. K. 2005. Low oxygen tension during *in vitro* maturation of porcine follicular oocytes improves parthenogenetic activation and subsequent development to the blastocyst stage. *Theriogenology*, **63:** 1277-1289.

Jin, Y., Li, J., Choi, S., Kim, T. and Cui, X. 2007. Heat shock inducing apoptosis related gene expression and apoptosis in porcine parthenotes developing *in vitro*. *Animal Reproduction,* **100:** 118-127.

Ju, J.C. and Tseng, J.K. 2004. Nuclear and cytoskeletal alterations of in vitro matured porcine oocytes under hyperthermia. *Molecular reproduction and development*, **68(1):** 125-133.

Kala, C., Syed, S.A., Abid, M., Sweety, R. and Najam, A.K. 2015. Protection Against FCA Induced Oxidative Stress Induced DNA Damage as a Model of Arthritis and In vitro Anti-arthritic Potential of Costus speciosus Rhizome Extract. *International Journal of Pharmacognosy and Phytochemical Research,* **7 (2):** 383-389.

Kawarsky, S.J. and King, W.A. 2001. Expression and localisation of heat shock protein 70 in cultured bovine oocytes and embryos. *Zygote*, ***9*(01):** 39-50.

Kere, M., Siriboon, C., Lo, N.W., Nguyen, N.T. and Cherng, J.U. 2014. Ascorbic Acid Improves the Developmental Competence of Porcine Oocytes After Parthenogenetic Activation and Somatic Cell Nuclear Transplantation. *Journal of Reproduction and Development*, **59:** 78-84.

Khatun, M., Bhuiyan, M.M.U., Ahmed, J.U., Haque, A., Rahman, M.B. and Shamsuddin, M., 2011. *In vitro* maturation and fertilization of prepubertal and pubertal black Bengal goat oocytes. *Journal of Veterinary science*, **12(1):** 75-82.

Kim, H., Rafiuddin-Shah, M., Tu, H.C., Jeffers, J.R., Zambetti, G.P. and Hsieh, J. J. 2006. Hierarchical regulation of mitochondrion-dependent apoptosis by BCL 2 sub families. *Nature Cell Biology*, **8:** 1348-1358.

Knudson, C.M., Tung, K.S.K. and Tourtellotte, W.G. 1995. Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science,* **270:** 96-99.

Kodama, H., Kuribayashi, Y. and Gagnon, C. 1996.Effect of sperm lipid peroxidation on fertilization. *Journal of Andrology,* **17:** 151-157.

Kowaltowski, A.J. and Vercesi, A.E. 1999. Mitochondrial damage induced by conditions of oxidative stress. *Free Radical Biology and Medicine*, **26:** 463-471.

Krininger, C.E., Stephens, S.H. and Hansen, P.J. 2002. Developmental changes in inhibitory effects of arsenic and heat shock on growth of pre-implantation bovine embryos. *Molecular Reproduction and Development,* **63:** 335-340.

Kumar, B.S., Singh, G. and Meur, S.K. 2010. Effects of addition of electrolyte and ascorbic acid in feed during heat stress in buffaloes. *Asian Australas Journal of Animal Science*, **23(7):** 880-8.

Kumar, D. and Anand, T. 2012. In vitro embryo production in buffalo**:** Basic concepts. *Journal of Buffalo Science*, **1(1):** 50.

Lamirande, E.D. and Gagnon, C. 1993. A positive role for the superoxide anion in triggering hyperactivation and capacitation of human spermatozoa. *International Journal of Andrology*, **16:** 21-2.

Leibfried, L. and First, N.L. 1979. Characterization of bovine follicular oocytes and their ability to mature *in vitro*. *Journal of Animal Science* **48:** 76-86.

Li, H.J., Liu, D.J., Cang, M., Wang, L.M., Jin, M.Z., Ma, Y.Z. and Shorgan, B. 2009. Early apoptosis is associated with improved developmental potential in bovine oocytes. *Animal reproduction science*, **114(1):** 89-98.

Liang, X., Zhang, X., Yang, B., Cheng, M., Huang, F., Pang, C., Qing, G., Liao, C., Wei, S., Senatore, E.M., Bella, A. and Presicce, G.A. 2007. Pregnancy and calving rates following transfer of *in vitro* produced river and F1 (river x swamp) buffalo (*Bubalus bubalis*) embryos in recipients on natural oestrus or synchronized for ovulation. *Reproduction, Fertility and Development*, **19:** 670-676.

Liu, Z. and Foote, R.H. 1995. Development of bovine embryos in KSOM with added superoxide dismutase and taurine and with five and twenty percent O_2. *Biology of Reproduction*, **53:** 786-790.

Loos de, F., Kastrop, P., van Maurik. P., van Beneden, T.H. and Kruip, Th. A.M. 1991. Heterologous cell contacts and metabolic coupling in bovine cumulus oocyte complexes. *Molecular Reproduction and Development,* **28:** 255-259.

Madan, M.L., Singla, S.K., Chauhan, M.S., Manik, R.S. 1994a. *In vitro* production and transfer of embryos in buffaloes. *Theriogenology,* **41:** 139-143.

Madan, M.L., Chauhan, M.S., Singla, S.K., Manik, R.S. 1994b. Pregnancies established from water buffalo (*Bubalus bubalis*) blastocysts derived from *in vitro* matured, *in vitro* fertilized oocytes and co-cultured with cumulus and oviductal cells. *Theriogenology,* **42:** 591-600.

Matsuzuka, T., Ozawa, M., Nakamura, A., Ushitani, A., Hirabayashi, M. and Kanai, Y. 2005. Effects of heat stress on the redox status in the oviduct and early embryonic development in mice. *Journal of Reproduction and Development*, **51:** 281-287.

Meister, A. 1988. Glutathione metabolism and its selective modification. *Journal of Biological Chemistry,* **263(33):** 17205-17208.

Michels, J., Johnson, P.W. and Packham, G. 2005. Mcl-1. *The international journal of Biochemistry & Cell Biology*, **37(2):** 267-271.

Munne, S. and Estop, A. 1991. Superoxide anion increases after sperm storage and produces chromosome abnormalities. *Biology of Reproduction,* **44:** 681-687.

Murray, A.A., Molinek, M.D., Baker, S.J., Kojima, F.N., Smith, M.F., Hillier, 1.S. and Spears, N. 2001. Role of ascorbic acid in promoting follicle integrity and survival in intact mouse ovarian follicles in vitro. *Reproduction,* **121(1):** 89-96.

Nagao, Y., Saeki, M.H. and Kainuma, H. 1994. Effects of oxygen concentration and oviductal tissue on the development of *in vitro* matured and fertilized bovine oocytes cultured in protein-free medium. *Theriogenology,* **41:** 681-687.

Nandi, S., Chauhan, M.S. and Palta, P. 1998. Influence of cumulus cells and sperm concentration on cleavage rate and subsequent embryonic development of buffalo (*Bubalus bubalis*) oocytes matured and fertilized *in vitro*. *Theriogenology,* **50 (8):** 1251-1262.

Nandi, S., Chauhan, M.S. and Palta, P. 2000. Effect of a corpus luteum on the recovery and developmental potential of buffalo oocytes. *Veterinary Record,* **147 (20):** 580-581.

Nandi, S., Chauhan, M.S. and Palta, P. 2001. Effect of environmental temperature on quality and developmental competence *in vitro* of buffalo oocytes. *Veterinary Record,* **148:** 278-279.

Narula, A., Taneja, M. and Totey, S.M. 1996. Morphological development, cell number and allocation of cells to trophectoderm and inner cell mass of *in vitro* fertilized and parthenogenetically developed buffalo embryos**:** the effect of IGF-I. *Molecular Reproduction and Development*, **44:** 343-351.

Oh, C., Li, M., Kim, E.H., Park, J.S., Lee, J.C. and Ham, S.W. 2011. Antioxidant and Radical Scavenging Activities of Ascorbic Acid Derivatives Conjugated with Organogermanium. *ChemInform*, **42:** 16.

Oliva, R. 2006. Protamines and male infertility. *Human reproduction update*, ***12*****(4)**.

Oliveira De, A.T.D., Lopes, R.F.F. and Rodrigues, J.L. 2005. Gene expression and developmental competence of bovine embryos produced in vitro under varying embryo density conditions. *Theriogenology*, **64(7):** 1559-1572.

Otsuka, F. and Shimasaki, S. 2002. A novel function of bone morphogenetic protein-15 in the pituitary**:** selective synthesis and secretion of FSH by gonadotropes. *Endocrinology,* **143:** 4938-4941.

Oyamada, T. and Fukui, Y. 2004. Oxygen tension and medium supplements for in vitro maturation of bovine oocytes cultured individually in a chemically defined medium. *Journal of Reproduction and Development*, **50(1):** 107-117.

Ozawa, M., Hirabayashi, M. and Kanai, Y. 2002 . Developmental competence and oxidative state of mouse zygotes heat-stressed maternally or *in vitro*. *Reproduction,* **124:** 683-689.

Ozawa, M., Tabayashi, D., Latief, T. A., Shimizu, T., Oshima, I. and Kanai, Y. 2005. Alterations in follicular dynamics and steroidogenic abilities induced by heat. *Reproduction,* **129:** 621-30.

Palmer, J.S., Zhao, Z.Z., Hoekstra, C., Hayward, N.K., Webb, P.M., Whiteman, D.C., Martin, N.G., Boomsma, D.I., Duffy, D.L. and Montgomery, G.W. 2006. Novel variants in growth differentiation factor 9 in mothers of dizygotic twins. *The Journal of Clinical Endocrinology & Metabolism,* **91:** 4713-4716.

Palta, P. and Chauhan, M.S. 1998. Laboratory production of buffalo (*Bubalus bubalis*) embryos. *Reproduction, Fertility and Development*, **10:** 379-391.

Parrish, J.J. 2014. Bovine in vitro fertilization**:** in vitro oocyte maturation and sperm capacitation with heparin. *Theriogenology*, **81(1):** 67-7.

Perez-Crespo, M., Ramirez, M.A., Fernandez, G.R., Rizos, D., Lonergan, P., Pintado, B. and Gutierrez A.A. 2005. Differential sensitivity of male and female mouse embryos to oxidative induced heat-stress is mediated by glucose-6-phosphate dehydrogenase gene expression. *Molecular Reproduction and Development*, **72:** 502-510.

Picco, S.J., Abba, M.C., Mattioli, G.A., Fazzio, L.E., Rosa, D., De Luca, J.C. and Dulout, F.N., 2004. Association between copper deficiency and DNA damage in cattle. *Mutagenesis*, **19(6):** 453-456.

Pontes, J.H.F., Nonato-Junior, I., Sanches, B.V., Ereno-Junior, J.C., Uvo, S., Barreiros, T.R.R., Oliveira, J.A., Hasler, J.F. and Seneda, M.M. 2009. Comparison of embryo yield and pregnancy rate between in vivo and in vitro methods in the same Nelore (Bos indicus) donor cows. *Theriogenology*, **71(4):** 690-697.

Putney, D.J., Drost, M. and Thatcher, W.W. 1989. Influence of summer heat stress on pregnancy rates of lactating dairy cattle following embryo transfer or artificial insemination. *Theriogenology*, **31:** 765–778.

Putney, D.J., Mullins, S., Thatcher, W.W., Drost, M. and Gross, T.S. 1989. Embryonic development in superovulated dairy cattle exposed to elevated ambient temperatures between the onset of estrus and insemination. *Animal Reproduction Science*, **19(1-2):** 37-51.

Ray, P.D., Huang, B.W. and Tsuji, Y. 2012. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cellular signalling*, **24(5):** 981-990.

Rensis, F.D. and Scaramuzzi, R.J. 2003. Heat stress and seasonal effects on reproduction in the dairy cow-a review. *Theriogenology*, **60(6):** 1139-51.

Roth, Z., Meidan, R., Braw-Tal, R. and Wolfenson, D. 2000. Immediate and delayed effects of heat stress on follicular development and its association with plasma FSH and inhibin concentration in cows. *Journal of Reproduction and Fertil*ity, **120:** 83-90.

Roth, Z., Meidan, R., Shaham, A.A., Braw, T.R. and Wolfenson, D. 2001a. Delayed effect of heat stress on steroid production in medium-sized and preovulatory. *Reproduction*, **121**(5)**:** 745-751.

Roth, Z., Arav, A., Bor, A., Zeron, Y., Braw, T.R. and Wolfenson, D. 2001b. Improvement of quality of oocytes collected in the autumn by enhanced removal of impaired follicles from previously heat-stressed cows. *Reproduction,* **122:** 737-744.

Roth, Z. and Hansen, P.J. 2004. Involvement of apoptosis in disruption of developmental competence of bovine oocytes by heat shock during maturation. *Biology of Reproduction,* **71:** 1898-1906.

Ruder, E.H., Hartman, T.J. and Goldman, M.B., 2009. Impact of oxidative stress on female fertility. *Current opinion in obstetrics & gynecology*, ***21*(3):** 219.

Saikhun, K., Faisaikarm, T., Ming, Z., Lu, K.H. and Kitiyanant, Y. 2008. α-Tocopherol and l-ascorbic acid increase the in vitro development of IVM/IVF swamp buffalo (*Bubalus bubalis*) embryos, **2(10):** 1486-90.

Sakamoto, N., Ozawa M, Yokotani-Tomita K, Morimoto A, Matsuzuka T, Ijiri D, Hirabayashi M, Ushitani A, Kanai Y. 2008. DL-alpha-tocopherol acetate mitigates maternal hyperthermia-induced pre-implantation embryonic death accompanied by a reduction of physiological oxidative stress in mice. *Reproduction*, **135:** 489-96.

Sakatani, M., Kobayashi, S. and Takahashi, M. 2004. Effects of heat shock on *in vitro* development and intracellular oxidative state of bovine preimplantation embryos. *Molecular Reproduction and Development*, **67:** 77–82.

Sakatani, M., Yamanaka, K., Kobayashi, S. and Takahashi, M. 2008. Heat shock-derived reactive oxygen species induce embryonic mortality in *in vitro* early stage bovine embryos. *Journal of Reproduction and Development*, **54(6):** 496-501.

Salvetti, N.R., Stangaferro, M.L., Palomar, M.M., Alfaro, N.S., Rey, F., Gimeno, E.J. and Ortega, H.H. 2010. Cell proliferation and survival mechanisms underlying the abnormal persistence of follicular cysts in bovines with cystic ovarian disease induced by ACTH. *Animal Reproduction Science*, **122(1):** 98-110.

Samad, H.A., Khan, I.Q., Rehman, N.U. and Ahmad, N. 1998. The recovery, *in vitro* maturation and fertilization of Nili-Ravi buffalo follicular oocytes. *Asian-Australasian Journal of Animal Sciences,* **11:** 491-497.

Sartori, R., Sartor, B.R., Mertens, S.A., Guenther, J.N., Parrish, J.J. and Wiltbank, M.C. 2002. Fertilization and early embryonic development in heifers and lactating cows in summer and lactating and dry cows in winter. *Journal of Reproduction & Infertility*, **85:** 2803-2812.

Schrock, G.E., Saxton, A.M., Schrick, F.N. and Edwards, J.L., 2007. Early in vitro fertilization improves development of bovine ova heat stressed during in vitro maturation. *Journal of Dairy Science*, **90(9):** 4297-4303.

Sharma, G., Nath, A., Prasad, S., Singhal, S., Singh, N., Gade, N., Dubey, P. and Saikumar, G. 2012. Expression and characterization of constitutive Heat Shock Protein 70.1 (HSPA-1A) gene in in vitro produced and in vivo-derived buffalo (bubalus bubalis) embryos. *Reproduction in Domestic Animals*, **47(6):** 975-983.

Shimizu, S., Narita, M. and Tsujimoto, Y. 1999. Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. *Nature*, **399(6735):** 483-487.

Shkolnik, K.,Tadmor, A., Ben-Dor, S.,Nevo, N., Galiani, D. and Dekel, N. 2011. Reactive oxygen species are indispensable in ovulation. *Proceedings of the National Academy of Sciences*, **108(4):** 1462-1467.

Silva, J.R., Van den, H.R., Van tol, H.T., Roelen, B.A. and Figueiredo, J.R. 2005. Expression of growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15) and BMP receptors in the ovaries of goats. *Molecular Reproduction and Development,* **70:** 11-19.

Sirard, M.A., Richard, F., Blondin, P. and Robert, C. 2006. Contribution of the oocyte to embryo quality. *Theriogenology*, **65(1):** 126-136.

Somero, G.N. 2002. Thermal physiology and vertical zonation of intertidal animals**,** optima, limits, and costs of living. *Integrative and Comparative Biology*, **42(4):** 780-789.

Sreenivas, D., Kaladhar, D.S.V.G.K., Sastry, N. and Preethi, R. 2014. *In vitro* Production of Sheep Embryos in CR1aa Medium Supplemented with L-Ascorbic Acid. *Journal of Tissue Science and Engineering*, **5:** 1.

Takahashi, M., Nagai, T., Hamano, S., Kuwayama, M., Okamura, N. and Okano, A. 1993. Effect of thiol compounds on *in vitro* development and intracellular glutathione content of bovine embryos. *Biology of Reproduction*, **49:** 228-232.

Tao, Y., Chen, H., Tian, N.N., Huo, D.T., Li, G., Zhang, Y.H., Liu, Y., Fang, F.G., Ding, J.P. and Zhang, X.R. 2010. Effects of l-Ascorbic Acid, α-Tocopherol and Co-culture on In Vitro Developmental Potential of Porcine Cumulus Cells Free Oocytes. *Reproduction in Domestic Animals*, **45(1):** 19-25.

Tatemoto, H., Ootaki, K., Shigeta, K. and Muto, N. 2001. Enhancement of developmental competence after in vitro fertilization of porcine oocytes by treatment with ascorbic acid 2-O-α-glucoside during in vitro maturation. *Biology of Reproduction*, **65(6):** 1800-1806.

Taylor-Robinson, A.W., Walton, S., Swain, D.L., Walsh, K.B. and Vajta, G. 2014. The potential for modification in cloning and vitrification technology to enhance genetic progress in beef cattle in Northern Australia. *Animal Reproduction Science*, **148(3):** 91-96.

Thompson, J.G., Simpson, A.C., Pugh, P.A., Donnelly, P.E. and Tervit, H.R. 1990. Effect of oxygen concentration on in-vitro development of preimplantation sheep and cattle embryos. *Journal* of *Reproduction and Fertility*, **89 (2):** 573-578.

Tompkins, E.C., Heidenreich, C.J. and Stob, M. 1967. Effect of post-breeding thermal stress on embyronic mortality in swine. *Journal of Animal Sciences,* **26:** 377-380.

Totey, S.M., Singh, G., Taneja, M., Pawshe, C.H. Talwar, G.P. 1992. *In vitro* maturation, fertilization and development of follicular oocytes from buffalo *(Bubalus bubalis). Jounal of Reproduction and Fertilization,* **95:** 597-607.

Totey, S.M., Pawshe, C.H., Singh, G.P. 1993. *In vitro* maturation and fertilization of buffalo oocytes *(Bubalus bubalis)***:** Effects of media, hormones and sera. *Theriogenology,* **39:** 1153-1171.

Totey, S.M., Daliri, M., Appa Rao, K.B.C., Pawshe, C.H., Taneja, M. and Chillar, R.S. 1996. Differential cleavage and developmental rates and their correlation with cell numbers and sex ratios in buffalo embryos generated *in vitro*. *Theriogenology,* **45:** 521-533.

Tseng, J.K., Tang, P.C. and Ju, J.C. 2006. *In vitro* thermal stress induces apoptosis and reduces development of porcine parthenotes. *Theriogenology,* **66:** 1073-1082.

Umaoka, Y., Noda, Y., Narimoto, K. and Mori, T. 1991. Developmental potentiality of embryos cultured under low oxygen tension with superoxide dismutase. *In vitro Fertilization and Embryo Transfer,* **8(5):** 245-249.

Umaoka, Y., Noda, Y., Narimoto, K. and Mori T. 1992. Effects of oxygen toxicity on early development of mouse embryos. *Molecular Reproduction and Development,* **31:** 28-33.

Van Soom, A., Vandaele, L., Goossens, K., de Kruif, A. and Peelman,L., 2007. *Gamete origin in relation to early embryo development. Theriogenology*, **68:** S131-S137.

Vitt, U.A., Hayashi, M., Klein, C. and Hsueh, A.J. 2000. Growth differentiation factor-9 stimulates proliferation but suppresses the follicle-stimulating hormone-induced differentiation of cultured granulosa cells from small antral and preovulatory rat follicles. *Biology of Reproduction*, **62:** 370-377.

Wang, X., Falcone, T., Attaran, M., Goldberg, J.M., Agarwal, A. and Sharma, R.K. 2002. Vitamin C and Vitamin E supplementation reduce oxidative stress-induced embryo toxicity and improve the blastocyst development rate. *Fertility and Sterility*, ***78*(6):** 1272-1277.

Warriach, H.M., McGill, D.M., Bush, R.D., Wynn, P.C. and Chohan, K.R., 2015. A review of recent developments in buffalo reproduction-a review. *Asian-Australasian Journal of Animal Sciences*, **28(3):** 451-455.

Wolfenson, D., Lew, B.J., Thatcher, W.W., Graber, Y. and Meidan, R. 1997. Seasonal and acute heat stress effects on steroid production by dominant follicles in cows. *Animal Reproduction Sciences,* **47:** 9-19.

Wu, Y.T., Tang, L., Cai, J., Lu, X.E., Xu, J., Zhu, X.M., Luo, Q. and Huang, H.F. 2007. High bone morphogenetic protein-15 level in follicular fluid is associated with high quality oocyte and subsequent embryonic development. *Human Reproduction*, **22:** 1526-1531.

Yadav, A., Singh, K.P., Singh, M.K., Saini, N., Palta, P., Manik, R.S., Singla, S.K., Upadhyay, R.C. and Chauhan, M.S. 2013. Effect of physiologically relevant heat shock on development, apoptosis and expression of some genes in buffalo (*Bubalus bubalis*) embryos produced in vitro. *Reproduction in Domestic Animals*, **48(5):** 858-865.

Yan, C., Wang, P., de Mayo, F.J., Elvin, J.A., Carino, C., Prasad, S.V., Skinner, S.S., Dunbar, B.S. and Dube, J.L. 2001. Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. *Molecular Endocrinology,* **15:** 854-866.

Yang, X., Kubota, C., Suzuki, H., Taneja, M., Bols, P.E. and Presicce, G.A. 1998a. Control of oocyte maturation in cows- biological factors. *Theriogenology,* **49:** 471-482.

Yang, H.W., Hwang, K.J. and Kwon, H.C. 1998b. Detection of reactive oxygen species (ROS) and apoptosis in human fragmented embryos. *Human Reproduction,* **13:** 998-1002.

Yang, M.Y. and Rajamahendran, R. 2002. Expression of Bcl-2 and Bax proteins in relation to quality of bovine oocytes and embryos produced in vitro. *Animal Reproduction Science,* **70:** 159-169.

Yoshino, O., McMahon, H.E., Sharma, S. and Shimasaki, S. 2006. A unique preovulatory expression pattern plays a key role in the physiological functions of BMP-15 in the mouse. *Proceedings of the National Academy of Sciences,* **103:** 10678-10683.

Yu, Y., Gao, Q., Zhao, H.C., Li, R., Gao, J.M., Ding, T., Bao, S.Y., Zhao, Y., Sun, X.F., Fan, Y. and Qiao, J., 2015. Ascorbic acid improves pluripotency of human parthenogenetic embryonic stem cells through modifying imprinted gene expression in the Dlk1-Dio3 region. *Stem Cell Research and Therapy,* ***6*****(1):** 180.

Zalata, A.A., Ahmed, A.H., Allamaneni, S.S., Comhaire, F.H. and Agarwal, A. 2004. Relationship between acrosin activity of human spermatozoa and oxidative stress. *Asian Journal Andrology*, ***6*****(4):** 313-318.

Zeron, Y., Ocheretny, A., Kedar, O., Borochov, A., Sklan, D. and Arav, A. 2001. Seasonal changes in bovine fertility**:** relation to developmental competence of oocytes, membrane properties and fatty acid composition of follicles. *Reproduction,* **121:** 447-454.

Zhu, B., Walker, S.K., Oakey, H., Setchell, B.P. and Maddocks, S. 2004. Effect of paternal heat stress on the development *in vitro* of preimplantation embryos in the mouse. *Andrologia,* **36(6):** 384-94.

ANNEXURE

ANNEXURE

1.1 OOCYTE COLLECTION AND MATURATION MEDIA

Normal saline containing antibiotics

Composition		**Volume (1000ml)**
Sodium chloride	:	9.0 g
Streptomycin	:	0.1 g
Distilled water	:	1000 ml

Aspiration Medium (For 200-250 ovaries)

Composition		**Volume (40 ml)**
TCM-199 (HEPES modified)	:	50 ml
BSA	:	0.15 g
L-glutamine	:	0.004g
Gentamicin sulfate	:	50μg/ml

Washing Medium

Composition		**Volume (40 ml)**
TCM-199 (HEPES modified)	:	36 ml
FBS @10%	:	4 ml
Sodium pyruvate	:	0.0036 g
L-glutamine	:	0.004g
Gentamicin sulfate	:	50 μg/ml

Maturation Medium

Composition		**Volume (10 ml)**
Washing medium	:	9.0 ml
Porcine FSH	:	5 μg/ml
Follicular Fluid @10%	:	1 ml
Estradiol 17-β	:	1 μg/ml
Ascorbic acid	:	50 μM/100 μM

1.2 *IN VITRO* FERTILIZATION MEDIA

Brackett and Oliphant (BO) Medium

Solution -A (Stock)

Sodium chloride	:	4.3092 g
Potassium chloride	:	0.1974 g
Calcium chloride dihydrate	:	0.2171 g
Magnesium chloride- hexahydrate	:	0.0697 g
Sodium hydrogen phosphate-dihydrate	:	0.0840 g

Dissolve the above components in 500 ml of distilled water. Mix 0.05 ml of 0.5% phenol red for coloring and indication of pH of the solution. A yellowish color will appear after addition of phenol red. Add penicillin (50 I.U/ ml) and streptomycin (5 µg/ml) for preservation for longer periods.

Solution- B (Stock)

Sodium bicarbonate	:	2.5873 g

Dissolve it in 200 ml of distilled water. Mix 0.01 ml of 0.5% phenol red for indication of pH of the solution. Pink color will appear. Add penicillin/ streptomycin as given above.

Working BO medium

Composition		**Volume (50 ml)**
Solution -A	:	38 ml
Solution- B	:	12 ml
Heparin	:	10 µg/ml
Sodium pyruvate	:	0.0068 g
Caffeine sodium benzoate	:	0.0971 g

(Heparin can be used 50-100 µg/ml solution)

BO Medium for Capacitation and Fertilization

Composition		**Volume (10 ml)**
Working BO media	:	10 ml
BSA (Fatty acid free) [FAF]	:	0.1 g

1.3 *IN VITRO* EMBRYO CULTURE MEDIA

Washing Medium for Presumed Zygotes

Composition		**Volume (20 ml)**
mCR_2aa	:	20 ml
BSA (Fraction V) @ 0.6%	:	0.12 g
Gentamicin sulfate	:	50 μg/ml

***In Vitro* Culture Medium**

Composition		**Volume(10ml)**
mCR_2aa	:	4.5 ml
FBS	:	0.5 ml
Gentamicin sulfate	:	50 μg/ml
Ascorbic acid	:	50 μM

Composition of Modified Charles Rosenkrans-2 Medium with Amino Acids

The Modified Charles Rosenkrans-2 Medium with Amino Acids (mCR2aa) medium is prepared in 100 ml aliquots as per the compositions given below. The pH and the osmolarity of the media are checked.

Composition of mCR_2aa

	Component		For 100 ml	For 200 ml
1.	Water(To make final volume)	:	100 ml	200 ml
2.	NaCl	:	0.6329 g	1.2658 g
3.	$NaHCO_3$	:	0.2091 g	0.4182 g
4.	NEAA	:	1 ml	2 ml
5.	EAA	:	2 ml	4 ml
6.	Glutamine	:	0.0146 g	0.0292g
7.	KCl	:	0.0216 g	0.0432 g
8.	Hemicalcium lactate	:	0.0272 g	0.0544 g
9.	Sodium pyruvate	:	0.0055 g	0.0110 g
10.	Glycine	:	0.0037g	0.0074 g
11.	Alanine	:	0.0044g	0.0088g
12.	Glucose	:	0.0180 g	0.0360 g
13.	Gentamicin	:	Add at time of use 50 μg/ml	
14.	BSA (Fraction-V)	:	Add at time of use @ 0.6%	
15.	Phenol red		Mix 0.01 ml of 0.5%	