UNIVERSITA DEGLI STUDI DI SASSARI

SCUOLA DI DOTTORATO in

RIPRODUZIONE, PRODUZIONE, BENESSERE ANIMALE E SICUREZZA DEGLI ALIMENTI DI ORIGINE ANIMALE

Direttore Prof. Giovanni Garippa

INDIRIZZO IN'. Riproduzione, Produzione e Benessere Animale (XXII CICLO)

(coordinatore: Prof. Sergio Ledda)

STUDIES OF THE EFFECT OF SEMI-DEFINED DILUENTS ON THE CRYO-TOLERANCE OF SPERMATOZOA AND IN VITRO PRODUCTION EMBRYOS

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ANNO ACCADEMICO 2009- 2010

To my lovely Angels in my Life

My Lovely Parents

& My wings in this life my lovely sisters "Luma & Rasha"

& My close friend, my lovely Brother

"Zaid"

& My Sweety "Hanin &Jojo"

& To my life love……

Special thanks from all my heart for unconditional love, generosity, and keeping me from getting off track. Thank you for all the help & support I couldn't have got this far without you…….. My love with sweet kisses to all

Ammar

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ABSTRACTS

First Experiment:

Can Semi- defined Diluents be used Successfully for Improvement of Frozen- thawed Spermatozoa and IVP Sheep Embryos?

Abstract

In last few years many studies have been conducted that the duration of sperm- oocyte co-incubation in *in vitro* produced embryos have a positive effects on early cleavage, blastocyst formation, and results in faster embryonic development with superior morphological quality, implantation rates, and skewed sex ratio than the general accepted overnight IVF protocol.

The purpose of this study were to examine on large series, whether very short exposure of mature sheep oocytes to frozen- thawed semen prepared from different concentrations of bovine serum albumin (BSA) may affect the fertilization, subsequent development, and embryo quality before and after freezing.

Sarda ewe oocytes were collected and matured for 24h. Matured oocytes were partially denuded of cumulus cells and divided randomly into four different gamete oocytes co-incubation times, fertilized using frozen-thawed ram semen diluted with 10, 15, 20% BSA (single ejaculate). Presumptive zygotes were washed, removed from fertilization wells at 1, 2, 3 and 20 hours (h) and placed in culture medium. The numbers of cleavage, and expanded blastocysts/ group 144- 192 hours post inseminations (hpi) were recorded, and evaluate before and after freezing.

Among all co- incubation times effect, the cleavage rate increased highly with gamete co-incubation time. In 10% BSA group, there was significant difference (PO.01) in cleavage rate between 3h and standard co-incubation time (93/110, 84.5 %), (89/125, 71.2 %), respectively. While in 20% BSA group, there was highly significant difference in cleavage rate between 2h and standard co-incubation time (96/109, 88.1%), (92/124, 74.2%), respectively. Moreover, regardless the method used better blastocysts rate were obtained when sperm and oocytes co-incubated for 3h versus brief co-incubation time, and the prolongation of the time up to 20h had no positive effects on fertilization and blastocysts rates. Whereas, among different concentration of BSA groups, the cleavage rates increased significantly after 1, and 2h co-incubation time. Although, the blastocysts rates were statistically similar among all BSA groups. A slight but insignificantly were observed concerning good embryo quality rate in short compared to standard insemination.

We have concluded that: (1) this is the first study in sheep demonstrating that spermatozoa-oocyte interaction takes place within lh co-incubation time, and (2) both cleavage and blastocyst rate increase linearly with gamete co-incubation time with superior results when sperm and oocytes were co-incubated for 3h and the prolongation of that time had no additive effects on fertilization and blastocysts rates, and (3) no evidence of polyspermy insemination.

Second Experiment:

THE EFFECT OF REDUCED SPERM- OOCYTE EXPOUSER TIME ON OVINE EMBRYO PRODUCTION

Abstract

In the last few years, several authors reported that the short duration of sperm- oocyte co-incubation time can affect early cleavage, blastocyst formation and quality, implantation rates, and skewed sex ratio compared with the generally accepted overnight protocol. The purposes of this study were to examine the effects on large series how very short exposure of mature oocytes to frozen- thawed semen prepared from different concentrations of bovine serum albumin (BSA) affect the fertilization, subsequent development, and embryo quality before and after freezing.

Sarda ewe oocytes were collected and matured for 24h. Matured oocytes were partially denuded of cumulus cells, divided randomly into four different gamete oocyte co-incubation times, and fertilized using frozen-thawed ram semen diluted with 10, 15, and 20% BSA (single ejaculate). Presumptive zygotes were washed, removed from fertilization wells at 1, 2, 3 and 20 hours (h) and placed in culture medium. The numbers of cleavage, and expanded blastocysts/ group 144- 192 hours post inseminations (hpi) were recorded, and evaluated before and after freezing.

Among all co- incubation times, the cleavage rate increased highly with gamete co-incubation time. In the 10% BSA group, there was a significant difference (PO.01) in cleavage rate after 3h *vs.* overnight co-incubation time (93/110, 84.5 %), (89/125, 71.2 %) respectively, while in the 20% BSA group, there was a highly significant difference in cleavage rate after 2h *vs.* overnight co-incubation time (53/109, 48.6%), (92/124, 74.2%), respectively. Regardless of the method used, better blastocyst rates were obtained when sperm and oocytes co-incubated for 3h versus a brief co-incubation time, and the prolongation of the time up to 20h had no positive effects on fertilization or blastocyst rates. Whereas, among different concentrations of BSA groups, the cleavage rates increased significantly after 1 and 2h co-incubation times. However, the blastocyst rates were statistically similar among all BSA groups.

We have concluded that: (1) this is the first study in sheep demonstrating that spermatozoa-oocyte interaction takes place within a lh co-incubation time, and (2) both cleavage and blastocyst rates increased linearly with gamete co-incubation time with superior results when sperm and oocytes were co-incubated for 3h, and the prolongation of that time had no additive effects on fertilization and blastocyst rates, and (3) no evidence of polyspermy insemination.

Third Experiment:

STUDIES ON THE EFFECT OF SUPPLEMENTING SEMEN DILUENTS WITH DIFFERENT EGG YOLK TYPES ON POST-THAW SPERM QUALITY AND IVP SHEEP EMBRYOS

Abstract

Cryopreservation of sperm in domestic animals has been used for artificial insemination (AI) and in vitro embryos production (IVP). Egg yolk (EY) is one of the common compounds used as cryoprotectants. The major role of EY is to prevent sperm damage during the cooling and freezing process. Indeed, a different composition of yolks (cholesterol, fatty acids, and phospholipids) gives different levels of protection. The aim of this study was to evaluate the effectiveness of different types of EY [partridge (PEY), duck (DEY), turkey (TUREY), tortoise (TOREY), ostrich (OEY)], and hen egg yolks (HEY) on the quality of cryopreserved semen and IVP sheep embryos.

Ejaculates were collected from a Sarda breed ram through 11 weeks (replicates; *n*= 11) and diluted in tris- citric and glycerol extender containing 20% of either PEY, DEY, TUREY, TOREY, OEY and HEY at 37 °C. Extended semen was cooled to 4 °C and preserved as a 0.25 mL pellet in LN_2. After thawin, sperm were examined for progressive motility, viability,

morphology, acrosome integrity and ATP. Secondly, matured sheep oocytes were divided randomly into six different groups, fertilized by the frozen-thawed semen prepared as described above, and cultured. Cleavage rates and blastocyst formation (day 6- 8) were recorded.

The results were two-fold. First, our results showed that partridge EY have the best cryoprotective effects on cryopreservation of ram sperm in term of the highest sperm motility, membrane integrity, and acrosome integrity compared with egg yolk of hen, duck, turkey, ostrich, or tortoise. Second, the cleavage rates were higher after fertilization with partridge and duck egg yolk compared with hen EY. There was significant difference ($P< 0.05$) between frozen-thawed semen prepared with partridge EY than hen EY (105/ 131, 80.1%), (189/270, 70.0%), respectively. Subsequently, the blastocysts rates were higher in partridge and duck EY treatment compared to hen EY. There was significantly difference ($P<0.05$) in blastocysts rate between duck and hen EY (60/125, 48.0%), (99/270, 36.7%), respectively. In summary, our results have suggested that supplementation of partridge EY improve the cryo-tolerance of sheep semen, and could be used as an alternative to hen egg yolk in extender. Moreover, the duck EY offers advantages over hen EY in production of sheep embryos.

ACKNOWLEDGMENTS

The author would like to thank the following peoples:

My monitor Prof. Dr. S. Naitana, sincere thanks for all the love and generosity, ... all the support given to me during my study. Special Thanks for taking me in and cultivating my mind through this period. Thank you for always guiding me in the right direction, supporting and standing by me the whole time.

Prof.ssa Rina Mazzette, for all the generosity, and for all help given in difficult time. Really, I am proud to have friend like you

My dear's friend Dott. Paolo Andrea Soro & Dr.ssa Maria Luisa Delogu, thanks from my heart for all love generosity and big support during my thesis experiment and until now. Really, I couldn't have got this PhD without your help.

Great thanks to whole Veterinary Medicine Faculty family for showing wide interest and generous in love and kindness towards me and my research, but most importantly giving me the most kind, beautiful and intelligent sense..... special thanks to:

Prof. Giovanni Garippa & Prof. Sergio Ledda, for generous and big support during my thesis experiment and until now.

My lovely friend "Valeria " for kindness support during the difficult time, I am proud that I have great friend like you *My dear friends "Giovani, Sara, Daniela, Mannuela, Augusta, Vlaentina and Davide* for all their help, technical assistance, care, fun, and great friendship along the way.

..... *& thanks to all my friends.*

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LIST OF ABBREVATIONS

The following abbreviations were used throughout this thesis:

Aero some reaction	(AR)
Adenosine diphosphate	(ADP)
Adenosine triphosphate	(ATP)
Artificial insemination	(AI)
Blastocel cavity	(BC)
Bovine serum albumin	(BSA)
Breakdown of the germinal vesicle	(BGV)
Cyclic adenosine monophosphate	(cAMP)
Day	(d)
Deoxyribonucleic acid	(DNA)
Dimethysulfoxide	(DMSO)
Duck egg yolk	(DEY)
Egg yolk	(EY)
Fluorescein isothiocynate labelled Pisum Sativum agglutinin	(FITC-PSA)
Fetal calf serum	(FCS)
Germinal vesicle	(GV)
Germinal vesicle breakdown	(GVB)
Glutamic-oxaloacetic transaminase	(GOT)
Glutathione	(GSH)
Glycosaminoglycans	(GAGs)
Gram	(gm)
Hen egg yolk	(HEY)
High ionic strength	(HIS)
High ionic strength	(HIS)
Hours	(h)
Hour post insemination	(hpi)
Hyaluronic acid	(HA)
Hypo-osmotic swelling test	(HOST)
Inner cell mass	(ICM)
In vitro culture	(IVC)
In vitro fertilization	(IVF)
In vitro maturation	(IVM)
In vitro fertilization	(IVF)
In vitro production	(IVP)
Liquid nitrogen	(LN_2)
Low- density lipoprotein	(LDL)

Lutenizing hormone	(LH)
Male pronucleus	(MPN)
Metaphase I	(MI)
Metaphase II	(Mil)
Microgram(10^{-6}g)	(|ig)
Milligram (10^{-3}g)	(mg)
Minimum essential medium (non essential amino acid)	(MEM)
Minute	(min)
Monounsaturated fatty acids	(MUFA)
Non-essential amino acids	(MEM)
Ostrich egg yolk	(OEY)
Osmolarity	(Osm)
Oxidative stress	(OS)
Partridge	(PEY)
Phosphate buffered saline solution	(PBS)
Polar body	(PB)
Polyvinyl alcohol	(PVA)
Primordial germ cells	(PGCs)
Propidium iodide	(PI)
Reactive oxygen species	(ROS)
Secound	(s)
Synthetic oviduct fluid	(SOF)
Tyrode's albumin lactate pyruvate medium	(TALP)
Tortoise egg yolk	(TOREY
Two pronuclei	(2PN)
Turkey egg yolk	(TUREY)
Virus	(vs.)
Zona pellucida	(ZP)

CHAPTER I GENERAL INTRODUCTION

Semen cryopreservation is considered to be an optimal handling procedure for efficient and safe breeding as well as introduction of new genetic material, which means that distance between male and females, including overseas transit, can be overcome. It also facilitates sanitary quarantine, semen deposition. However, several researchers were reported that frozen- thawed mammalian spermatozoa are greatly damaged during the freezing and thawing processes, the freezing process produces physical and chemical stress on the sperm membrane which related to reduction in cell motility, viability and fertilizing capacity compared to fresh semen (Liu et al, 2004; Aisen et al., 2005; Gillan et al., 1997; Holt et al., 2000; Songsasen et al., 2002; Watson et al., 2000). Thus, any improvements in the post-thaw sperm survival will reduce the cost of an artificial breeding program and increase the efficiency of production embryos *in vitro* and improving fertility.

Over the past 70 years, the cryoprotective media used to dilute semen for sperm storage have continuously revised but the basic ingredients of the media remain unchanged. Egg yolk (EY) (Philips, 1939) or milk (Jones and Foote, 1972) and glycerol (Polge et al., 1949) represent the indispensable compounds of practically media used for sperm preservation in liquid or frozen states. Tris- citrate- EY based diluents have been widely used to provide adequate sperm cryopreservation (Salamon and Maxwell, 2000) for several farm animals' species, including the sheep (Anderson et al., 1973;

Salamon and Maxwell, 1995a), despite the species- to species differences that exist (Holt, 2000).

The beneficial role of EY in the cryopreservation of sperm can be attributed to a resistance factor which helps to protect against cold shock, and a storage factor, which helps to maintain viability (Kampschmidt et al, 1953). The cholesterol (Kampschmidt et al., 1953; Darin- Bennet and White, 1977; Coombes et al., 2000), phospholipids (Kampschmidt et al., 1953; Lanz et al., 1965), and low density lipoproteins (LDL) content in EY may be the factors which afford protection to the sperm against cold shock during the freeze-thaw process (Kampschmidt et al., 1953; Foulkes, 1977; Pace and Grahan, 1974; Watson, 1976; Panda and Singh, 1990). The higher levels of these factors may improve the protection of the sperm during the freeze- thaw processes resulting in higher sperm motility after thawing.

Several authors were suggested that LDL adhere to sperm membrane and provide protection to sperm by stabilizing the membrane. In another hand, other hypothesis suggests that phospholipids present in LDL protect sperm by forming a protective film on the sperm surface or by replacing sperm membrane phospholipids that are lost or damaged during the cryopreservation process (Foulkes et al., 1980; Quinn et al., 1980; Graham and Foote, 1987). Whereas, previous reports have observed the addition of LDLs from other sources than EY failed to afford the similar protection as egg yolk (Miller and Mayer 1960; Gebauer et al., 1970). This was probably because the LDL fraction of yolk contains many other compounds, particularly proteins that work in conjunction to afford protection to the sperm (Watson, 1981). While, the third suggestion mechanism of protection suggests that LDL seizes the deleterious proteins present in the seminal plasma thus improving the

freezability of spermatozoa (Manjunath et al., 2002; Bergeron and Manjunath, 2006). However, until today the exact mechanism by which EY helps preserve the spermatozoa during the freeze-thaw process is however unknown.Moreover, the different chemical composition of the yolk types, particularly the amount of cholesterol, fatty acids and phospholipids contents (Bair and Marion, 1978; Surai et al., 1999), were thought to potentially afford different levels of sperm protection against damage during freezing and thawing (Trimeche et al., 1997; Bathgate et al., 2006). Therefore, in last few years numerous workers reported that using the eggs from other avian species resulted in significantly higher motilities and longevities of frozen- thawed stallion, buffalo bull, boar, goat and Poitou donkey sperm (Trimeche et al., 1997; 1998; Clulow et al., 2004; Eiman et al., 2004; Bathgate et al., 2006; Humes and Webb, 2006; Andrabi et al., 2008; Kulaksiz et al., 2010). The beneficial role of other types of egg yolk can be attributed to the high level of phospholipids, cholesterol, monounsaturated fatty acids (MUFA); phosphotidylinositol; LDLs content and compared to hen EY (Turk and Barnett 1971; Prasard et al., 1988; Maurice et al., 1994; Surai et al. 1999; Choi et al., 2001; Bathgate et al., 2006). Even within the same species of bird, egg yolk composition can vary, and the breed (Stepinska et al., 1993) and diet (Milinsk et al., 2003) can have notable effects.However, preparation of uniform semen diluents containing EY is quite difficult, because individual EY quality may vary depending on the number of days after lying and the storage conditions. Moreover, semen diluents containing EY as a cryoprotectant may pose hygienic risks and are difficult to standardize, or could contain some deleterious components which are potent to reduce semen motility (Moussa et al., 2002). Therefore, there have been

many attempts to find out which component in egg yolk provides cell protection with the aim to prepare chemically defined extender, a well-defined and pathogen- free substitute of non-animal origin for EY would be preferable. Several authors have observed that the addition of some external cryoprotectant (proteins, phospholipids, sugars) or LDL could increase cell protection during freezing and thawing, because it is play an important role in the membrane structure stabilization and that they reduce damage caused by cold shock or osmotic stress (Cabrita et al., 2001, Watson and Morries, 1987; Moussa et al., 2002). For these reasons, partial or total EY substitution in extender has been tried (bull, Muller- Schlosser et al., 1995; Moussa et al., 2002; turkey, Bakst et al., 1992; rainbow, Cabrita et al., 2001; monkey, Li et al., 2005; ram, Partida et al., 1998; Matsuoka et al., 2006) with different level of success.Bovine serum albumin (BSA) is one of the proteins available for replacement of EY. Different evidence has shown their ability to protect sperm membrane integrity from heat shock during freezing- thawing semen. In addition, bovine serum albumin has been considered as a lipid peroxidation inhibitor (Alvarez et al., 1993) or an emuulisifying substrate (De Leuw et al., 1993), and is known to eliminate free radicals generations by oxidative stress, and therefore to protect the membrane integrity of sperm cells from lipid peroxidation (Lewis et al., 1997; Uysal et al., 2007). Previous works were reported that add human serum albumin in freezing media acts as a powerful antioxidant to prevent oxidative stress- induced damaged (Ericsson et al., 1973; Armastrong et al., 1998; Aitken et al., 1994). Indeed, the antioxidants play an important role in decreasing apoptosis during spermatogenesis, and sperm storage through provide defence mechanisms through prevention,

interception, repair (Sikka et al., 2004), and usually the spermatozoa used the antioxidants that present in seminal plasma as available protection against reactive oxygen species (ROS) effect (Sharma and Agarwal, 1996; Sikka, 2001). However, this antioxidant capacity in sperm cells may be insufficient in preventing lipid peroxidation during the freeze- thawing process. Because, the freezing- thawing process decreased the antioxidant defence capacity of sperm cell, the loss of superoxide dismutase activity and the decrease in glutathione levels with 78% given fresh semen (Lasso et al., 1994). Hence, several researchers tried to insert additional antioxidant to semen diluents (Upreti et al., 1997; Graaf et al., 2007).Reproductive technologies are constantly pushing the physiological limits. Therefore male and female reproductive potential are the subject of constant research. In vitro production procedures permits the production of large number of offspring from living/ or slaughtered animals (Gorden, 1997), and provide an excellent source of low-cost embryos for basic research on development biology and physiology and commercial application of the emerging biotechnology such as nuclear transfer and trasngenesis (Baldassare et al., 2002). In vitro production (IVP) embryos involves three main steps: maturation of primary oocytes from large antral follicles, fertilization of the matured secondary oocytes with fresh/ or frozen- thawed semen and culture of the putative embryos for 6- 8 days until formation of blastocysts that can be transferred to recipients or cryo-preserved for future use. However, the efficiency of IVP embryos is still low and inconsistent, mainly due to the inadequate oocyte maturation, inappropriate timing of fertilization and a large number of spermatozoa with oocytes *in vitro* versus *in vivo,* leads to exposure

of oocytes and embryos to toxic effects associated with excessive generation of reactive oxygen species (ROS) released by spermatozoa (Quinn et al, 1998; Baker et al., 2004). The high levels of ROS endanger sperm motility, viability, and decreased fertilization rate (Kessopoulou et al., 1992; Mammoto et al., 1996). In addition, these products would induce peroxidation of the membrane lipids reducing membrane fluidity and impairing sperm function (Mortimer, 1994). Free oxygen radicals may initiate preoxidate polyunsaturated fatty acids in cell membranes, causing a decrease in the flexibility and fluidity of membrane (Aitken, 1987; 1994 a; b) and cause hardening of the zona pellucid, which may have detrimental effects on embryonic development (Dirnfeld et al., 1999; Gianaroli et al., 1996a), and negatively influence the implantation potential of embryo (Waldenstrom et al., 1993; Gianaroli et al., 1996a; b; Dirnfeld et al., 2003). Hence, several reports emphasized that unnecessary long insemination expose the oocytes and the zygotes to the detrimental effect of ROS released by the spermatozoa. Therefore, it is possible to reduced co-incubation time of gamete in IVF protocol that established by Cheng, (1985).Therefore, the aims of the present thesis was conducted to improve defined semen diluents to reduce the damage resulting from freezing and thawing process by establishes new semi- defined composition of sheep semen quality, via using different concentration of bovine serum albumin (BSA) in sheep diluents lacking egg yolk, and uses it successfully in production of embryos in vitro. Moreover, try to investigate the effects of different sperm- oocytes co-incubation times, and the effect of different concentration of BSA with different co-incubation times on cleavage rates,

blastocysts formation, and embryos quality before and after freezing. While, the last goal was evaluate and compare of substitution of hen EY with other egg yolk species on cryodiluent and freezability, post-thaw sperm quality and production embryos *in vitro*.

CHAPTER II
LITERATURE REVIEW

2.1 Mechanism of in Vivo Maturation and Fertilization

In mammals, the ovary is the female gonad responsible for the differentiation and release of mature oocytes for fertilization, and successful propagation of the species. The ovary is an endocrine organ that produces steroids to allow the development of female secondary characteristics and support pregnancy. The outermost layer covering the ovary consists of germinal epithelium (Fig. 1). Directly underneath the germinal epithelium there is a layer of dense connective tissue known as the tunica albuginea. The ovarian follicles, in conjunction with surrounding fibroblast, collagen and elastic fibers, form the ovarian cortex located under tunica albuginea. The ovarian medulla contains the blood vessels, lymphatic vessels and nerves. The formation of a functional ovary depends on three major events taking place during early stages of gonadogenesis: the initiation of meiosis, the formation of follicles, and the differentiation of steroid producing cells.

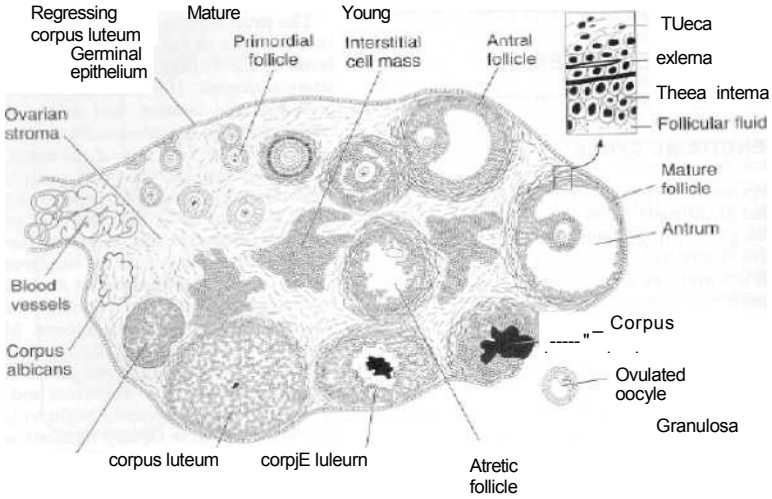

Fig. 1. Diagram of mammalian ovary.

An ovarian follicle is a highly complex unit consisting of distinct cell types. The ovarian follicle is comprised of several layers of somatic cells surrounding a fluid-filled cavity called the antrum in which the oocytes are surrounded by somatic cells. The fluid found in the antrum is known as follicular fluid. The follicles provide a microenvironment for oocyte growth and are responsible for the production of hormones (Gorden, 1994). The oocyte and its companion somatic cells comprising the follicular unit maintain close association throughout development from primordial to preovulatory stages. The walls of mature preovulatory follicles consist of granulosa cells, theca interna and theca externa. Granulosa cells are cells of epithelial origin essential for growth and survival of the oocyte. Granulose cells are not a homogeneous tissue but rather specialized subpopulations consisting of the corona radiata, cumulus cells, mural and antral granulosa cells. The cumulus cells are the subpopulation of ovarian granulosa cells that surrounds the oocyte. The cumulus cells in contact with the oocyte are known corona radiata. They are in close contact with the oocyte through cytoplasmic extension across the zona pellucida (ZP). The corona radiata allows the oocyte

to communicate with other cumulus cells through the gap junctions thus forming an electrophysical syncytium (De Loos et al, 1991). The cumulus cells nourish the oocyte (Buccione et al., 1990), and participate in the formation of the ZP. In addition, cumulus cells have also been implicated in the modulation or generation of oocyte maturation inhibitors (Tsafriri et al., 1982; Eppig and Downs, 1984).

The granulosa cells closest to the basement membrane are known as mural granulosa cells, while those closest to the follicular are known as antral granulosa cells. The follicular antrum contains the follicular fluid which is made of blood exudates modified by local secretions and metabolism (Gordon and Lu, 1990). The subpopulations of granulosa cells differ in their distribution of receptors and steroidogenie characteristics (Rouillier et al., 1998). The theca interna and theca externa are stromal or fibroblastic cells that constitute the outermost coat of the preovulatory follicle. An extensive capillary network irrigates theca cells. The theca interna is the major source of androgens during the final stage of development of the Graafian follicle (Moor, 1977). Follicles can be classified as primordial follicles, preantral follicles (primary and secondary follicles), antral and preovulatory follicles.

2.1.1 Oocyte maturation

Unlike spermatozoa, which are generated continuously from puberty onwards, there is finite population of oocytes. The total number of oocytes present in the adult ovary originates from a definite number of primordial germ cells (PGCs) that are formed in the yolk sac epithelium of the embryo. These cells reach the primitive ovary after migration through the gut mesentery and the gonadal ridges of mesonephros of the early embryo

(Byskov and Hoyer, 1994). Once PGCs have reached the developing ovary the cells begin to differentiate into oogonia. The population of oogonia proliferates until shortly before birth at which time the oogonia enter meiosis and are termed primary oocytes (Gosden and Bownes, 1995). The process of meiosis will halve the number of chromosomes resulting in the creation of haploid oocytes that are ready for fertilization by sperm carrying the other half of genome. However, primary oocytes progress only through part of meiosis and arrest at the dictyate stage (Picton, 2001). When an oocyte enters meiosis a single layer of flattened pre-granulosa cells enclose it, thus forming the primordial follicle. This is the first step of folliculogenesis: the process that leads to the formation and growth of the ovarian follicle. The follicle structure is completed by the appearance of several layers of stromal cells that will differentiate into the theca layers after follicle growth commences (Gougeon, 1996).

In vivo, follicular and oocyte growth has two phases. In the first phase growth of oocytes and follicle are highly correlated, while during the second phase of growth the follicles continues to grow while the oocyte remains arrested until it reaches full adult size. During the second growth phase, oocytes depend on somatic cell (granulosa)-oocyte interactions. Once established, the follicular unit helps to maintain the oocyte in a controlled environment and isolates the cell from any potentially harmful substances circulating in bloodstream. Granulosa (cumulus) cells provide nucleotides, amino acids, phospholipids, substrates for energy utilization, and maintain the ionic balance of the oocyte. Cumulus cells also protect oocytes against oxidative stress- induced apoptosis (Tatemoto et al, 2000), and granulosa

cells also act as paracrine centers for the oocytes and follicles by producing estrogen and other proliferative factors.

Oocyes that do not become incorporated into primordial follicles will degenerate. The majority of primary oocytes are not incorporated into primordial follicles resulting in a loss of up to 90% in cows (Erickson, 1966), more than 90% in ewes, and 60% in sows (Guthrie and Garrett, 2001) of the original oocyte pool at the time of birth. The number of oocytes that reach ovulation is obviously limited to the number of offspring that each species can bring to term in the uterus. From the several thousand primary oocytes available at birth, the number of oocytes that will be fertilized and develop to term following natural mating or artificial insemination (AI) is reduced to only a few. Primordial follicles constitute the store of germ cells in the postnatal ovary. The number of primordial follicles varies between species, and with the age of the animal. Picton, (2001) demonstrated that the number ranges from approximately 135,000 in cows, 82,000 in ewes, and 500,000 in pigs. As soon as the primordial follicle store is established, follicle recruitment begins and continues without interruption until the animal is slaughtered or until the ovary is depleted. Cohorts of follicles are selected for growth, and follicular growth occurs in a wave- like pattern during oestrus cycles. Follicular growth is continuous and in most cases ends with degeneration (atresia) of the follicle and its oocyte. Once follicles form an antrum and reach a certain diameter (2-4 mm in sheep, and 4-6 mm in cattle) the growth of the follicle becomes gonadotrophin dependent (Draincourt, 2001). When a follicular wave is exposed to adequate hormonal milieu, selection takes place and the number of growing follicles in a wave is reduced to a predefined species-specific number that will ovulate. Evans, (2003),

observed that the wave of follicular development occurs before puberty and during pregnancy, but in these circumstances ovulation does not occur because hormonal levels are not appropriate.

However, oocytes are not all equal. In sheep, follicles with a large diameter have been shown to contain oocytes with higher developmental potential (Lonergan et al. 2003). Oocyte diameter is directly proportional to follicle diameter, and as both increase the developmental capability of oocytes improves. This indicates that oocyte competence is acquired within the ovary during the developmental stage that precedes ovulation through a process referred to as oocyte capacitation (Hyttel et al., 1997). Though the precise mechanisms are unclear, it can be hypothesised that during capacitation oocytes become equipped for future embryonic development, and during maturation an appropriate signal must be provided in order to trigger the developmental programme acquired during capacitation (Moor and Gandolifi, 1987).

2.1.2 Oocyte fertilization

The meeting of spermatozoon with the oocyte is a complex process that starts at the time of insemination with deposition of millions of spermatozoa into the female reproductive tract. Thousands of spermatozoa pass through the uterotubal junction into the oviductal isthmus, where they form a reservoir by binding to fucosylated molecules on the oviductal epithelium (Hunter and Wilmut, 1984; Lefebvre et al., 1997). The oviduct is the site of fertilization and early embryo development, the oviductal environment can support embryonic growth up to the blastocyst stage across a wide range of species, while the culture system is the major determinant of blasyocyst quality,

irrespective of the origin of the oocyte. The culture environment can also have a significant effect on embryo metabolism which may have implications for embryo quality (Wrenzycki et al., 1999; Rizos et al., 2002). In 1991 Drobnis and Katz observed by using videomicroscopy spermatozoa that had undergone the acrosomal reaction on the ZP were able to work through their acrosomal shroud and penetrate into the oocyte while those recovered from female reproductive tracts prior to reaching the oocyte had not undergone the acrosome reaction (Suarez et al., 1983; Kopf and Gerton, 1991).

In order for fertilization to occur, the spermatozoa must first undergo capacitation in the female reproductive tract, in which the protective plasma coating covering the spermatozoa surface molecules is removed to allow sperm to be able to bind to the oocyte. This adhesion is mediated by fucose-binding sites on the sperm surface, which are exposed after removal of seminal plasma. Then spermatozoa adhere to the oocyte's ZP to induce the acrosome reaction, in which the fusion of the spermatozoal plasma membrane and the outer acrosomal membrane occurs to allow the acrosomal enzymes to be released. Fertilization occurs only when a spermatozoon penetrates the oocyte, and only after these two critical events are completed can fusion of the two gametes occur.

In most mammals, include sheep, the fully mature oocyte released from ovary at ovulation is surrounded by both the cumulus oophorous and the ZP, through which the spermatozoon must pass before it can bind to and fuse with oolemma (Yanagimachi and Cheng, 1994). The cumulus oophorous is defined as a group of closely associated granulosa cells, called cumulus cells, which surrounds the oocyte in the antral ovarian follicle. Approximately 3,000 cumulus cells surrounding the oocyte are embedded in a thick extra cellular

matrix (Lin et al, 1994) which presents an unavoidable obstacle for spermatozoa. One of the significant components comprising the extracellular matrix is a specific disaccharide, hyaluronic acid (HA). Spermatozoa possess an enzyme called hyaluronidase which breaks down HA found throughout the extracellular matrix surrounding the oocyte, thereby creating a pathway so that spermatozoa can travel towards and adhere to the ZP.

In response to the preovulatory lutenizing hormone (LH) surge, the oocyte resumes meiosis and the cumulus cells synthesize a large amount of HA which is then organized between the cells to form a nucoelastic matrix. This matrix is deposited into the intracellular space, and stabilized by accessory proteins in a process called cumulus expansion (Ball et al., 1982; mammals; Mattioli, 1994; Zhuo and Kimata, 2001). This matrix facilitates the extrusion of the oocyte at ovulation (Chen et al., 1993), its capture by the ciliated epithelial cells of the infundibulum (oocyte pick up) and its transport to the fertilization site (Mahi-Brown and Yanagimachi, 1983). The granules and filaments in the extra cellular matrix are responsible for adhesion between the oocyte and cilia of the oviduct (Lam et al., 2000).

The fully developed cumulus oophorous exerts three important biological functions: before ovulation, the cumulus oophorus supports oocyte maturation (Wassarman, 1988), during ovulation, it conducts the oocyte into the oviduct (Mahi-Brown and Yanagimachi, 1983), and shortly after ovulation it participates in the complex mechanisms controlling the access of spermatozoa to the oocyte (Tesarik, 1990) (Fig. 2).

The roles of the cumulus oophorus during mammalian fertilization still need to be clarified. In most mammals the cumulus oophours is still present at the time of fertilization in the oviduct (Yanagimachi and Cheng, 1994), and

disappears between 3-6 h after ovulation. It is therefore probably not present it at the time of fertilization (ovine; Dziuk, 1965; bovine; Lorton and First, 1979). Also, the cumulus oophorous could exert a beneficial effect on fertilization by selecting morphologically normal spermatozoa (human; Carrell et al., 1993) by retaining non-capacitated and acrosome-reacted spermatozoa (hamster; Cherr et al., 1986; Cummins and Yanagimachi, 1986), and by guiding hyperactivated spermatozoa to the oocyte surface (mammals; Katz et al., 1989; Saling, 1989; Cox et al., 1993). Tanghe, (2005) proposed the following beneficial effects of the cumulus oophorus on fertilization:

a) Facilitating fertilization, by attracting, trapping and/ or selecting spermatozoa.
b) Facilitating sperm capacitation, acrosome reaction and/ or penetration.
c) Preventing precocious hardening of the ZP.

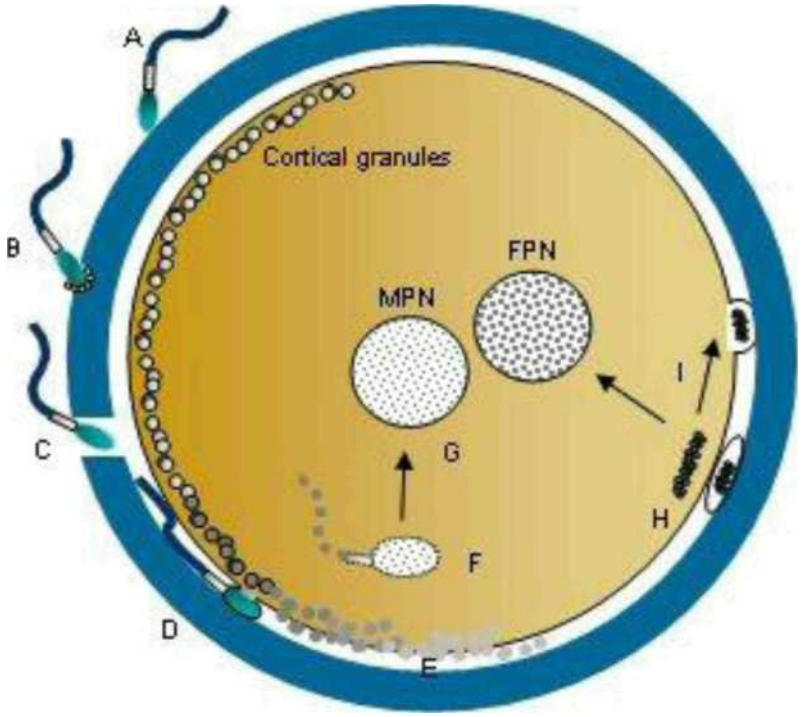

Fig.2. A schematic mechanism of fertilization

(A) Acrosome intact, capacitated sperm bind to the zona pellucida, thereby
(B) Triggering the acrosome reaction. The enzymatic acrosomal contents lyse the ZP
(C) Thus enabling the now hypermotile sperm cell to enter the perivitelline space, fuse with the oolemma and
(D) Activate the oocyte. Upon activation, release of cortical granule content takes place
(E) Which prevents the penetration of supplementary sperm. The sperm head, now incorporated within the ooplasm, begins to swell and decondense.
(F) Eventually forming the male pronucleus
(G) The oocyte, until now arrested in metaphase II
(H) Commences progression through meiosis II to extrude the second polar body (I) and form the female pronucleus (I). The male and female pronuclei are now in opposition, as a final prelude to syngamy.

The cumulus oophorus could increase the number of fertilizing spermatozoa around the oocyte by attracting and trapping spermatozoa. Sperm

may be attracted to the cumulus oophorus by a kind of chemotaxis which stimulates sperm transport in the oviduct (Ito et al., 1991; Eisenbach, 1999).

Around the time of ovulation, spermatozoa become capacitated, are released from the reservoir by removal or modification of the fucose-binding site and swim up to the ampulla's. At the same time, mannose-binding sites on the sperm surface are revealed or activated (Revah et al., 2000). Capacitation of spermatozoa by biochemical changes, such as removal of surface molecules to allow sperm to bind to the oocyte, changes in membrane lipid composition and internal pH, increases in membrane permeability and plasma membrane fluidity and metabolism, all render spermatozoa capable of fertilizing the oocyte. Sperm acquire many of these capabilities as they pass through the epididymis. In order to fertilize the oocyte, capacitated spermatozoa need to find a passage through the cumulus extra cellular matrix. They do this by disrupting the macromolecular structure of the matrix (Yudin et al., 1988) and by displaying the hyaluronidase activity of the protein, PH-20, which is present on the plasma membrane of mammalian sperm (human; Sabeur et al., 1998; macaque; Cherr et al., 2001). During capacitation, PH-20 migrates from the tail to the acrosomal domain and undergoes endoproteolytic cleavage to optimize hyaluronidase activity (Seaton et al., 2000). A glycoprotein, PH-20, found on the head of mammalian spermatozoa is homologous to that of hyaluronidase (Lathrop et al., 1990). Lin et al., (1994) also demonstrated that PH-20 has hyaluronidase activity and spermatozoa without the PH-20 on their membrane cannot traverse the cumulus cells. In addition, Deng et al., (1999) demonstrated that the activation of PH-20 occurs during spermatozoan transport and is regulated by deglycosylation. After the capacitation of spermatozoa in the female reproductive tract, the spermatozoa adhere to the

oocyte's ZP to induce the aero some reaction, in which the fusion of the spermatozoal plasma membrane and the outer acrosomal membrane occurs to allow the acrosomal enzymes to be released.

Capacitated spermatozoa lose their ability to bind to oviductal epithelium, are released from the isthmus and begin to display a specific movement which is characterized by vigorous beatings of the tail with the sperm head tracing an erratic figure eight. This non-progressive movement is interspersed by brief liner movements. Both types of movement are referred to as hyper activation and provide spermatozoa with strong thrusting power. Hyper activated motility seems to be beneficial, not only for the migration of spermatozoa from the oviductal ishmus to the oviductal ampulla where fertilization takes place, but also for sperm penetration through the oocyte investments. Although enormous numbers of spermatozoa are released in the female reproductive tract, only one will successfully fertilize the oocyte (Yangimachi and Chang, 1994; Revah et al, 2000; Tanghe, 2005).

Bedford and Kim, (1993) demonstrated in an *in vivo* study that the rat cumulus oophorus traps and brings the few spermatozoa reaching the ampulla's to the immediate vicinity of the oocyte. Sperm trapping may be caused by entanglement in the HA matrix between the cumulus cells. Furthermore, they suggested that the cumulus oophorous prevents polyspermy in rats. It has also been shown that very few spermatozoa are present near the oocyte during fertilization in the rabbit (Overstreet et al., 1978) and that an intact cumulus oophorus does not protect against polyspermy (Hunter and Leglise, 1971a; b). Moore and Bedford (1978) observed that the state of the oocyte itself is probably a critical factor in establishing an effective block against polyspermy.

2.2 Cryopreservation and storage of semen

Sperm cryopreservation contributed to the expansion of reproductive techniques, such as artificial insemination (AI) and in vitro fertilization (IVF). This could be achieved by methods that reduced or arrested the metabolisum of spermatozoa and thereby prolonged their fertile life. Previous works were reported storage of smen in 1) liquid (unfrozen) state, using reduced temperature or other mean to depress sperm metabolisim; and 2) in frozen state which involved storage at sub-zero temperatures

2.2.1 Diluents types

Originally, semen was diluted to protect spermatozoa during cooling, freezing and thawing. Several diluents have been used for preservation of mammalian semen, and generally should have adequate pH and buffereing capacity, suitable osmolality, and should protect spermatozoa from cryodamage injury (Salamon and Maxwell, 2000), can be grouped according to their chronological use or development

- *Citrate- sugar based diluents:*

Opinions of researchers differed on the *"suitable "* type of sugar to be included in citrate medium. Due to decrease in osmotic pressure caused by the glycerol in the extender. The invistigators have chosen arabinose, fructose or glucose, as components of the citate diluent. Ram spermatozoa can tolerate twice the normal glucose or fructose concentration because their capacity to permeate spermatozoa and equalise the osmotic gradient (Salamon and Maxwell, 2000). The citrate- sugar-based diluents were seldom used for freezing ram semen after the late 1960s.

- *Milk diluents:*

Skimmed milk protection is based in the protein fraction, namely caseins (Medeiros et al, 2002). Milk has been adapted for freezing mammalian semen, mostly in reconstituted form combined with arabinose, fructose or egg yolk. However, addition of egg yolk to heated homogenized milk did not increase post-thaw sperm survival (Salamon and Maxwell, 2000). In some laboratory studies, a milk preparation has been included in synthetic extenders containing sugars and electrolytes and it have been used in practice to the dilution and freezing of semen. Skim milk has been used as both portions of the two- step dilution, and freezing of semen for practical use in Sweden (Soderquist et al., 1996).

- *Lactose- based diluents:*

Successful use of lactose, as the main component of diluents for freezing bull semen, stimulated its application for other mammalian semen. Lactose-yolk was used for both the nonglycerolised and glycerolated diluent's portions, or only for the non- glycerolated portion followed by glycerolated INRA medium (Salamon and Maxwell, 2000).

- *Saccharose based diluents:*

Saccharose has been used as the principal component of synthetic extenders because it protects the acrosome integrity of sperm better than glucose, fructose or lactose (Milovanov and Sokolovskaja, 1980). Synthetic antioxidants have been used in saccharose extenders to inhibit peroxydation of sperm phospholipids particularly unsaturated fatty acids. Lipid peroxydation

is also detected in thawed semen. Synthetic tocopherol (Vitamin E) has been used as antioxidant in freezing extenders (Salamon and Maxwell, 2000).

- *Raffinose based diluents:*

In 1964, Nagase and Graham first mentioned to use raffinose as a diluents compenent with bull semen that sugar of high molecular weight provides better protection to spermatozoa during fast freezing than those of low molecular weight. In ram and bull freezing extenders some authors have detected better cryoprotective effect with trisaccharides than with mono or disaccharides in stabilizing protein- lipid complex of the sperm membrane (Salamon and Maxwell 2000).

- *Tris based diluents:*

In 1970 Tris diluents was reported for freezing ram semen by several workers. Moreover, Tris extenders are frequently used for semen cryopreservation of bulls, and bucks (Purdy, 2006). Ram spermatozoa tolerate Tris concentrations from 250 to 400 mM, and glucose is a better sugar component in Tris medium than fructose, lactose or raffinose (Salamon and Maxwell, 2000). Fischer et al., (1987) found that Tris with an osmolality of 375 mOsm/ kg containing 2% egg yolk was the best in preserving acrosomal integrity and motility after thawing. Semen frozen in Triladyl® (a Tris based diluent) gave rise to reasonable fertility after sheep transcervical insemination. This extender gave better in vitro results than lactose- yolk and saccharose-lactose- yolk, and the addition of 2% bovine serum albumin improved its protective effect on acrosome integrity. Tris based extenders used for semen freezing (buck and ram) normally contains fructose or lactose, lowering their normal concentrations compared to other extenders (Purdy, 2006).

- *Other diluents:*

Zwitterion buffers which are synthetic, such as Tes, hepes, and pipes have been used with varying success as the basis of diluents for freezing ram semen. Fertility of semen extended in Test medium (Tes titrated with Tris) varied from poor to good results (13- 67%). Fertility was lower in semen frozen in zwitterions buffers than in Tris- glucose- yolk (Salamon and Maxwell, 2000).

2.2.2 Cryoprotective agents

Many compounds have been tested for their efficacy as sperm cryoprotectants but most semen preservation protocols still favour egg yolk and glycerol in the cryoprotective media, following the example set by (Philips, 1939; Polge et al, 1949). Originally, the aim to add the cryoprotectants in diluents media to reduce the physical and chemical stresses derived from cooling, freezing and thawing of sperm cells (Gao et al., 1997; Purdy, 2006).

Cryoprotectants are classified as either penetrating/ or none penetrating.Penetrating cryoprotectants (glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol) cause membrane lipid and protein rearrangement, resulting in increased membrane fluidity, greater dehydration at lower temperatures, reduced intracellular ice formation, and increased survival to cryopreservation (Holt, 2000). Additionally, penetrating cryoprotectants are

solvents that dissolve sugars and salts in the cryopreservation medium (Purdy, 2006).

Glycerol is frequently used as a cryoprotectant for freezing ram semen. Glycerol or dimethyl sulfoxide can induce osmotic stress and toxic effects to spermatozoa, but the extent of the damage varies according to the species and depends on its concentration in the extender solution (Purdy, 2006).

Several experiments were done to calculate the optimal level of glycerol in semen extenders, combining cooling/ freezing rates, extender composition and type of glycerol addition. In semen frozen by the slow conventional method and using mainly hypertonic extenders, glycerol is frequently used within the range of 6- 8%. Upper levels cause's damages to sperm cells lowering post thawing survival of spermatozoa. Best results were obtained with 4- 6% glycerol and a freezing rate of 10- 100°C/ min (Byrne et al. 2000; Anel et al, 2003). In the formulation of semen extenders, glycerol may be joined initially or later in a separated fraction (glycerolated fraction), after semen refrigeration. In the first situation, the complete extender is joined after semen collection (one step method); in the second situation a fraction of the extender (without glycerol) is joined after semen collection, and the remaining portion (with glycerol) is joined after refrigeration and before semen freezing (two step method) (Evans and Maxwell, 1987). This point is controversial, and some authors don't find glycerol variations effects associated with the moment of glycerol addition. By example some workers (Evans and Maxwell, 1987) have published that one step addition of glycerol at 30°C is a practical and widely used method for ram semen freezing, however French workers (Colas and Brice, 1975) recommended the two step method, for instance joining glycerol at 5°C, in two or three times. Effective

cryoprotection after short (5- 10s) contact with glycerol, has been demonstrated for bull, boar and ram semen (0- 5 min), which proves that penetration of glycerol into the cell is not essential for sperm protection, being this a controversial subject.

Removal of glycerol from thawed semen by centrifugation or by dialysis had no effect on lambing rate. Some authors have been succeeded to freeze ram semen without glycerol, extending semen at 5°C, 3 h after collection, with hypertonic test buffer containing 25- 30% egg yolk and 10% maltose monohydrate but these experiments must be repeated. Others cryoprotectants may be used in semen extenders, namely dimethysulfoxide (DMSO), ethylenoglycol, albumin, high concentrations of sugars of various types, compatible solutes (proline, glycine, betaine, taurine) and antifreeze proteins from polar fish, but best results were always attained with glycerol (Salamon and Maxwell, 2000).

A non penetrating cryoprotectant (egg yolk, non fat skimmed milk, trehalose, aminoacids, dextrans, sucrose) doesn't cross plasma membrane and only acts extracellularly (Aisen et al., 2000). Therefore, non penetrating cryoprotectant may alter the plasma membrane, or act as a solute, lowering the freezing temperature of the medium and decreasing the extracellular ice formation (Amman, 1999; Kundu et al., 2002).

Egg yolk is a normal component of semen extenders, protecting the sperm cell against cold shock and the cell membrane during freezing and thawing. The protective mechanisms are determined by the phospholipids (lecithin) and low density lipoproteins (Medeiros et al., 2002; Purdy, 2006). Egg yolk acts on the cell membrane, having a greater effect in bull than ram spermatozoa. For freezing ram semen in ampoules, egg yolk is joined at 3-

6%, but for straws and pellet freezing greater concentrations are used (15-17%), although the effect are dependent on extender composition (Salamon and Maxwell, 2000).

Previous works were examined egg yolk concentrations as low as 1.5-3.75 %, but that low levels were unsatisfactory (Graham et al., 1978). On another hand, it seems that increased concentrations of egg yolk in the extender may reduce the glycerol levels (Abdelhakeam et al., 1991). However, egg yolk, although sometimes partially replaced by substance(s) with similar activity, is still likely to remain an important component of diluents for freezing semen, particularly due to its protective effect on the plasma membrane.

2.2.3 Processing and semen cryopreservation

There are two methods can be used for gamete cryopreservation: slow freezing and vitrification. Slow freezing uses low concentrations of cryoprotectants which are associated with chemical toxicity and osmotic shock. Vitrification is a rapid method that decreases cold shock, does not need expensive freezing equipment and the method (vitrification/ warming) only takes a few seconds (Isachenko, 2003). Classical vitrification requires a high proportion of permeable cryoprotectants in the medium (30- 50% compared with 5- 7% for slow freezing) and seems to be inadequate for sperm cells, due to lethal osmotic effects and possible chemical alterations.

The cryopreservation method includes temperature reduction, cellular dehydration, freezing and thawing (Medeiros et al., 2002). The lowering from normal temperature to 4 °C reduces cellular metabolic activity and increases the life span of sperm cells. Cryopreservation stops cells cellular activity,

restarting its normal functions after thawing (Mazur, 1984). The freezing rate must be slow enough to allow water to leave the cells by osmosis, preventing intracellular ice formation which causes irreversible damages to sperm cells (Fisher and Fairfull, 1986).

During semen refrigeration with slow cooling rates (0.5- l°C/min), temperature reduction induces stresses on membranes, probably ascribed to the phase changes in lipids bilayer and altered functional state of membranes (Watson, 2000). The stresses caused by ice crystal formation are associated with the osmotic pressure changes in the unfrozen solution (Medeiros et al., 2002). Membrane permeability is increased after cooling and this may be a consequence of increased membrane leakiness and specific protein channels. Calcium regulation is affected by cooling and this has severe consequences in cell function, inclusively cell death. The uptake of calcium during cooling influences capacitation changes and fusion events between plasma membrane and acrosomal membrane. Sperm membrane is a structure that undergoes reorganization during capacitation. Cold shock reduces membrane permeability to water and solutes and injures acrosomal membranes (Purdy, 2006). While, the fast cooling between 30 and 0°C causes cell injuries in domestic species sperm cells called *"cold chock"*\ that is dependent on the cooling rate and temperature interval (Gilmore et al., 1998; Watson, 2000).

In frozen-thawed semen, motility of sperm cells is better preserved than its morphological integrity. Plasma and outer acrosome membranes are the most cryosensitive. Biochemical changes have also been detected, including the release of glutamic-oxaloacetic transaminase (GOT), losses of lipoproteins and amino acids, decrease in phosphatase activity, decrease in loosely bound cholesterol protein, increase in sodium and decrease in potassium content,

inactivation of hyaluronidase and acrosin enzyme, loss of prostaglandins, reduction of ATP and ADP synthesis and decrease in acrosomal proteolytic activity (Salamon and Maxwell, 1995b).

The cryopreservation protocol causes several damages to sperm by the influence of several factors, namely the dramatic changes in temperatures, submission to osmotic and toxic stresses derived from exposure to molar concentrations of cryoprotectants and finally the formation and dissolution of ice in the intracellular and extracellular environment (Medeiros et al, 2002). The main changes that occurs during freezing are mainly ultrastructural, biochemical and functional, which impairs sperm transport and survival in the female reproductive tract and reduces fertility in domestic species (Salamon and Maxwell, 2000). The ultra structural damage is greater in ram than bull spermatozoa. Greater damages have been detected in plasma and acrosome membranes, mitochondrial sheath and axoneme (Salamon and Maxwell, 2000).

The damaging effects of cooling and freezing upon sperm membrane varies among domestic species and is influenced by several elements namely cholesterol/ phospholipids ratio, content of lipids in the bilayer, degree of hydrocarbon chain saturation and protein/ phospholipid ratio (Medeiros et al., 2002). In 1997 Parks divided the mammalians depends on theri sensitive to cryodamage effects. The rabbit, human, and rooster are *"less sensitive";* dog and cat are *"somewhat sensitive";* **bull, ram and stallion are** *"very sensitive ";* while, boar sperm is the *"most sensitive";* to cold shock. The male animals can be classified as "*good freezers"* or " *bad freezers"* depending on the aptitude of their semen to support freezing with little cryodamage. Previous works were obsereved that there are some characteristics of the membrane structure,

which may depend on individual genetic variation, allowing better resistance of sperm cells to cryopreservation (Shamsuddin and Larsson 1993; Watson 2000). Indeed, Tada et al, (1993) demonstrated that the cryosensitive damage can be regulated by genetic factors, suggested by the fact that some mouse strains have better post thaw motility and in vitro fertility when compared to others.

2.3 In Vitro Production Embryo (IVP)

In vitro production (IVP) embryos have become routine and is increasingly available as a commercial service to meat, wool, and dairy producers. Production embryos in small ruminants provides an excellent source of low- cost embryos for basic research in developmental biology and physiology and for commercial application of emerging biotechnologies such as nuclear transfer and transgenesis (Baldassare et al., 2002). However, the efficiency of producing viable embryos and development of such embryos after transfer to recipients is perceived to be inferior to that which occurs in vivo. Production embryos systems are specially designed to supply the needs of each developmental stage, and involves three main steps:

- Maturation of primary oocytes from large antral follicles,
- Fertilization of the matured oocytes with fresh/ or frozen-thawed semen.
- Culture of putative embryos until the formation of blastocysts that can be transferred to recipients/ or cryopreserved for future use.

2.3.1 In vitro maturation (IVM)

Maturation oocyte in vitro is designed to closely resemble that which occurs in vivo, by increasing the chance of oocyte maturation occurring without the presence of the follicle. The germinal vesicle breakdown (GVB) and resumption of meiosis were first recognized in vitro by Edwards (1965), who observed that the maturation in vitro after lutinizing hormone (LH) supplementation was similar to that in vivo and hypothesized that the observable blocks to meiosis and maturation were due to inadequacies of the IVM medium.

Follicular oocytes arrested in the primordial oocyte phase are surrounded by the primordial follicle, a single layer of granulosa cells, and represent a prolonged diplotene stage of the first meiotic division also known as the germinal vesicle stage (GV). The immature oocyte will resume meiosis and complete its final maturation after being liberated from its follicular environment in vitro (Pincus and Enzmann, 1935), and usually characterized by condensation of the chromatin, breakdown of the germinal vesicle (BGV) and organization of the chromosomes into the metaphase I (MI) plate and spindle. At this stage the oocyte enters into meiosis II, and upon extrusion of the first polar body (PB), remains arrested in the metaphase II (Mil) stage until activation of the oocyte takes place at fertilization. Parallel with nuclear maturation, cytoplasmic maturation/ capacitation of the oocyte progresses and is characterized by migration of the cortical granules to the periphery of the ooplasm, migration of the mitochondria to the inner cytoplasm, and increased synthesis of glutathione (GSH).

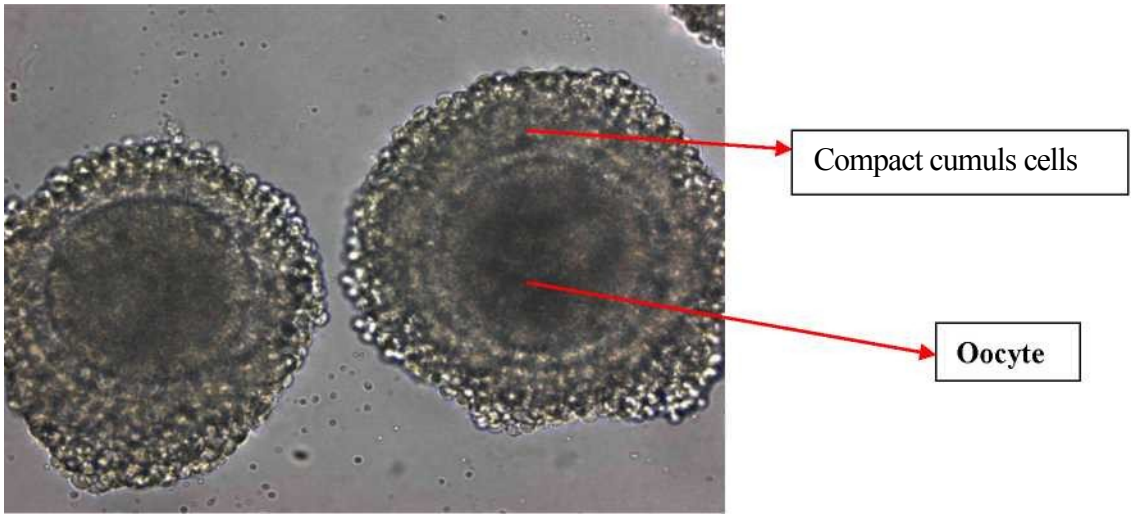

Fig.3 Ovine cumulus-oocyte complex (COC) with layers of cumulus cells before IVM.

Oocyte maturation is a complex phenomenon during which the oocyte progresses from the diplotene to the metaphase II stage (nuclear maturation). Maturation of oocytes correlates with follicular maturation. Prmordial oocytes are surrounded by the primordial follicle, a single layer of granulosa cells. Follicular and oocyte growth have two phases: in the first phase, growth of oocytes and follicle are highly correlated, while in the second phase, the follicle continues to grow but the oocyte remains arrested until it reaches full adult size. Through the second growth phase oocytes depend on granulosa cell-oocyte interactions. The granulosa (cumulus) cells provide nucleotides, amino acids, phospholipids, substrates for energy utilization, and maintain the ionic balance of the oocyte. Cumulus expansion is a result of synthesis and accumulation of extracellular matrix component such as hyaluronan (Kimura et al., 2002). The oocytes secrete cumulus- expansion enabling factor that help regulate cumulus expansion via FSH or EGF which is mediated by cyclic adenosine monophosphate (cAMP) (Coskun et al., 1995). In addition, cumulus cells also protect oocytes against oxidative stress-induced apoptosis

(Tatemoto et al., 2000), and granulosa cells also act as paracrine centers for the oocytes and follicles by producing estrogen and other proliferative factors. As the follicle and oocyte mature, the number of binding sites for FSH and the binding capacity of FSH to the granulosa cells significantly increase (La Barbera and Ryan, 1981). Additionally, the oocytes secretes steroidogenic inhibitory factors that reduce the effect of gonadotrophins on the oocyte eliminating the possibility of premature resumption of meiosis and separation between the granulosa cells and the oocytes prior to the LH surge (Coskun et al., 1995).

"Nuclear maturation" refers to the progression of the oocyte nucleus from the germinal vesicle to the metaphase II stage. Nuclear maturation involves GVBD, condensation of chromosomes, organization of the chromosomes into the metaphase I plate and spindle formation, and separation of the homologous chromosomes (at which stage the oocyte enters into meiosis II). Upon extrusion of the first polar PB, the oocyte will remain arrested at metaphase II of meiosis II until the sperm-induced resumption of meiosis occurs, producing a haploid chromosomal complement from the previous diploid state (Kubelka et al., 1988). The nuclear membrane starts to fold, the nuclear pores disappear and then the nuclear membrane undergoes fragmentation and rapidly disappears (Szollosi et al., 1972). Twenty-four hours are necessary for the oocyte to complete nuclear maturation (Sirard et al., 1989). In cattle; Hyttel et al., (19866) observed that nuclear maturation follows the same pattern in vivo and in vitro. Nuclear maturation can be evaluated by simple staining methods, such as hoechst and aceto-orcein or 4', 6'-diamidino-2-phenylindole dihydrochloride (DAPI) and examined under fluorescent microscopy (Liu et al., 2002; Chohan and Hunter, 2003). In 1998

Yang and co-workers observed that the completion of nuclear maturation alone does not guarantee subsequent embryo development.

[LL] *Cytoplasmic maturation"* can be described as processes modifying the oocyte cytoplasm that are essential for fertilization and preimplantation embryonic developmental competence. Pivotal to the concept of cytoplasmic maturation are the production and presence of specific factors, the relocation of cytoplasmic organelles, and the post-transcriptional modification of mRNAs that have been accumulated during oogenesis plus the ultrastructural changes that take place in the oocyte from the GV to the Mil stage in the nucleus (Calarco, 1995; Duranthon and Renard, 2001). The efficiency of cytoplasmic maturation includes also the ability of the oocyte to block the penetration of more than one sperm (Ducibella, 1998), and also to support the decondensation of the sperm head within the ooplasm of the fertilized oocyte. Cytoplasmic maturation is indirectly and retroactively assessed as the ability of the mature oocyte to undergo normal fertilization, cleavage and blastocyst development. Other indirect morphological parameters taken into account to evaluate cytoplasmic maturation include cumulus cell expansion, expulsion of the PB and an increased perivitelline space (Kruip et al., 1983). In addition, cytoplasmic maturation can be determined by indirect means such as the blastocyst yield and cell number, or the ability of oocytes to decondense sperm nuclei after sperm penetration, glutathione content of the oocyte and the percentage of male pronucleus (MPN) formation (Liu et al., 2002).

All these processes (nuclear and cytoplasmic maturation, with capacitaion of the oocyte) are believed to progress in parallel. Nuclear and cytoplasmic regulations of oocyte maturation are important, because there are intricate interactions between these two processes that must occur in order to

support the entire oocyte maturation process. Nuclear maturation, cytoplasmic maturation, and capacitation of the oocyte are concluded by migration of cortical granules to the periphery of the ooplasm, migration of mitochondria to the inner cytoplasm, and increased synthesis of glutathione. Synchronization of nuclear and cytoplasmic maturation is essential for establishing optimal oocyte developmental potential.

2.3.2 In vitro fertilization (IVF)

Fertilization is a process that involves the participation of two highly specialized cells, the sperm and oocyte. Several steps have to proceed in a precise sequence for fertilization to be successful.

2.3.2.1 Fertilization in vitro

Fertilizing a mammalian ovum with spermatozoa outside the female and transferring the resulting embryo to recipients is a relatively recent accomplishment. The first successful report of implantation and pregnancy following the use of this technique in farm animals was published during the 1980s. Since then, the techniques have become known as in vitro fertilization (IVF).

Early claims of successful IVF are widely disputed, but it was not until 1951 that there was unequivocal evidence of the need for sperm capacitation in order for fertilization to occur. It was described separately by Austin and Chang, (1951). Capacitation is a physical change that the spermatozoon must undergo before it is capable of penetrating the ovum. In essence, it involves

changes to the structure and physical properties of the membranes of the sperm head, resulting in the acrosome reaction and hyperactive sperm movement, together with the release of chemicals capable of breaking down the gel-like ZP enclosing the ovum contents. It was Chang, (1959) who overcame the difficulty of differentiating fertilization from parthenogenic activation. In these experiments, spermatozoa were taken from males with specific genetic traits not present in the females donating the ova.

During the last century, little success had been achieved in fertilizing ovine oocytes in vitro. Thibault and Dauziert, (1961) reported 4/78 ovulated oocytes fertilized in vitro by spermatozoa recovered from the uterus of a previously inseminated ewe. Kraemer, (1966) also used spermatozoa capacitated in the reproductive tract of a previously inseminated ewe and reported 4/23 oocytes penetrated. Bondioli and Wright, (1980) capacitated ovine spermatozoa in vitro by pre-incubation in media with protein supplements and achieved a 14% fertilization rate. Dahlhausen et al, (1980) observed that 4/6 follicular oocytes were penetrated by ram spermatozoa capacitated by in vitro incubation in medium containing lamb serum. In 1982 Brackett and co-workers demonstrated the first successful domestic IVP experiments and achieved a 40% fertilization rate, with the first live calves born following IVF. Pugh et al., (1991) reported the birth of the first lamb produced in vitro from oocytes collected outside of the breeding season.

The in vitro fertilization portion of an IVP system is designed to closely resemble the steps that occur in vivo around the time of fertilization, as evidenced by low rates of MPN formation and a high incidence of polyspermy (Hunter, 2000). Successful IVF requires appropriate preparation of sperm and

oocyte, as well as culture conditions that are favourable to the metabolic activity of the male and female gametes.

2.3.2.2 Fertilization media

As a result of successfully transferring embryos between rabbits (Heape, 1891), scientists became interested in the possibility of culturing embryos in the laboratory, thereby enabling the study of early embryonic development. The IVF system consists of preparing the spermatozoa and the oocytes for syngamy and the co-incubation of oocytes and spermatozoa for a certain period of time. To enhance successful fertilization of the oocytes, sperm cells must be motile and have the ability to undergo capacitation and express the acrosome reaction. The sperm must also possess the capacity to bind to the ZP and vitelline membrane by acquiring the correct binding proteins during maturation, and exposing these binding sites to the oocyte at the appropriate time. In addition, Pavlok et al, (1988) observed that ejaculated spermatozoa are more suitable than sperm collected from the epididymus for IVF, and give rise to more normal embryos, but they have to undergo a capacitation treatment prior to fertilization. Spermatozoa capacitation occurs by incubating the spermatozoa in media for 2-4 h prior to IVF. The cumulus cells surrounding the oocytes are removed by mixing them with 0.1% hyaluronidase, thus reducing the number of spermatozoa needed by allowing the spermatozoa to reach the egg faster and more efficiently (Greve and Madison, 1991).

Normally, sperm capacitation in vivo takes place in the female genital tract. In vitro capacitation procedures are aimed at reproducing the sequence of events that normally occurs in the female. Capacitation and acrosome

reaction appear to be a general phenomenon in all mammals (Trounson and Gardner, 2000). Sperm capacitation involves a complex series of biochemical and physiological reactions, but an important part of this process is the gradual removal or alteration of the seminal proteins and other substances that coat the sperm membrane of the ejaculated semen, especially in the region of the acrosome. Removal or alteration of this coat permits exposure of receptor sites, allowing sperm to interact with oocyte receptors. To do this, sperm cells are subjected to thorough washing and exposure to media with elevated ionic strength.

Several chemical systems have been used to induce capacitation of either fresh or frozen spermatozoa in vitro (Cheng et al .,1986: Pugh et al., 1991), such as washing with high ionic strength (HIS) solution (Brackett et al., 1982), treatment with bovine follicular fluid (Fukui et al., 1983), or oviduct fluid (Parrish et al., 19896), co-culture into oviduct cell monolayers (Guyader and Chupin, 1991), caffeine (Niwa, et al., 1988), heparin (Parrish et al., 1988), or caffeine plus heparin (Niwa and Ohgoda, 1988), and incubation with Ca^{+2} ionophere A23187 (Cognie et al., 1991), or caffeine plus calcium ionophore A23187 (Aoyagi et al., 1988). Despite significant progress, the technology of capacitation of ram semen is still rather empirical and more knowledge of gamete biology is necessary for routine application of IVF in sheep.

A variety of media have been used for IVF, most of them based on blood plasma, serum, or undefined biological fluids. In 1949, John Hammond Jr. devised a complex medium that permitted 8-cell mouse embryos to develop into blastocysts. Whitten, (1956) showed that simpler, chemically-defined medium could do the same job. Soon after, McLaren and Biggers

(1958), combining the culture fluid of Whitten with the transfer techniques of Heape, cultured mouse embryos from the 8-cell to the blastocyst stage, and transferred them to the uterus of a foster-mother where they developed normally. In general, bovine fertilization medium used for sperm/ oocyte co-culture is Tyrode's albumin lactate pyruvate medium (TALP), supplemented with several capacitating and motility-stimulating agents such as heparin, epinephrine, hypotaurine and penicillamine (Parrish et al., 1986). This medium was originally developed for mouse and hamster IVF (Bavister and Yanagimachi, 1977; Yanagimachi and Chang, 1994). Parrish et al., (1985) adapted this medium for use in the cow. Berger and Horton, (1988) demonstrated that Tris-buffered medium without antibiotics with the addition of 5 mM of caffeine and 5 mM of Ca^{+2} increased the rate of sperm penetration (Wang et al., 1991), and that removal of glucose improved the level of fertilization (Parrish et al., 1989a).

Bicarbonate has been reported to be essential for fertilization of oocytes and suggested to be necessary for Ca influx into spermatozoa to induce the acrosome reaction (mouse; Lee and Storey, 1986). The bicarbonate also acts to increase the intracellular pH of caput and caudal spermatozoa (bovine; Vijayarghavan et al., 1985) and therefore is effective for maintaining sperm motility, one of the important factors supporting penetration of spermatozoa into oocytes (Fraser and Ahuja, 1988). Ca^{+2} ionophore (A23187) is used to bypass early stages of capacitation of sperm by increasing the Ca^{2+} content of the cell and inducing the acrosome reaction (AR), and has also been used as a capacitation agent, replacing the need for heparin (Yang et al., 1993). Brackett and Oliphant (1975) developed rabbit IVF "Brackett and Oliphant's defined medium" for use in rabbit IVF. Their technique includes washing and

incubating spermatozoa at high ionic concentrations. These media have been used for IVF in cows and goats (Brackett et al, 1980; 1982; Younis et al., 1991), but few attempts have been made to fertilize ovine oocytes. Treatments with HIS medium have been shown to capacitate bovine (Brackett et al., 1980) and ovine spermatozoa (Bondioli and Wright, 1983) by removal of proteins from the spermatozoa surface.

Both caffeine and heparin are known to induce capacitation of spermatozoa. Caffeine is a cyclic nucleotide phosphodiesterase inhibitor that has been employed as a motility- stimulating agent in bull sperm. It achieves its effect by inhibiting phosphodiesterase, which results in an intracellular accumulation of cAMP that activates respiration and sperm motility. Little work has been conducted with capacitating agents such as heparin or other glycosaminoglycans for ovine IVF, despite their reported effectiveness at stimulating the acrosome reaction in vitro (Thompson and Cummins, 1986). Heparin, as well as other glycosaminoglycans (GAGs), is present in the female genital tract (Lenz et al., 1982). All evidence suggests that heparin-binding proteins attach to the sperm surface, enabling heparin-like GAGs in the female tract to induce capacitation, resulting in an improved fertilization rate. Parrish et al., (1988) demonstrated that heparin is able to capacitate fresh bovine sperm within 4h, and thawed sperm within 15 min (Parrish et al., 1986). Another study pointed out that semen from different bulls reacts differently to heparin treatment, and that the optimal heparin concentration varies from 0.05-100 [ig/ml (Fukui et al., 1990). As a consequence, each bull and perhaps each ejaculate must be carefully tested for optimal heparin dosage and/ or optimal sperm concentration before use in IVF (Mermillod et al., 1990). Egg penetration by sperm has been improved by supplementation of

the capacitation medium with 5 [ig/ml heparin (frozen semen) or by a short incubation (15 min) of fresh semen in a capacitating medium containing 0.5 mM 8-brom-cAMP, 10 [ig/ml heparin and 100 nM ionomycin (fresh semen; Wang et al., 2002). Sterol-binding proteins from serum are involved in the removal of cholesterol from plasma membranes during in vitro capacitation (Langlais et al., 1988). In order to increase egg penetration in goats, thawed spermatozoa are incubated for lh in SOF supplemented with 10% sheep serum and 0.5 (j,g/ml heparin (Cognie et al., 2003). Spermatozoa are incubated with oocytes for 17 h based on the time required for sperm capacitation inside the female reproductive tract and for observation of pronuclei. Motile spermatozoa are obtained by centrifugation of thawed semen on a Percoll gradient (45- 90 %) for 10 min at 500 xg at room temperature. Percoll density gradient centrifugation seems superior to the other procedures for separating live spermatozoa from dead in thawed semen (Rho et al., 2001). Fresh sheep semen is washed twice in 9 ml of Hepes-buffered synthetic oviduct fluid (H-SOF) containing 4 mg/ ml BSA and centrifuged at 200 xg for 5 min (Ptak et al., 1999). Accord et al., (2004) modified this procedure by washing fresh semen kept at room temperature for up to 2h in fertilization medium 3 times, then washed the semen in SOF, centrifuged it twice at 200 xg for 5 min and added it directly to the fertilization medium.

Cheng, (1985) and Crozet et al., (1987) reported a high fertilization rate of sheep oocytes matured in vitro when the semen was re-suspended in medium containing oestrus sheep serum, and serum still remains the most effective capacitating factor in sheep. Different concentrations of heat-inactivated oestrus serum are used for capacitation media, varying between

2% in thawed semen (O'Brien et al., 1997) and 20% in fresh semen (Comizzoli et al., 2001; Berg et al., 2002).

Cognie and co-workers (2003) used 10% (v/v) oestrous sheep serum in SOF medium for both sheep and goats. Ram ova are penetrated 2.5- 3 h after insemination with fresh semen capacitated in the presence of serum (Huneau et al., 1994). The alteration of the plasma membrane of spermatozoa due to freezing induces a lower resistance to a prolonged period of capacitation, and 1 h for capacitation of frozen ram semen seems to be optimal (Guerin et al., 1989). Capacitated sperm are added to the fertilization drop at a final concentration of 1 x 10^6 spermatozoa/ml and plates are incubated for 17 h at 39 °C under an atmosphere of 5% CO_2 in air with maximum humidity (Cognie et al., 2004), although the mechanism of action is not yet understood (Thompson, 1997; Accordo et al., 2004).

Sera introduce unknown substances and contaminants making necessary to control every batch used for IVF. It is very important to use standardized source of proteins such as certified bovine serum albumin (BSA). BSA plays an important role in inducing capacitation and the acrosome reaction of spermatozoa. It removes cholesterol and/or zinc from sperm due to its considerable binding capacity for both molecules, which are known to stabilize cell membranes (Byrd, 1981). To enhance capacitation and the acrosome reaction, cell membranes of both oocytes and sperm must be destabilized. However, a low fertilization rate is observed when spermatozoa are pre-incubated in medium containing BSA (Bondioli and Wright, 1980).

On the other hand, polyvinyl alcohol (PVA) has been reported to decrease the spontaneous acrosome reaction in hamster (Bavister, 1981) and mouse (Fraser, 1985) spermatozoa. In cattle, the medium containing heparin

and PVA can stimulate sperm capacitation, but can barely support the fertilization of cumulus-free oocytes as compared to BSA (Parrish et al., 1989a). In addition, other researchers have shown that penetration of oocytes in vitro is possible in medium without protein when cumulus cells are present (hamster; Bavister, 1982; mouse; Fraser, 1985; and bovine; Tajik et al., 1993). Many other factors also affect penetration of oocytes, such as semen concentration (rat; Niwa and Chang, 1974; pig; Nagai et al., 1984; and human; Wolf et al., 1984), and number of spermatozoa (Wang et al., 1991).

- *Sperm- oocyte co-incubation time*

In vitro fertilization (IVF) techniques are continuously progressing since 1980s. The actual standard IVF protocol recommends an overnight gamete co-incubation. Fertilization is assessed by the observation of two pronuclei after 18- 20h. However, several studied on gamete kinetic interaction in vivo have shown that gamete contact and fertilization occur within 20 min post coitus in mammals (Wassarman, 1988), and an average number of 15 spermatozoa are observed in vitro in the cumulus after 15 min of gamete contact. Furthermore, 80% of oocytes are fertilized after lh exposure to spermatozoa (Gianaroli et al., 1996a; Aitken et al., 1987).

Eighteen hours of incubation with spermatozoa was originally established for practical reasons, and corresponds to the time required for observation of two-pronuclei, (2PN). This long period of co-incubation, however, has been shown to create problems with high levels of reactive oxygen species (ROS) which may affect fatty acid peroxydation causing a decrease in membrane fluidity (Aitken et al., 1994; Twigg et al., 1998), embryo developmental arrest (Nasr- Esfahani et al., 1990), and the quality of

embryos (Gianaroli et al, 1996b; Dirnfeld et al, 1999; Bedaiwy et al, 2004), and cause hardening of the zona pellucid (ZP), known to negatively influence the viability, and implantation potential of the embryo (Waldenstram et al., 1993; Gianaroli et al., 1996a; b; Dirnfeld et al., 2003; Bungum et al., 2006). Moreover ROS is one of the main sources to DNA fragmentation (Twigg et al., 1998), and strand breaks in sperm (Aitken and Clarkson, 1987; Aitken et al., 1989a; b). Although, DNA-damaged sperm are able to fertilize oocytes (Ahmadi and Ng, 1999). Although, the high rates of DNA breaks are known to negatively influence fertility in vivo (Evenson et al., 1999; Spano et al., 2000) as well as in vitro (Morris et al., 2001; Bungum et al., 2004; Gandini et al., 2004; Virroetal., 2004).

In 1998, Kochhar and King reported that a 9-10 h gamete co-incubation longer is required to achieve optimal cleavage rates and blastocyst yield when spermatozoa are added to cumulus-oocyte complexes in sheep; or cattle (Ward et al., 2002). In goat IVF, Cognie et al., (2004) observed very early cleaving embryos develop at low rates when they are separated out before 17-24 hours post-insemination (h.p.i.). Guerin et al., (1989) demonstrated that the alteration of the plasma membrane of spermatozoa due to freezing induces a lower resistance to a prolonged period of capacitation, and that lh for capacitation of frozen ram semen seems to be optimal.

Based on these observations, some investigators tried to test the beneficial effect of a shortened gamete contact on embryo quality and pregnancy rate. Some of them have shown that both penetration and blastocyst rates were improved by reducing the co-incubation time from 5 h to 10 min (Grupen and Nottle, 2000; Gil et al., 2004) and enhanced embryo quality and pregnancy rate after a brief gamete contact of 1 to 4 h (Gianaroli et al., 1996a;

Dirnfeld et al, 1999; Kattera et al, 2003). In front, others have described an equivalent embryo morphology or pregnancy rate compared to 18h incubation (Cosken et al., 1998; Quinn et al., 1998; Lin- Shau et al., 2000; Lundqvist et al., 2001; Swenson et al., 2000; Kattera and Chen, 2003; Bungum et al., 2006).

On the other hand, Cognie et al., (2003) observed that lh co-incubation with spermatozoa, only 10% of oocytes are fertilized, but the cleavage rate and blastocyst yield following a 3h co-incubation are similar to a 17 h co-incubation, i.e. 71.50 % at 3 h and 78.53% at 17 h. While Lundqvist et al., (2001) observed a decreased fertilization rate in the short insemination group compared to the standard one. These results, obtained with varying IVF protocols and study designs, are contradictory and suggest that the improvement of IVF outcome by a reduction of the oocytes insemination duration is still an open debate.

Several studies are required to determine whether reducing the time of sperm-oocyte interaction affects embryo development, and improves the implantation rate after embryo transfer in domestic animals, as has been observed for human IVF (Gianaroli et al., 1996).

2.3.2.3 Separation of Viable Sperm and Prevention of Polyspermy

Semen is known to contain factors that can prevent capacitation and/ or fertilization, like abnormal spermatozoa, dead cells, enzymes and bacteria that can cause IVF failure or otherwise reduce fertility. Numerous techniques have been proposed for removing undesirable semen components and concentrating the motile sperm fraction in a suspension of known concentration. The most conventional way of controlling sperm concentration is the swim-up method

(Parrish et al., 1986; Parrish and Foote, 1987) but other methods have been described involving either centrifugation on BSA (Wall et al., 1984) Percoll density gradients (Bolton and Braude, 1984) or Sephadex column separation (Drobins and Katz, 1991). Centrifugation on Percoll density gradients is the most commonly used method for human IVF, improving the pregnancy rate from 18% with swim-up to 31.5% after Percoll separation (Guerin et al., 1989). The use of Percoll gradients reduced the rate of polyspermy, which was the major cause of IVF failure in cattle (Mermillod et al., 1990; 1992).

"*Polyspermy* is the entry of more than one sperm into the oocyte due to failure of the zona reaction to block the entry of multiple sperm. The natural block against polyspermy starts after the spermatozoon has penetrated the oocyte; the cortical granules release their contents exocytotically into the PVS surrounding the oocyte. The contents of these cortical granules alter the properties of the ZP, creating a block against polyspermy (Hunter, 1991; Dandekar and Talbot, 1992). Bedford and Kim, (1993) observed that the rat cumulus oophorus prevents polyspermy, but Hunter and Leglise (1971a; b) demonstrated that pig and rabbit cumulus oophorus do not. The size of the PVS has also been linked to the ability of the oocyte to prevent polyspermy, and ovulated oocytes have a much wider PVS than those matured in vitro (Wang et al., 1998). Cran and Cheng, (1986) observed that the cortical granules in the PVS were not dispersed and remained circular after IVF, contrary to in vivo fertilized oocytes. Kim et al., (1996) and Kano et al., (1994) demonstrated that insufficient modification of the ZP and cortical granules in vitro may be due to the lack of a secreted oviductal factor. Han et al., (1999) observed that polyspermic zygotes have fewer inner cell mass (ICM) numbers and abnormal cleavage patterns. The size of the mitochondria

and cortical granules in pigs are small, and glutamine metabolism is lower in prepubertal than in adult oocytes. In pig, the level of polyspermy in prepubertal oocytes is much greater compared to adult oocytes (Funahashi et al., 1994). Maximum penetration and the minimum incidence of polyspermy occurred after 6 h *vs.* 3 or 12 h co-incubation (Abeydeera and Day, 1997b).

The cause(s) of failure to block polyspermic penetration in vitro are unknown. During the last few years various strategies for the reduction of polyspermy have been developed, focusing on enhancement of either oocyte cytoplasmic maturation or modulation of sperm and IVF treatments. These have included co-culture of the male and/or female gamete with oviductal secretions or proteins, supplementation of fertilization media with glycosaminoglycan or thiol compounds, and the presence of cumulus cells. Culturing the oocytes in low NaCL medium results in a wider PVS and a reduction in polyspermy (Abeydeera, 2002). Dubuc and Sirard, (1995) demonstrated that co-culturing the spermatozoa with oviductal cells significantly reduces the incidence of polyspermy (Nagai and Moor, 1990).

The success of the IVF process involves several critical steps: sperm selection, sperm capacitation and fertilization itself. IVF can be a key point for development of new technologies such as production of embryos of known sex by sperm selection (Upreti et al., 1988) the production of transgenic offspring obtained by sperm electroporation with foreign DNA (Gagne et al., 1991), or programmed DNA pronuclear microinjection (Baldassare et al., 2002). IVF of prepubertal oocytes has the potential to shorten the interval between generations and increase production of genetically valuable females (Ptak et al., 1999a; Baldassare et al., 2003). 2.3.3 In vitro culture (IVC)

The final step of an in vitro production system (IVP) is in vitro culture (IVC). The major purpose of embryo culture is to sustain the zygote in a suitable environment until it reaches the blastocyst stage, ultimately resulting in the production of a healthy newborn following transfer to a recipient. Several major developmental events occur between zygote and blastocyst formation including: the first cleavage division, the activation of the embryonic genome at the 8-16 cell stage (Lonergan et al., 1999a: Memili and First, 2000), the compaction of the morula (Boni et al., 1999) and the formation of the blastocyst accompanied by the emergence of the first two embryonic cell lines, the trophectoderm and the inner cell mass (ICM) (Watson, 1992).

Many factors within the female reproductive tract affect embryonic development and interaction (Gandolfi, 1994), and thus far, in vitro conditions have not been able to replicate the dynamic metabolic changes occurring in the oviduct and uterus. (Bavister, 1995). There is a need to optimize the IVC system to obtain embryos of equal quality to those obtained naturally in the oviduct (in vivo).

In 1974 Tervit and Rowson developed a simple culture medium called synthetic oviduct fluid (SOF) based upon the concentrations of ions and carbohydrates present in sheep oviduct fluid, and similar to several "simple" media (Restall and Wales, 1966). Walker et al., (1992) developed a culture system supplemented with serum that produced viable blastocysts from early cleavage stage embryos of sheep and cattle. Gardner et al., (1994) used amino acid supplementation and Cognie (1999) used SOF medium with 5-10% FCS at 2-3 day post-insemination (pi) to promote a higher viability after transfer of IVP embryos. In most of these systems the embryos are cultured in micro

drops under paraffin oil or mineral oil, and incubation is performed at 38.5 C° in 5% CO_2, 5% O_2, and 90% N_2, under humidified atmosphere.

The presences of amino acids are important in sheep embryonic development. They might serve as an energy substrate, in metabolism, and as regulators of intracellular pH (hamster; Carney and Bavister, 1987; Gardner and Lane, 19976), and may increase the endogenous amino acid pool size and/or provide a pool for de novo protein synthesis when added to embryo culture media (Rozenkrans and First, 1994). The significance of pooled groups of amino acids, especially the non-essential amino acids (e.g., alanine, aspartate, asparagines, glycine, glutamate, and glutamine) was demonstrated in sheep embryos (Thompson et al, 19926; Gardner and Lane, 1993a; Gardner et al., 1994).

- *Evaluation of embryo*

Oocyte maturation, fertilization, and embryo development are complex and dynamic events and it is difficult to pin-point a single moment in development that will identify the embryo destined for implantation. One of the most important steps in embryo technology is their evaluation.

Some methods which have been used to judge embryo viability include: metabolism, gene expression and apoptosis (Van Soom and Boerjan, 2002) measures of enzymes activity (O'Fallon and Wright, 1986; Sugawara and Takeuchi, 1973), glucose uptake (Friedhandler, 1961; Sugawara and Takeuchi, 1973), and staining (Purcell et al., 1985; Schilling et al., 1979). Gross morphology (Shea, 1981; Wright, 1981; Linder and Wright, 1983b) total and inner cell mass cell number, kinetics of development, and post-

cryopreservation survival, are the most often used criterion to assess embryo viability.

By definition, a *"blastocyst"* is an embryo that has divided into hundred of cell and is composed of two parts. The outer sphere of the blastocyst is called the trophoblast, and the inner portion of the blastocyst is filled with fluid. Inside the trophoblast, there is a clump of cells called the inner cell mass (ICM). The ICM is the portion of the blastocyst that actually becomes the new born. The percentage of zygotes cleaved to 4-8 cell stage 48 h after IVF, can be used as a fast gauge to estimate the potential of cleaved zygotes to develop to the blastocyst stage. Those embryos that consist only of evenly sized blastomeres are believed to have a better chance of further development than zygotes with cytoplasmic fragments, which frequently contain no nuclei. Not all fragmentation appears to be detrimental to embryo development, but the pattern of fragmentation has a profound effect on embryo's developmental potential. Large fragments formed at the 2 to 4-cell stage appear more detrimental due to the depletion of essential organelles such as mitochondria involved in exogenous protein uptake (Alikani et al., 1999). The presence of small fragments does not appear to effect developmental rates to the smae degree as large fragments. The formation of small fragments may represent incomplete cytokinesis. Implantation rates are similar between embryos without fragmentation and those with moderate fragmentation.

Embryo development to the blastocyst stage on d 7-8 of culture and blastocyst cell numbers are considered as an indicator of embryo quality (Ellington et al., 1990). Generally, a good quality blastocyst contains a well-expanded blastocoleic cavity, and homogenous trophoblast with multiple cell-cell contacts and distinct nuclei, and an ICM that is clearly visible and intact.

Marquant-Le Guienne et al. (1989) demonstrated that embryo quality could be investigated by total embryonic cell count using histological or fluorescent techniques (Ellington et al. 1990). More accurate results may be obtained by cell number allocation between ICM and trophectoderm (Iwasaki et al., 1990).

A large number of studies conclude that if, good quality blastocyst is available for transfer, and pregnancy rates are high as 60 % (Langley et al., 2001; Gardner et al., 2000a; b). Selection of the "golden" blastocyst is also not well understood, some investigators strongly believe that extending the culture of embryos to blastocyst represents a type of natural selection of those embryos that will be genetically normal and viable in uterus.

Assessment of embryo viability in culture is rather subjective: gross embryos morphology is used as the most common method for selecting embryos for transfer. The ability to identify the most viable embryos from within a given cohort should increase the overall success of assisted reproductive procedures. Embryo morphology is based on a series of dynamic processes that can change dramatically from moment to moment. Some researcher believes that embryo assessment should begin with the ovary and follicle (Van Blerkom, 2000; Bhal et al., 1999). It is important to realize that morphologic assessment cannot be accomplished by a one-time observation. The visible features that help us select the *"golden"* embryo may appear gradually, starting with increased follicular size, continuing with a healthy, mature oocytes that will fertilizes normally and an embryo that will divide regularly with blastomeres that are homogeneous, intact and have one distinct nucleus. Depending on the day of transfer, the chance of success can be further influenced with selection of the most competent blastocyst and an uneventful and atraumatic embryo transfer.

Mrphological parameters have been commonly used to evaluate embryo quality including the shape, color of cytoplasm, number and compactness of cells, size of the perivitelline space, number of extruded or degenerated cells, and the frequency and size of cytoplasmic vesicles. Indeed, morphological scores of embryos are highly correlated with pregnancy rates (Hsler et al., 1987; Funahashi et al., 1994c). Lindner and Wright, (1983b) suggested a four-category system to judge bovine embryo quality which can be applied to all farm species. Quality of individual embryos were determined by the following criteria: 1) Excellent: an ideal embryo, spherical, symmetrical whith cells of uniform size, colour and texture; 2) Good: trivial imperfections such as a few extruded blastomeres, irregular shape, few vesicles; 3) Fair: defined but no severe problems, presence of extruded blastomeres, vesiculation few degenerated cells; and 4) Poor: severe problems, numerous extruded blastomere's and degenerated cells, cells of varying sizes, large numerous vesicles but a viable appearing embryo mass. Lane et al., (2003) demonstrated that blastocyst morphology was assessed after 144 h of culture. Blastocysts were assessed as either early blastocysts, blastocysts, expanded blastocysts or hatching/ hatched blastocysts. Early blastocyst were observed to have a disyinct blastocoel cavity which less than 2/3 of the volume of the blastocyst; blastocysts were those which contained a blstocoel cavity at least 2/3 of the volume of the blastocyst but where the ZP had no thinned; expanded blastocysts contained a full blastocoel and were expanded beyond the original diameter of the oocye and had thinned the ZP; hatching /hatched blastocysts, had partially or completely breached the ZP. Moreover, Palasz et al., (2006) demonstrated that blastocysts developmental stage were evaluated by the size of blastocel cavity (BC): early blastocyst, clearly visible BC; blastocyst, BC <

2/3 volume expanded blstocyst, BC occupied whole volume of the embryos with visible thinning of the ZP: hatching blastocyst, ZP open and part of embryo proper out of the zona; and hatched blastocyst, entire is out the ZP. Furthermore, freeze resistant may be used as an indicator of embryo viability (Greve et al, 1993; Furnus et al, 1997). Gradner et al, (1996) demonstrated that blastocysts surviving the freeze-thaw procedure had a significantly higher glucose uptake and lactate production than those embryos that did not re-expand and subsequently died. The significance of this study is that there was no overlap in the distribution of glucose uptake by the viable and non-viable embryos.

Various procedures are available depending on the species and the intended use, but the most simple, quick and inexpensive method remains the evaluation by morphological methods. The disadvantages are grading is subjective, and depends on the experience of the evaluator, and metabolic, genetic or epigenetic disorders of embryos are not detectable. In conclusion when we will be able to relate quality to structure, the level of success achieved with embryo transfer could be improved, this still remains a large challenge.

CHAPTER III
MATERIAL & METHODS

3.1 Animal and Semen Collection

Semen were collected from one Sarda breed ram 3-4 years old, with an average body weight of 70 kg, was housed at Sassari University, Faculty of Veterinary Medicine, Department of Animal Biology- Italy, under natural light and maintained under a uniform and constant nutritional regime.

Pooled of semen were obtained with the aid of an artificial vagina through breeding and non breeding season. Immediately after collection, the ejaculate was immersed in incubator at 33- 35 °C until their assessment in the laboratory. Only ejaculates of between 1- 2 ml in volume, with >75 % sperm progressive motility and an ejaculate concentration of higher than $3* 10^9$ sperm/ ml, was accepted and include in this experiment.

3.2 Assessment of Frozen- thawed Spermatozoa

3.2.1 Sperm motility

After thawing, the sperm samples were kept in a CO_2 incubator at 37°C without further dilution and were immediately examined. The sperm motility was defined as a sperm displaying vigorous forward motion, and the percentage of sperm motility was assessed by using a phase- contrast microscope (100* magnification) Diaphot (Nikon, Japan), with a warm stage maintained at 37°C. A wet semen mount was made using 5 [il drop of semen placed directly on a microscope slide and covered by a cover slip. For each sample, at least five slide fields were examined by the same observer; the mean of the three estimations was recorded as the final motility score.

3.2.2 Assessment of viability and acrosome integrity

The viability of sperm as determined by membrane integrity staining, the sample was assessed by incubating sperm cells with specific fluorochrome propidium iodide (PI), which is excluded from vital cells. Acrosome integrity was evaluated by incubating spermatozoa with fluorescein isothiocynate labelled Pisum Sativum agglutinin (FITC- PSA), which is a non-permeable lectin membrane that binds to glycoconjugates of the acrosome content (Naitana et al., 1998). The aliquots (100 [il) of sperm suspension, after addition of 5 [il of a 0.1 mg/ml solution of FITC-PSA and 1.4 [il of a 1 mg/ml solution of propidium iodide (PI), were incubated for 15 min at 39 °C. In order to reduce background fluorescence, unbound PSA and propidium were removed by adding 200 [il of phosphate buffered saline solution (PBS) and spermatozoa were washed by centrifugation in a microcentrifuge for 2min. The supernatant was aspirated and the pellet resuspended in 100 [il of PBS. After washing, a 10 jal sample was put on a slide and cover slipped. The slide was immediately dried by leaving at 37 °C for 10 min for immobilization of

sperm cells. To evaluate the stained sperm cells, at least 200 cells were counted in duplicate for each sample, using a Diaphot (Nikon, Japan) epifluorescence microscope.

Stained spermatozoa were classified according to the specific PI and FITC-PSA fluorescence exhibited. Dead sperm cells (plasmatic membrane damaged spermatozoa) were unable to exclude the propidium vital stain and therefore showed fluorescent red, while live spermatozoa did not show fluorescence. Besides, acrosome- damaged spermatozoa appeared green in the acrosomal region because the fluorescein isothiocyanate labelled agglutinin of the Pisum Sativum was able to gain the inner acrosome region and bind to the glycoconjugates of the inner acrosome content; the spermatozoa with acrosome integrity, on the other hand, showed no green fluorescence. Only viable sperm were evaluated for their acrosome status.

3.2.3 Assessment of morphological abnormality

The morphological abnormality of spermatozoa was assessed by means of eosin- nigrosin stain (Evans and Maxwell, 1987). The stain was prepared as eosin-Y 1.67 g, nigrosin 10 g, sodium citrate 2.9 g, dissolved in 100 ml distilled water. The sperm smears were prepared by mixing a drop of semen with two drops of stain on a warm slide and spreading the stain immediately with the aid of a second slide. Spermatozoa were examined for morphological abnormality (detached heads, acrosomal aberrations, abnormal in mid-pieces and tail defects) by counting 200 sperm cells with bright-field microscopy (400x) (Nikon, Japan) microscope. The percentage of sperm abnormality was calculated using a formula: number of abnormal spermatozoa/ 200 x 100.

3.2.4 Assessment of membrane integrity

The hypo-osmotic swelling test (HOST) was used as complementary test to evaluate the functional integrity of the sperm membrane. HOST relies on the resistance of the membrane to the loss of permeability barriers under stress conditions of stretching in a hypoosmotic medium (Revell and Morode, 1994; Buckett et al, 1997). Sperm cells with resistant membranes exhibited a curled and swollen around the tails, such that the flagella become curled and the membrane maintained a swollen "bubble" around the curled flagellum. The assay was performed by mixing 30 [il semen in a 300 [il of a 100 mOsm/ kg hypo-osmotic solution (9 gm fructose plus 4.9 gm sodium citrate per liter of distilled water) at 37°C for 60 min (Revell and Morode, 1994). After incubation, 0.2 ml of the mixture was spread with a cover slip on a warm slide. Sperm were evaluated immediately (x 400 magnification) under the phase-contrast microscope. A total of 200 spermatozoa were counted in at least five different slide fields. The percentages of sperm with swollen or coiled tails were recorded.

3.2.5 Assessment of adenosine triphosphate concentration

Each sample analyzed for Adenosine triphosphate (ATP) concentration was also assessed for spermatozoa viability, as previously described, in order to correlate these two variables. Fifty of frozen/thawed spermatozoa (approximately 1.1* 10^9 cells/ ml, 20.000 total cells) from each group were washed twice with 0.1 ml of cold physiological solution. For the extraction of nucleotides, 0.1 ml of ice-cold 0.6 M perchloric acid were added to each eppendorf containing spermatozoa and kept for 15 min; after the suspension was centrifuged in an Eppendorf Microfuge (3 minutes at 10000 rpm) and the

supernatant was neutralized with 15 ul of 3.5 M K_2CO_3 (Balestri et al, 2007). ATP levels were measured spectrophotometrically at 340 nm using NADH-linked enzyme-coupled assays (Bergmeyer, 1974) modified for being adapted to our system.

The enzymatic spectrophotometric ATP assay was carried out at 37 C with a Beckman DU-7 spectrophotometer, and performed used the coupling enzymes, glucose 6 phosphate dehydrogenase (G6PD) and hexokinase (HK). Addition of excess HK (2 ul from 2mg/ml) and G6PD (2 ul from lmg/ ml) in the presence of excess glucose (8 ul from 18 mg/ml) and Nicotinamide adenine dinucleotide phosphate (NADP+) (8 ul from 20 mg/ml) to perchloric extract (25 ul) and to 400 ul of TRAP buffer (0,lM, pH 7.6), the reaction begins and ATP was determined from the formation of NADPH.

3.3 In vitro production embryos

3.3.1 Oocyte collection

Ovaries of Sarda breed sheep ovaries were recovered at a local slaughterhouse and transported to the laboratory within 1-2 h in Dulbecco's PBS at temperature between 25 and 35°C. After washing in fresh medium, ovaries were sliced using a micro- blade and the follicle content released in medium TCM199 (with Earle's salts and bicarbonate) supplemented with 25 mmol Hepes, penicillin and streptomycin and 0.1% (w/v) polyvinyl alcohol (PVA), only the follicular oocytes that covered at least 2 layers of granulose cells and evenly granulated cytoplasm were selected for in vitro maturation (IVM).

3.3.2 In vitro maturation (IVM)

All the oocytes were selected and washed in the same fresh medium, were in vitro matured in TCM199 supplemented with 10% heat- treated fetal calf serum (FCS), 10 *iV* ml of FSH/ LH and 100 jiM cysteamine, 30- 35 COCs were put in 600 [il of the maturation medium in a four-well Petri dish (Nunclon, Nalge Nunc International, Denmark), layered with 300 [il mineral oil and cultured for 24 h in 5% CO_2 in air at 39°C.

3.3.3 Sperm preparation and in vitro fertilization (IVF)

After maturation, the COCs were partially stripped of the granulose cells and fertilized in vitro at 39°C and 5% CO_2, 5% O_2 and 90% N_2 atmosphere in four- well Petri dishes (Nunclon). Matured oocytes were divided randomly to six different groups and frozen thawed prepared from different types of egg yolk were used to fertilize them. The fertilization system was composed of 300 [il of synthetic oviduct fluid (SOF) medium supplemented with 2% of estrus sheep serum and swim-up derived motile spermatozoa at 1 x 10^6 spermatozoa/ ml concentration layered with mineral oil.

3.3.4 In vitro culture (IVC)

After 20-22 h, presumptive zygotes were mechanically denuded of their cumulus cells and cultured in four- well Petri dishes containing SOF+ essential and non-essential amino acids at oviductal concentration+ 0.4% BSA

under mineral oil in maximum humidified atmosphere with 5% CO_2, 5% O_2, 90% N_2 to blastocyst stage. On the 3rd and 5th d the cultured medium was replenished for all groups. The culture was continued until 8d post-fertilization. The numbers of expanded blastocysts/ group (144- 192 hpi) were recorded and vitrified.

3.4 First Experiment:

CAN SEMI- DEFINED DILUENTS BE USED SUCCESSFULLY FOR IMPROVEMENT OF FROZEN- THAWED RAM SPERMATOZOA?

3.4.1 Preparation of extender and cryopreservation procedures

A Tris- based extender (297.58 mM Tris, 96.32 mM citric acid, 82.66 mM fructose pH 7.2 and, 375 mOsm was used as the basic semen diluents (Vivanco et al, 1987), all extenders were used at the same day as they were prepared. The pooled samples were kept at 30°C and the semen volume was diluted to a final concentration of 400 xlO^6 sperm/ ml, with the extender containing 4% (v/v) glycerol and 10, 15, and 20% (v/v) BSA versus traditional extender with 20% egg yolk, and cooled to 4°C over a period of 2 h

and equilibrated for 20 min before freezing. Finally, semen was frozen in pellet form (0.25 ml) on dry ice and then plunged into LN_2. After storage at least 7 days in LN_2, pellets were thawed rapidly by plunging a sterilized glass falcon tube containing the pellet in a 39°C water bath for less than 30s.

3.4.2 Semen collection

Pooled of semen were obtained with the aid of an artificial vagina through (March 2008- March 2009).

3.4.3 In vitro production embryos

Ovaries of prepubertal Sarda breed lambs (less than 40 days, and 6-10kg) and adult sheep ovaries were used in this experiment. Six hundred and twenty five lambs plus one thousand and one hundred seventy five ewe oocytes were selected and washed in the same fresh medium before matured them. The same IVM protocol was used to mature all oocytes, and was explained previously.

After maturation, the COCs were partially stripped of the granulose cells and fertilized in vitro at 39°C and 5% CO_2, 5% O_2 and 90% N_2 atmosphere in four- well Petri dishes (Nunclon). Frozen thawed spermatozoa from the same ejaculate of one ram with 10, 15, and 20% BSA were used across all experimental procedures, plus standard diluents with 20% egg yolk. The fertilization system was composed of 300 [il of synthetic oviduct fluid (SOF) medium supplemented with 2% of oestrus sheep serum and swim-up derived motile spermatozoa at $lxlO^6$ spermatozoa/ ml concentration layered with mineral oil.

After 20-22 h, presumptive zygotes were mechanically denuded of their cumulus cells and cultured in same IVC protocol as explained previously.

3.5 Second Experiment:

THE EFFECT OF REDUCED SPERM- OOCYTES EXPOUSER TIME ON OVINE EMBRYO PRODUCTION

3.5.1 Preparation of extender and semen cryopreservation procedures

The total pellets were prepared in the first experiment and were used in this experiment also.

3.5.2 In vitro production embryos

Thirteen hundred and forty two oocytes were used in this experiment. After maturation, the COCs were partially denuded of cumulus cells and divided into three main groups (10, 15, and 20% BSA), each group was divided equally into four sperm- oocytes exposure times 1, 2, 3 h (short) and 20 h as standard insemination group. Four pellets from each main group were transferred in sterilized conical glass tube below 1 ml of warmed IVF- SOF supplemented with 2% of estrus sheep serum and incubated at 39°C in humidified atmosphere at 5% CO_2 in air for 15 min. Swim- up derived motile spermatozoa 1* 10^6 sperm/ ml final concentration layered with mineral oil were used to fertilized matured oocytes.

After 1, 2, 3 and 20 hours post insemination (hpi), the presumptive zygotes were washed, and removed from in vitro fertilization and cultured in four- well Petri dishes containing SOF+ essential and non-essential amino acids at oviduct concentration +0.4% BSA under mineral oil in humidified atmosphere with 5% CO_2, 5% O_2, 90% N_2 to blastocyst stage. On the 3rd and 5th d the cultured medium was replenished for all groups. The culture was continued until 8d post- fertilization. The numbers of expanded blastocysts/ group (144- 192 hpi) were recorded and vitrified.

3.5.3 Vitrification and warming embryos

Vitrification and warming media were prepared using PBS supplemented with 20% (v/v) FCS as base media. Embryos were vitrified according to a simple method (Leoni et al., 2002). Briefly, blastocysts were put into 200 [il drops of 1.4 M glycerol for 5 min, then into 200 [il drops of 1.4 M glycerol and 3.6 M ethylene glycol for 5 min before being transferred into a 15 [il column of 3.4 M glycerol and 4.6 M ethylene glycol, and loaded

into the centre of 0.25 ml plastic insemination straws using a fine glass capillary pipette. In the straws, the embryos and vitrification media were separated from two columns of 0.5 M sucrose solution. After sealing, the straws were transferred directly into LN_2 and kept in it.

For warming to a biological temperature, the straws were transferred from LN_2 into a water bath at 35 °C for 10s. The content of each straw was expelled into a Petri dish and stirred gently to facilitate the mixture of the two solutions. The embryos were retrieved and transferred into 200 [il drops of 0.25 M sucrose solution supplemented with 20 % FCS or 0.1% PVA for 3 min to allow for removal of intracellular cryoprotectants. Embryos were held 10 min in corresponding media of PBS containing 20% FCS or 0.1 % PVA for rehydration and equilibration.

To determine their viability in vitro the embryos were cultured in TCM199 with 10% FCS in humidified atmosphere 5% CO_2 in air at 39 °C. They were then examined at 12 h intervals for 60 h. The embryos that re-expanded the blastocoelic cavity were considered to be viable.

3.5.4 Embryo evaluation

Embryo evaluation was based on the method of Hill et al., (1989) before and after freezing. Grade A embryos were the best embryos containing even- sized, symmetrical blastomeres with no obvious fragmentation. Grade B embryos had blastomeres of uneven size or the total cytoplasm mass contained < 10% fragmentation; grade C embryos had a maximum of 50% of their cytoplasm fragmented and uneven blastomeres; and grade D embryos showed >50% cytoplasm fragmentation.

3.6 Third Experiment:

STUDIES ON THE EFFECT OF SUPPLEMENTING SHEEP SEMEN DILUENTS WITH DIFFERENT TYPES OF EGG YOLK ON THE QUALITY OF FROZEN- THAWED SHEEP SEMEN AND IVP EMBRYOS

3.6.1 Preparation of extender and cryopreservation procedures

In order to evaluate which type of egg yolk can provide the best cryoprotective actions on ram sperm cryopreservation, six different types of EY were used to prepare six different extenders.

All fresh laid eggs from domestic duck, *(Anatidae anas platyhynchos),* turkey, *(Meleagris gallopavo),* ostrich, *(Struthio camelus),* Barbary partridge, *(Alectoris Barbara),* and Marginated tortoise, *(Testudo marginata sarda)* versus traditional extender with domestic hen, *(Gallus domesticus)* were purchased from local farmer farm, and used on the same day of laying for preparation of extender.

A Tris- based extender (297.58 mM Tris, 96.32 mM citric acid, 82.66 mM fructose pH 7.2 and, 375 mOsm was used as the basic semen diluents (Vivanco et al., 1987), all extenders were used at the same day as they were prepared. The pooled samples were kept at 30°C and the semen volume was diluted to a final concentration of 400 xlO^6 sperm/ ml, with the extender containing 4% (v/v) glycerol and 20% (v/v) from each of five different egg yolk types domestic duck (DEY), turkey, (TUREY), Marginated tortoise, (TOREY), Ostrich, (OEY), and partridge, (PEY) versus traditional extender with domestic hen, (HEY), and cooled to 4°C over a period of 2h and equilibrated for 20 min before freezing. Finally, semen was frozen in pellet form (0.25 ml) on dry ice and then plunged into LN_2. After storage at least 7 days in LN_2, pellets were thawed rapidly by plunging a sterilized glass falcon tube containing the pellet in a 39 °C water bath for less than 30s.

3.6.2 In vitro production embryos

Nine hundred and thirteen oocytes were selected and washed in the same fresh medium, were in vitro matured in TCM199 supplemented with

10% heat- treated fetal calf serum (FCS), 10 ^1/ ml of FSH/ LH and 100 jiM cysteamine, 30- 35 COCs were put in 600 [il of the maturation medium in a four-well Petri dish (Nunclon, Nalge Nunc International, Denmark), layered with 300 \i\ mineral oil and cultured for 24 h in 5% CO_2 in air at 39°C.

After maturation, the COCs were partially stripped of the granulose cells and fertilized in vitro at 39°C and 5% CO_2, 5% O_2 and 90% N_2 atmosphere in four- well Petri dishes (Nunclon). Matured oocytes were divided randomly to six different groups and frozen thawed semen prepared from different types of egg yolk was used to fertilize them. The fertilization system was composed of 300 [il of synthetic oviduct fluid (SOF) medium supplemented with 2% of estrus sheep serum and swim-up derived motile spermatozoa at 1 x 10^6 spermatozoa/ ml concentration layered with mineral oil. After 20-22 h, presumptive zygotes were mechanically denuded of their cumulus cells and cultured in four- well Petri dishes containing SOF+ essential and non-essential amino acids at oviductal concentration+ 0.4% BSA under mineral oil in maximum humidified atmosphere with 5% CO_2, 5% O_2, 90% N_2 to blastocyst stage. On the 3rd and 5th d the cultured medium was replenished for all groups. The culture was continued until 8d post-fertilization. The numbers of expanded blastocysts/ group (144- 192 hpi) were recorded.

3.7 Analysis of Data

The analysis of variance test (ANOVA) was used to compare the motility, viability, morphological abnormality, HOST, ATP concentration, and acrosome integrity. The chi- square tests (SAS/ STAT User's Guide, 6.03

Edition, SAS Institute Inc., Cary, NC) was used to compare the cleavage and blastocysts rate after fertilization with frozen-thawed semen.

CHAPTER IV
RESULTS

4.1 First Experiment:

CAN SEMI- DEFINED DILUENTS BE USED SUCCESSFULLY FOR IMPROVEMENT OF FROZEN- THAWED RAM SPERMATOZOA?

4.1.1 Sperm quality

Table 1 summarized all spermatological characteristics of frozen-thawed semen prepared from different diluents.

Among all treatment groups, the treatment with 10 % BSA was highest significantly in cell motility compared with 15, 20 % BSA and control group (48.61± 0.66%) *vs.* (45.83± 0.55%), (41.01± 0.47%) and (45.29± 0.66%), respectively. In addition, there was significant difference ($P<0.05$) between treatment with 15 and 20% BSA. On the other hand, the treatment with 20% BSA was lower significantly in cell motility compared with control group (41.01±0.47%), (45.29± 0.66%), respectively.

The treatment with 15% BSA was higher in sperm viability rate compared to other groups. There were significant difference ($P <0.05$) between 15 and 20% BSA, and control group (53.23± 0.48%) *vs.* (51.10± 0.63%) and (51.08± 0.52%), respectively. While there was no significant difference among other groups. Moreover, the treatment with 10 and 15% BSA were significantly ($P<0.05$) lower in percentage of sperm morphological abnormality than 20% BSA and control group (6.34± 0.06%), (6.4± 0.09%) *vs.* (9.31± 0.21%), and (9. 18± 0.12%), respectively. In front, no significant differences between treatment with 10 and 15% BSA group.

Swelling of the sperm tail test (HOST), the treatment with 10 % BSA was significant higher compared to 20% BSA and control group (25.81± 0.72%), (21.84± 0.64%), and (21.92± 0.65%), respectively. In addition, the

treatment with 15% BSA was higher than 20% BSA and control group (27.42± 0.66%), (21.84± 0.64%), and (21.92± 0.65%), respectively.

Subsequently, the treatment with 10, and 15% BSA were higher significantly (P<0.05) in spermatozoa with an intact acrosome compared with 20% BSA and control groups (65.36± 0.94%), (68.72± 0.9 %) versus (61.36± 0.50 %), and (61.81± 0.6%), respectively. In addition, the treatment with 15% BSA was higher significantly compared with 10% BSA.

Adenosine triphosphate (ATP) concentration, the treatment with 10% BSA was higher in ATP concentration compared to other groups. There was significant difference (p<0.05) between treatments with 10 and 20% BSA (73.95± 25.84), (9.63± 5.53) nmol ATP/ 10^6 spermatozoa, respectively. While there were no significant difference among other groups.

TABLE 1. Spermatological characteristics of frozen-thawed semen prepared from different diluents (mean± SEM%)

Semen diluents	**Progressive motility** (%)	**Viability** (%)	**Abnormal morphology** (%)	**HOST** (%)	**ATP concentration** (nmol ATP/10^6 spermatozoa)	**Acrosome integrity** (%)
10%BSA	48.61±0.66a	52.15±0.57a£	6.34±0.06a	25.81±0.72a	73.95±25.84a	65.36±0.94a
15%BSA	45.83±0.55£	53.23±0.48a	6.4±0.09a	27.42±0.66a	35.17±14.95a£	68.72±0.906
20%BSA	41.01±0.47c	51.10±0.63A	9.31±0.21A	21.84±0.64A	9.63±5.53A	61.36±0.50c
Control	45.29±0.66M	51.08±0.52A	9.18±0.12A	21.92±0.65A	48.57±8.96aA	61.81±0.60c

Different superscripts (a, *b, c)* within the same column of each test demonstrated significant differences (P <0.05).

4.1.2 In vitro production embryos

Table: 2 and 3 summarized all fertilizing rates and subsequent development of in vitro matured lambs, and adult oocytes using frozen-thawed sheep spermatozoa prepared from different diluents.

4.1.2.1 Pubertal oocytes

Among all groups, the BSA treatment groups were higher in cleavage rates compared with control group with 20% EY. There was high significant differences (PO.001) between 10% BSA and EY (116/130, 89.23%), (112/150, 74.66%), respectively. While the treatment with 10% BSA was higher significantly (PO.05) compared with 15% BSA (116/130, 89.23%), (131/164, 79.87%), respectively.

Subsequently, the treatments with BSA increased the blastocysts rate compared with control group. Interestingly, the treatment with 20% BSA was higher compared to 10, 15% BSA and control groups (37/148, 25.0%) *vs.* (24/130, 18.46%), (38/164, 23.17%), and (26/150, 17.33%), respectively. However, there was no significant difference in total blastocysts rate between treatments with BSA and control group.

4.1.2.2 Adult oocytes

Among all groups, the treatment with 10% BSA was higher in cleavage rates compared to other groups. The treatment with 10% BSA was significantly higher (PO.001) compared with 20% BSA (239/283, 84.45%), (245/344, 71.22%), respectively. There was significant differences (PO.05) between 10 and 15% BSA (239/283, 84.45%), (215/278, 77.33%), respectively. In addition, the control group was significantly higher (PO.001) compared with 20% EY (217/270, 80.3%) and (245/344, 71.2%), respectively.

Subsequently, the treatment with 10% BSA was higher in blastocysts rate compared with 10, 20% BSA and control group (117/283, 41.34%), (109/278, 39.20%), (127/344, 36.91%), and (99/270, 36.66%), respectively.

Although, there was no significant difference in blastocysts rate between treatments and control group.

However, the treatment with 15% BSA was higher in numbers of developed 6 and 7d blastocysts versus other groups. There was significant difference (P< 0.001) between 15% BSA and 10% BSA (18/109, 16.51%), (5/117, 4.27%), respectively. The treatment with 15% BSA was significantly higher (PO.001) in number of 6d balstocysts compared with 20% BSA (18/109, 16.51%), and (9/127, 7.08%), respectively. While, the treatment with 15% BSA was significantly higher in number of 7d blastocysts compared to control group (70/109, 64.22%) and (48/99, 48.48%), respectively.

TABLE 2. Fertilizing rates and subsequent development of in vitro matured lamb's oocytes using frozen-thawed sheep spermatozoa prepared from different diluents.

Semen diluent	**Number Oocytes**	**Cleavage Rates** (%)	**6(1 Blastocysts** (%)	**7d blastocysts** (%)	**8(1 Blastocysts** (%)	**Blastocysts rates** (%)
10% BSA	130	116/130a (89.23)	5/24 (20.83)	12/24 (50.0)	7/24 (29.16)	24/130 (18.46)
15% BSA	164	**131/1646*** (79.87)	8/38 (21.05)	16/38 (42.1)	14/38 (36.84)	38/164 (23.17)
20% BSA	**181**	148/181aA (81.76)	3/37 (8.10)	22/37 (59.45)	12/37 (32.43)	37/148 (25.0)
Control	150	112/1506** (74.66)	6/26 (23.07)	15/26 (57.69)	5/26 (19.23)	26/150 (17.33)

* Different superscripts (a, *b,)* within the same column of each test demonstrated significant differences (PO.05).

** Different superscripts (a, *b,)* within the same column of each test demonstrated significant differences (PO.001).

TABLE 3. Fertilizing rates and subsequent development of in vitro matured ewe's oocytes using frozen-thawed sheep spermatozoa prepared from different diluents.

Semen	**Number**	**Cleavage**	**6d**	**7d**	**8d**	**Blastocysts**

diluents	Oocytes	Rates	Blastocysts (%)	blastocysts (%)	Blastocysts (%)	rates (%)
10% BSA	283	239/283a* (84.45)	5/117a** (4.27)	66/1 *llab* (56.41)	46/117a** (39.31)	117/283 (41.34)
15% BSA	278	215/278Ac (77.33)	18/1096 (16.51)	70/109a* (64.22)	21/1096 (19.26)	109/278 (39.20)
20% BSA	344	245/344**c (71.22)	9/127a* (7.08)	78/127a6 (61.41)	40/127a* (31.49)	127/344 (36.91)
Control	270	217/270a6** (80.37)	10/99a6 (10.10)	48/996 (48.48)	41/99a** (41.41)	99/270 (36.66)

* Different superscripts (a, *b, c)* within the same column of each test demonstrated significant differences (PO.05).

** Different superscripts (a, *b, c)* within the same column of each test demonstrated significant differences (PO.05).

4.2 Second Experiment:

THE EFFECT OF REDUCED SPERM- OOCYTES EXPOUSER TIME ON OVINE EMBRYO PRODUCTION

4.2.1 Exposure times effects

4.2.1.1 Cleavage rate

Among all exposure times' treatments, three hours co-incubation time was higher in cleavage rate compared with 1, 2 and overnight co-incubation time in both 10 and 15% BSA groups. In front, overnight hours co-incubation time was higher in cleavage rate compared with other co-incubation times in 20% BSA group.

In 10% BSA group, three hours co-incubation times was significantly higher in cleavage rate compared to 1, 2 h and overnight co-incubation time (93/110, 84.4%), (42/100, 42.0%), (61/101, 60.4%),and (89/125, 71.2%), respectively. In addition, two hours and overnight co-incubation time were significantly higher (P<0.001) compared to lh co-incubation time.

In 15% BSA group, one hour co-incubation time was significant lower in cleavage rate compared than 2, 3 hours and overnight co-incubation time (59/109, 54.1%), (91/114, 79.8%), (83/100, 83.0%) and (96/132, 72.7%), respectively. While, there were no significant differences between other exposure times.

Whereas in 20% BSA group, overnight co-incubation time was significantly higher (P<0.001) compared than 1 and 2 overnight (92/124, 74.2%), (39/107, 36.4%) and (53/109, 48.6%), respectively. In front, overnight co-incubation time was significantly higher (PO.01) compared than 3h co-incubation time (92/124, 74.2%) and (62/101, 61.4%), respectively.

TABLE 4. Cleavage rate and blastocysts yield with sheep oocytes co-incubated with frozen-thawed sperm prepared by using 10% BSA extender

Group	Co-incubation Time (h)	Total Ooctyes	Cleavage Rates (%)	6(1 (%)	7d (%)	8(1 (%)	Blastocycts Rates
10% BSA	**1**	100	42/100 (42.0)a***	/	15/31 (48.4)a6	16/31 (51.6)a6	31/100 (31.0)a***
	2	101	61/101 *(60 A)bd*	/	18/42 (42.8)a*	24/42 (57.1)a*	42/101 *(4l.6)ab*
	3	110	93/110 (84.5)c**	/	35/54 (64.8)6	19/54 (35.2)6	54/110 (49.1)6
	20	125	89/125 **(71.2)rf**	3/54 (5.5)	30/54 *(55.5)ab*	21/54 (38.9)a6	54/125 *(43.2)ab*

* Different superscripts (a, *b)* within the same column of each test demonstrated significant differences (P <0.05).

** Different superscripts *(c, d)* within the same column of each test demonstrated significant differences (P <0.01).

*** Different superscripts (a, *b, c, d)* within the same column of each test demonstrated significant differences (P O.001).

TABLE. 5. Cleavage rate and blastocysts yield with sheep oocytes co-incubated with frozen-thawed sperm prepared by using 15% BSA extender

Group	Co-incubation Time (h)	Total Ooctyes	Cleavage Rates (%)	6(1 (%)	7d (%)	8(1 (%)	Blastocycts Rates
15% BSA	**1**	109	59/109 (54.1)a***	/	12/30 (40.0)a*	18/30 (60.0)a***	30/109 (27.5)a**
	2	114	91/114 (79.8)6	9/49 (18.4)	17/49 (34.7)a***	23/49 (46.9)a	49/114 (42.9)6
	3	100	83/100 (83.0)6	10/49 (20.4)	19/49 (38.8)a**	20/49 (40.8)a*	49/100 (49.0)6c***
	20	132	96/132 (72.7)6	9/51 (17.6)	32/51 (62.7)6	10/51 (19.6)6	51/132 (38.6)a6c

* Different superscripts (a, 6) within the same column of each test demonstrated significant differences (P <0.05).

** Different superscripts (a, 6) within the same column of each test demonstrated significant differences (PO.01).

*** Different superscripts (a, 6) within the same column of each test demonstrated significant differences (P O.001).

TABLE 6. Cleavage rate and blastocysts yield with sheep oocytes co-incubated with frozen-thawed sperm prepared by using 20% BSA extender

Group	Co-incubation Time (h)	Total Ooctyes	Cleavage Rates (%)	6(1 (%)	7d (%)	8(1 (%)	astocycts Rates
20% BSA	1	107	39/107a*** (36.4)	/	9/23 (39.1)	14/23 (60.9)	23/107 (21.5)
	2	109	53/109 *ac* (48.6)	/	10/33 (30.3)	23/33 (69.7)	33/109 (30.3)
	3	101	62/101 *be* (61.4)	/	12/35 (34.3)	23/35 (65.7)	35/101 (34.6)
	20	124	92/124 *d* (74.2)	5/45 (11.1)	18/45 (40.0)	22/45 (48.9)	45/124 (36.3)

* Different superscripts (a, b, c) within the same column of each test demonstrated significant differences (P<0.05). ** Different superscripts (a, b) within the same column of each test demonstrated significant differences (P<0.01). *** Different superscripts (a, b, c, d) within the same column of each test demonstrated significant differences (P <0.001).

4.2.1.2 Blastocysts rate

Among all exposure times effect, three hours was higher in blastocysts rate compared to other exposure time treatments. In 10% BSA group, three hours co-incubation times was higher significantly compared with lh co-incubation time (54/110, 49.1%), (31/100, 31.0%), respectively. While, there were no significant differences in blastocysts rate among other exposure times. In addition, there was significant difference (P<0.01) in numbers of 7d blastocysts rate between 3 and 2 h co-incubation times (35/54, 64.8%) and (18/42, 42.8%). There was significant differences (PO.01) in numbers of 8d blastocysts rate between 2 and 3 h co-incubation time (24/42, 57.1%) and (19/54, 35.2%), respectively.

Whereas in 15% BSA group, the treatment with 3 and 2h exposure times were significantly higher in blastocysts rate compared to lh co-incubation time (49/100, 49.0%), (49/114, 42.9%), and (30/109, 27.5%),

respectively. While there were no significant differences among other exposure times. Moreover, the treatment with lh exposure time was significantly lower in numbers of 7d blastocysts compared to overnight co-incubation time (12/30, 40.0%) and (32/51, 62.7%), respectively. In addition, overnight co-incubation time was significant higher in number of 7d blastocysts compared with 2 and 3h exposure time (32/51, 62.7%), (17/49, 34.7%), and (19/49, 38.8%), respectively. While, there was no significant differences between 2 and 3h co-incubation times.

In 20% BSA group, overnight co-incubation time was significantly higher ($P<0.01$) in blastocysts rate compared to lh co-incubation time (45/124, 36.3%), (23/107, 21.5%), respectively. In addition, three hours co-incubation time was significantly higher ($P<0.05$) compared to lh co-incubation time. While there were no significant differences among other exposure times.

4.2.1.3 Embryo evaluation

Among all groups, the findings generated from this study showed that the proportion of good quality (grade A and B) embryos in all BSA groups were higher (insignificantly) in all brief exposure times compared with traditional IVF protocol (32/68, 47.0%), (45/67, 67.2%), (47/82, 57.3%) *vs.* (13/33, 39.4%), (25/41, 60.9%), (16/29, 55.2%), respectively. Similar finding were observed after embryos thawing. Among all treatments, no significant differences were observed between brief and long exposure times (TABLE. 7 and 8).

TABLE 7. Average morphological score of embryos produced by using brief and standard co-incubation time

Group	Insemination Treatment	Embryo grade A+ B C+ D (%)	(%)	Total
10% BSA	Short	32/68 (47.0)	36/68 (52.9)	68
	Long	13/33 (39.4)	20/33 (60.6)	33
15% BSA	Short	45/67 (67.2)	22/67 (32.8)	67
	Long	25/41 (60.9)	16/41 (39.0)	41
20% BSA	Short	47/82 (57.3)	35/82 (42.7)	82
	Long	16/29 (55.2)	13/29 (44.8)	29

TABLE 8. Average morphological score of embryos produced by using brief and standard co-incubation time

Group	Insemination Treatment	Embryo grade A+ B C+ D (%)	(%)	Total
10% BSA	Short	24/61 (39.3)	37/61 (60.6)	61
	Long	10/28 (35.7)	18/28 (64.3)	28
15% BSA	Short	31/59 (52.5)	28/59 (47.4)	59
	Long	22/38 (57.9)	18/38 (47.4)	38
20% BSA	Short	41/77 (53.2)	36/77 (46.7)	77
	Long	12/26 (46.1)	14/26 (53.8)	26

4.2.2 Bovine serum albumin concentration effects

4.2.2.1 Cleavage rate

Among all BSA groups, the treatment with 15% BSA group was significantly higher ($P<0.001$) in cleavage rate compared than 10, and 20% BSA after 1 and 2h co-incubation times (59/109, 54.1%), (39/107, 36.4%) and (91/114, 79.8%), (53//109, 48.6%), respectively. The treatments with 10, and 15 % BSA were significantly higher in cleavage rate compared to 20% BSA after 3h co-incubation time (93/110, 84.5%), (83/100, 83.0%), and (62/101, 61.4%), respectively. While, no significant difference between 10 and 15% BSA (TABLE. 9, 10, 11,12).

4.2.2.2 Blastocysts rate

Among all BSA groups, the treatments with 10, and 15% BSA was significantly higher ($P<0.05$) in blastocysts rate compared with 20% BSA after 3h co-incubation time (54/110, 49.1%), (49/100, 49.0%), and (35/101, 34.6%), respectively. While, there were no significant differences in blastocysts rate among other groups in 1, 2h and overnight co-incubation times.

After 3h co-incubation time, the treatment with 10% BSA was significantly higher in number of 7d blastocysts compared with 15 and 20% BSA (35/54, 64.8%), (19/49, 38.8%) and (12/35, 34.3%), respectively. While under overnight co-incubation time, the treatment with 15% BSA was significantly higher ($P<0.05$) in number of 7d blastocysts compared with 20% BSA group (32/51, 62.7%) and (18/45, 40.0), respectively.

TABLE 9. Cleavage rate and blastocysts yield with sheep oocytes co-incubated for lh with frozen-thawed sperm prepared by using different concentration of BSA.

Group	Co-incubation time (h)	Total Oocytes	Cleavage Rate (%)	7d (%)	8(1 (%)	Blastocysts Rate (%)
10% BSA	**1**	100	42/100 (42.0)a£	15/31 (48.4)	16/31 (51.6)	31/100 (31.0)
15% BSA	**1**	109	59/109 (54.1)a*	12/30 (40.0)	18/30 (60.0)	30/109 (27.5)
20% BSA	**1**	107	39/107 (36.4) *b*	9/23 (39.1)	14/23 (60.9)	23/107 (21.5)

* Different superscripts (a, b) within the same column of each test demonstrated significant differences (PO.001).

TABLE 10. Cleavage rate and bl astocysts yield with sheep oocytes co-incubated for 2h with frozen-thawed s Derm prepared by using different concentration of BSA.

Group	Co-incubation time (h)	Total Oocytes	Cleavage Rate (%)	6(1 (%)	7d (%)	8(1 (%)	Blastocysts Rate (%)
10% BSA	**2**	**101**	61/101 (60.4) *ab*	/	18/42 (42.8)	24/42 (57.1) aft	42/101 (41.6)
15% BSA	**2**	**114**	91/114 (79.8)a**	9/49 (18.4)	17/49 (34.7)	23/49 (46.9) a	49/114 (42.9)
20% BSA	**2**	**119**	53/109 (48.6)6	/	10/33 (30.3)	23/33 (69.7) ***b***	33/109 (30.3)

* Different superscripts (a, b, c) within the same column of each test demonstrated significant differences (P<0.05). ** Different superscripts (a, b, c, d) within the same column of each test demonstrated significant differences (P<0.001).

TABLE 11. Cleavage rate and bl astocysts yield with sheep oocytes co-incubated for 3h with frozen-thawed s] Derm prepared by using different concentration of BSA.

Group	Co-incubation time (h)	Total Oocytes	Cleavage Rate (%)	6(1 (%)	7d (%)	8(1 (%)	Blastocysts Rate (%)
10% BSA	***i***	**110**	93/110 (84.5) a**	/	35/54 (64.8) a**	19/54 (35.2) a**	54/110 a* (49.1)
15% BSA	***i***	100	83/100 (83.0) a	10/49 (20.4)	19/49 (38.8) *b*	20/49 (40.8) a*	49/100 a* (49.0)
20% BSA	***i***	**101**	62/101 (61.4) *b*	/	12/35 (34.3) *b*	23/35 (65.7) ***b***	35/101 ***b*** (34.6)

* Different superscripts (a, *b)* within the same column of each test demonstrated significant differences (P<0.05).

* * Different superscripts (a, *b)* within the same column of each test demonstrated significant differences (P O.001).

TABLE 12. Cleavage rate and blastocysts yield with sheep oocytes co-incubated for overnight with frozen-thawed sperm prepared by using different concentration ofBSA.

Group	**Co-incubation time (h)**	**Total Oocytes**	**Cleavage Rate** (%)	6(1 (%)	7d (%)	8(1 (%)	**Blastocysts Rate** (%)
10% BSA	**20**	**125**	89/125 (71.2)	3/54 (5.5)	30/54aft (55.5)	21/54 a* (38.9)	54/125 (43.2)
15% BSA	**20**	132	96/132 (72.7)	9/51 (17.6)	32/5 la* (62.7)	10/51A (19.6)	51/132 (38.6)
20% BSA	**20**	**124**	92/124 (74.2)	5/45 (11.1)	18/45 *b* (40.0)	22/45a** (48.9)	45/124 (36.3)

*Different superscripts (a, *b)* within the same column of each test demonstrated significant differences (P <0.05).

** Different superscripts (a, *b)* within the same column of each test demonstrated significant differences (P O.001).

4.3 Third Experiment:

STUDIES ON THE EFFECT OF SUPPLEMENTING SHEEP SEMEN DILUENTS WITH DIFFERENT TYPES OF EGG YOLK ON THE QUALITY OF FROZEN- THAWED SHEEP SEMEN AND IVP EMBRYOS

4.3.1 Effect of using different types of egg yolk in diluents on sheep sperm cryopreservation

4.3.1.1 Sperm motility

All spermatological characteristics of frozen-thawed semen prepared from different diluents summarized in Table 13.

Among all treatments groups, the post-thaw sperm motility was highest in the partridge EY group (54.36± 0.24%) followed by the ostrich and turkey then hen EY (52.54± 0.38%), (52.09± 0.28%), and (51.90± 0.28%) respectively. While, the post-thaw sperm motility was lower in tortoise EY group (34.36± 0.43%) compared to all groups. There was significant difference ($P<0.05$) between partridge and turkey, duck, tortoise, and hen EY. There was significant difference ($P<0.05$) between ostrich and duck, and tortoise EY. Moreover, there was significant difference between turkey and duck, and tortoise EY. There was significant difference between duck and

tortoise, hen EY. In front, the post-thaw sperm motility with hen EY were higher significantly compared to duck, and tortoise EY (51.90± 0.28%), (49.81± 0. 74%), and (34.36± 0.43%), respectively.

4.3.1.2 Assessment of viability

The sperm viability rate (Table 13) of the partridge and ostrich EY were higher compared to other groups. There was significant difference between treatment with partridge EY compared to turkey, duck, and tortoise EY (52.90± 0.47%), (48.81± 0.37%), (48.81± 0.65%), and (35.18± 0.46%), respectively. In another hind, the treatment with tortoise EY was lower compared to all other types of EY (35.18± 0.64%). Moreover, the treatment with ostrich EY significantly higher compared to turkey, duck, tortoise EY. While, the treatment with turkey EY was higher significantly compared to tortoise EY.

4.3.1.3 Assessment of acrosome integrity

The rates of spermatozoa with an intact acrosome were higher in partridge and ostrich EY group compared to other groups. There was significant difference ($P<0.05$) between partridge and turkey, duck, tortoise, and hen EY (66.45± 1.10%), (63.18± 0.46%), (59.72± 0.44%), (42.81± 0.64%), and (61.54± 0.65%), respectively. Moreover, there were significant differences between ostrich and duck, tortoise EY. The treatment with turkey EY were higher significantly compared to duck, and tortoise EY. While the treatment with tortoise EY lower significantly compared to duck and hen EY.

4.3.1.4 Assessment of morphological abnormality

The sperm morphological abnormality rates of the partridge treatment group was lower compared to other groups. In compared, the treatment with tortoise EY observed higher percentage of morphological abnormality compared with other groups. There were significant difference between partridge, ostrich, turkey, duck and hen EY compared with tortoise EY (8.83± 0.18%), (9.02± 0.15%), (9.17± 0.10%), (9.19± 0.15%), and (9.45± 0.12%) *vs.* (14.51± 0.50%), respectively. While, there were no significant differences between other groups.

4.3.1.5 Assessment of membrane integrity

After incubation of spermatozoa in a hypoosmotic solution, cells with swollen tails were considered as spermatozoa with an intact plasma membrane. The treatment with partridge EY showed higher tolerance percentage compared to other groups. In front, the treatment with tortoise EY showed lower percentage of HOST compared to other groups.

There was significant difference ($P<0.05$) between partridge and ostrich, turkey, duck, tortoise, and hen EY (27.2± 0.21%), (25.89± 0.11%), (26.40± 0.21%), (25.12± 0.10%), (23.79± 0.18%), and (25.43± 0.07%), respectively. In addition, there were significant differences between ostrich and duck, tortoise EY. While, the treatment with turkey EY were higher significantly compared to duck, tortoise, and hen EY.

4.3.1.6 Assessment of adenosine triphosphate concentration

Among all groups, the treatment with tortoise EY give lower ATP concentration. The treatments with partridge, ostrich, and hen EY showed significant differences compared with tortoise EY (50.25± 5.88), (44.27±

6.94), (52.14± 8.57) *vs*. (15. 89± 4.18), respectively. While, there were no significant differences in ATP concentration between traditional extender with hen EY compared with partridge, duck, turkey, and ostrich EY.

4.3.2 In vitro production embryos

The total results of cleavage rate and blastocysts yield with sheep oocytes co-incubated for overnight with frozen-thawed sperm prepared by using different types of egg yolk presented in table 14.

4.3.2.1 Cleavage rate

Subsequently, the treatment with partridge egg yolk was higher in cleavage rate compared with other types of EY. Whereas the treatment with tortoise egg yolk showed minimum cleavage rate in front other groups. There was significant differences (PO.05) between PEY and HEN (105/131, 80.1%), (189/270, 70.0%), respectively. There was high significant difference (pO.OOl) between PEY and TOREY (105/131, 80.1%), (74/123, 60.2%), respectively. In addition, there was high significant (PO.OOl) differences between OEY and TOREY (97/125, 77.6%), (74/123, 60.2%), respectively. Moreover, there was high significant difference between DEY and TOREY (105/135, 77.8%), (74/123, 60.2%), respectively.

4.3.2.2 Blastocysts rate

Among all groups, the treatments with ostrich and partridge EY were higher in blastocysts rate compared to other groups. There was significant differences (PO.05) between OEY and HEY (60/125, 48.0%), (99/270,

36.7%), respectively. In addition, there was significant difference (PO.05) between OEY and TOREY (60/125, 48.0%), (43/123, 34.9%), respectively.

Furthermore, the addition of PEY, OEY, TUREY and DEY egg yolk were associated with increased significantly (PO.001) the proportion of zygotes that develop to blastocysts stage at 6d compared to hen EY (24/58, 41.4%), (19/60, 31.7%), (14/50, 28.0%) and (24/52, 46.1%) *vs*. (10/99, 10.1%), respectively. In addition, the treatment with PEY, OEY, TUREY, and DEY egg yolk were higher significantly (P< 0.001) compared to TOREY. Furthermore, there were no significant differences among other groups.

TABLE 13. Spermatological characteristics of frozen-thawed semen prepared from different diluents (mean ± SEM%)

Egg Yolk Source	**Progressive motility** (%)	**Viability** (%)	**Abnormal morphology** (%)	**HOST** (%)	**ATP concentration** (nmol ATP/10^6spermatozoa)	**Acrosome integrity** (%)
Partridge	54.36±0.24a	52.90±0.47a	8.83±0.18a	27.2±0.21a	50.25±5.88a	66.45±1.10a
Ostrich	52.54±0.38a	52.90±0.39a	9.02±0.15a	25.89±0.11Ae	44.27±6.94a	64.09±0.75a£e
Turkey	52.09±0.28A	48.81±0.37A	9.17±0.10aA	26.40±0.21A	40.43±3.98aft	63.18±0.46A
Duck	49.81±0.74c	48.81±0.65c	9.19±0.15a	25.12±0.10c	28.25±2.01aA	59.72±0.44c
Tortoise	34.36±0.43«"	35.18±0.64«"	14.51±0.30A	23.79±0.18M	15.89±4.18A	42.81±0.64«"
* Different differences						
Hen	51.90±0.28aA	50.90±0.43aftc	9.45±0.12a	25.43±0.07ce	52.14±8.57a	61.54±0.65Ac

superscripts (a, *b, c, d, e)* within the same column of each test demonstrated significant

TABLE 14. Cleavage rate and blastocysts yield with sheep oocytes co-incubated for overnight with frozen-thawed sperm prepared by using different types of egg yolk.

EY Source	**Oocytes number**	**Cleavage rates**	**astocysts 6d****	**Blastocysts 7d**	**Blastocysts 8d**	**Blastocysts rates**
Partridge	**131**	5/131a*** (80.1)	4/58 a*** (41.4)	18/58 (31.0)	16/58a*** (27.6)	58/13 *lab* (44.3)
Ostrich	**125**	7/125a*** (77.6)	9/60 a*** (31.7)	26/60 (43.3)	15/60a*** (25.0)	60/125a* (48.0)
Turkey	**129**	96/129a** (74.4)	4/50 a*** (28.0)	15/50 (30.0)	21/50aft* (42.0)	50/129aft (38.7)

Duck	135	105/135a*** (77.8)	4/52 a*** (46.1)	15/52 (28.8)	13/52 a6*** (25.0)	52/135a6 (38.5)
Tortoise	123	74/1236 (60.2)	3/43 6 (6.9)	14/43 (32.5)	26/436c (60.5)	43/1236 (34.9)
Hen	270	189/2706* (70.0)	10/996 (10.1)	30/99 (30.3)	59/99c (59.6)	99/2706 (36.7)

* Different superscripts *(a, b, c)* within the same column of each test demonstrated significant differences (P<0.05).

** Different superscripts *(a, b, c)* within the same column of each test demonstrated significant differences (P<0.05).

**Different superscripts *(a, b, c)* within the same column of each test demonstrated significant differences (P<0.001).

CHAPTER V

DISCUSSION & CONCLUSION

5.1 First Experiment:

"CAN SEMI- DEFINED DILUENTS BE USED SUCCESSFULLY FOR IMPROVEMENT OF FROZEN- THAWED RAM SPERMATOZOA?"

The major aims of our study were to establish semi- defined semen diluents able to improve sperm cryosurvival, fertilization rate and subsequent embryo development *in vitro*. Several researchers have attempted to improve sheep semen diluents and reduce the damage resulting from freezing and thawing process (Dronis et al, 1993; Aisen et al., 2000; 2005; Matsuoka et al, 2006). Hens' egg yolk is generally used as an agent in mammalian semen diluents for protection of spermatozoa against cold shock and the lipid- phase transition effect. However, preparation of uniform diluents containing EY is difficult, because individual EY quality may vary depending on the number of days after lying and the storage conditions. Moreover, semen diluents

containing EY as a cryoprotectant may pose hygienic risks and are difficult to standardize. Therefore, a well- defined and pathogen- free substitute of non-animal origin for EY would be preferable. Therefore, several published work have been conducted for establish semen diluents without egg yolk with different level of success (bull, Muller- Schlosser et al., 1995; turkey, Bakst et al, 1992; rainbow trout, Cabrita et al., 2001; monkey, Li et al., 2005).

The cryopreservation of mammalian semen related with damage of spermatozoa by rapid cooling from physiological temperature (37 °C) down to 5 °C is referred to as cold shock (Amann et al., 1987). The cold shock is a major cause of mitochondrial damage, decline post thaw motility and fertility (Ivanov- Kicheva et al., 1995; Cormier et al., 1997; Ruiz- Pesini et al., 1998; Ford, 2004; Rivlin et al., 2004; Schober et al., 2007). Freezing of sheep semen using Tris-fructose- EY as extender resulted in 39- 47% progressively motile sperm, >60% viability, and 13- 62% acrosomal integrity of spermatozoa stained with Spermac (Mtasuoka et al., 2006; Uysal and Buck, 2007; Valente etal.,2010).

Under our study, higher post-thaw motility were found for the spermatozoa prepared with 10, and 15% BSA compared to control group (48.61± 0.66%), (45.83± 0.55%), and (45.29± 0.66%), respectively. Plus, the treatment with 10, and 15% BSA increased the percentage of sperm viability compared to control group. In addition, the spermatozoa prepared with 10, and 15% BSA decrease the percentage of abnormal sperm morphology significantly compared with traditional extender with 20% EY. Very similar finding were subsequently reported in numerous species and with different concentrations of BSA (turkey, Bakst and Cecil, 1992; bull, Shenk and Amann, 1987; dog, Santos et al., 2007; rainbow trout, Cabrita et al., 2001).

These results were similar to Uysal and Buck, (2007), observed that treatment with 5, 10, 20 mg/ml increased significantly (PO.001) sperm viability compared to control group, and the treatment with 20 mg/ml decreased significantly (PO.001) sperm morphological abnormality compared to control group (11.8± 3.21), and (30.1± 5.30), respectively. In agreement, Anghel et al, (2010) observed that treatment with 5 mg/ml BSA was increased sperm motility (insignificantly) compared with control group (55.25± 0.07%), (50.00± 0.63%), respectively. Plus, increased significantly (PO.05) sperm viability compared to control group (54.51± 2.51%), (50.69± 2.53%), respectively. Rodrigues (1997), determined *in vitro* the influence of Tris- BSA extender on sperm viability during freezing of dog semen using different BSA concentrations (0.25, 0.5 or 1%) compared with each other and with Tris-EY extender. The author found a significant reduction in motility and vigor for semen diluted in Tris- EY compared to semen diluted in Tris- BSA. While, Matsuoka et al., (2006) reported that the post-thaw motility was increased significantly in the 15 and 10% BSA groups compared to control group (58.3± 6.7%), (55.0± 2.9%) and (41.7± 4.4%), respectively. In front, there were no significant differences between treatment with different concentration of and control. Cabrita et al., (2001) observed that the cell viability and fertility were not significantly different from those obtained with control extender. On the contrary, this observation is disagreement with the results of Anghel et al., (2010) observed that added 10 mg/ml BSA lead to a significantly decreased viability (with 13%) and membrane integrity compared to control group.

The acrosome is an important part of the spermatozoon and is essential for the mechanism of fecundation. However, the acrosome is very sensitive to aggressions caused by the process of cryopreservation. Although many factors

can cause acrosome damage, we believe that the extender is fundamental for preserving acrosomal integrity. Under our experiment, the treatment with 10, and 15% BSA were increased significantly the rates of swollen sperm tails (HOST) compared to 20% BSA and traditional extender with hen EY (25.81±0.72), (27.42± 0.66), (21.84± 0.64), and (21.92± 0.65), respectively. In addition, the treatment with 10, 15% BSA increased the percentage of intact acrosome compared to 20% and control group (63.36± 0.94), (68.72± 0.90), (61.36± 0.50) and (61.81± 0.60), respectively.

In general, previous investigators have concluded that phospholipids and lipoproteins act upon the sperm plasma membrane to render it more stable or through the actual alteration of sperm membrane properties by the modulation of its lipid composition (Holt and North, 1988). It is possible that albumin probably acts through the membrane surface, and the beneficial effects of albumin were caused might be through the active removal of sperm membrane lipids, phospholipids modulation, thereby changing the membrane composition (Go and Wolf, 1985; Holt and North, 1988). Given the regulatory role of the cholesterol level in membrane function.

In agreement with our results, Uysal and Buck, (2007) observed that the treatment with 5, 10 mg/ml BSA increased the percentage of swollen sperm tails compared to control group (41.5± 5.87), (48.5±6.40), (36.9± 4.40), respectively. Anghel et al, (2010) reported that treatment with 10 mg/ml BSA increased HOST (insignificantly) compared to control group (52.51± 0.70), (49.93± 1.68), respectively. While, Matsuoka et al., (2006) observed that there were no significant differences in HOST among the diluents with BSA and control group. In addition, the rates of spermatozoa with an intact acrosome in control group were significantly ($P<0.05$) lower than those 5, 10, and 15%

BSA groups. Conversely, Santo et al, (2007) observed that cryopreserved dog spermatozoa with Tris- EY better than Tris with different concentration of BSA.

Moreover, under our experiment no correlation between ATP concentration and sperm motility were observed. This finding contrast with the report by Parker and McDaniel, (2007) referred a positive correlation ship between sperm motility and ATP concentration. However, the treatment with 10% BSA was higher (insignificantly) in ATP concentration compared with control group (73.95± 25.84), (48.57± 8.96) nmol ATP/ 10^6 spermatozoa, respectively. The treatment with 10% BSA higher significantly ($P<0.05$) in ATP concentration compared with 20%BSA While, there were no significant differences between 10, 15% BSA and control group.

The second major aim of the present study was to evaluate the effect of the semi- defined semen diluents on fertilization rate and subsequent embryo development *in vitro*. Several works were reported that the motility of spermatozoa is a prerequisite to obtain high fertility rates. This basic physiological function achieved by continuous movement of the axial filament of spermatozoa and strongly depends on the availability of energy of mitochondrial energy metabolism. Therefore, a high motility of spermatozoa should correlate with high fertility rates. Whereas, sperm assessment like sperm motility in vitro have little/ or no correlation with the ability of the sperm to fertilize (Watson, 1979; Maxwell and Watson, 1996; Papadopoules et al., 2005), or for further development of resulting zygotes (Budworth et al., 1988; Eppleston and Maxwell, 1993; Evenson et al., 2002). Therefore, the selection of the most adequate fertility prediction parameter has been the goal of several authors. The percentage of swollen sperm tails (Matsuoka et al.,

2006) and sperm motility analyses (Morris et al., 2001) were recognized as useful methods. Some authors were unable to relate in vivo fertility with in vitro sperm functional tests or homologous IVF (Papadopoulos et al., 2005). Although in our study, a relatively high correlation was observed between post-thaw semen quality and IVF/ IVC or was obtained. The treatment with 10% BSA produced higher cleavage rate compared other groups in both lambs and ewes oocytes. The cleavage rate of lams oocytes was higher significantly in treatment with 10% BSA compared to control group. Subsequently, the blastocysts rate in 10% BSA higher (insignificantly) compared to other groups in both lamb and ewes. Moreover, the treatment with 15% BSA increased the numbers of 6 and 7d blastocysts versus other treatment. There was significant differences (PO.05) in number of 6d blastocysts between 15% compared to 10% BSA (18/109, 16.51%), (5/117, 4.27%), respectively. There was significant differences (PO.05) in number of 7d blastocysts compared with control group (70/109, 64.22%) and (48/99, 48.48%), respectively.

In conclusion, the sperm cryosurvival were improved by the presence of 10, and 15% BSA versus traditional diluents with 20% EY. These results were supported by in vitro production sheep embryos data. Therefore, could be substituted successfully as semi-defined diluents in place undefined extender with EY. This finding in contrast with the recent report by Valente et al., (2010) reported that EY is difficult to replace in ram semen extender. Furthermore, there is a positive correlation ship between sperm characteristics and production sheep embryos in vitro by using 10, and 15% BSA.

5.2 Second Experiment:

THE EFFECT OF REDUCED SPERM- OOCYTES EXPOUSER TIME ON OVINE EMBRYO PRODUCTION

The primary goal of our study was to investigate whether brief oocyte-sperm co-incubation time is long enough to achieve good fertilization and embryo sheep production *in vitro.* We select a co-incubation time as low as 1, 2 and 3h with the risk of failure sheep fertilization and embryonic development to be low. Data from previous publications have contributed to the set-up of this study. Firstly, the study was inspired by the data from human in vitro fertilization data demonstrating that the achievement of good fertilization rate within less than lh co- incubation time (Coskun et al, 1998;

Lin Shu et al., 2000; Bungum et al., 2006). Secondly, the previous data in first point were supported by porcine and ewes IVF data that the penetration and blastocysts rates where improved after short gamete co-incubation time (< 3h) (Alminana et al., 2008; Morton et al., 2005).

Our results showed that successful in vitro fertilization occurs within a brief period after exposures of the oocyte to spermatozoa in all groups. Among all brief co-incubation treatments, the cleavage rates were increased significantly high with gamete co-incubation time. A Three hours co-incubation time was superior significantly versus 1, and 2 h co-incubation times. Indeed, a three hours co-incubation was higher in cleavage rates than overnight co-incubation times. In addition, 3 h co-incubation times lead to higher percentage of blastocysts rate (insignificantly) compared to 1, 2h and standard co-incubation fertilization protocols. Which could be expected since the spermatozoa needs at least 3 h to penetrate matured sheep oocytes with acceptable fertilization rates. To our knowledge this is the first study demonstrating successful sheep fertilization within 1 h co-incubation period.

Very similar finding were subsequently reported in fertilization rate between brief or overnight co-incubation times (pigs, Grupen and Nottle, 2000; Gil et al., 2004; Alminana et al., 2008; bovine, Sumantri et al. 1997). Moreover, Dode et al., (2000) observed that the percentages of bovine cleavage increased progressively ($P< 0.05$) with co-incubation time. While other researcher were observed that the cleavage rates were similar between short and standard co-incubation times (Gianaroli et al., 1996a, b; Gomez and Diez, 2000; Anvari et al., 2009). Morton et al., (2005) observed that the oocyte penetration did not differ between duration co-incubation times. In front, our results were higher in cleavage rates than Morton and co-authors.

Especially, we used frozen- thawed semen plus 2, 3 h co-incubation times (79.8- 84.5%) *vs.* (42.9%), respectively. In our opinion these differences in IVF related to the presence of cumulus cells (partially *vs.* denuded oocytes), extender media and sheep type.

On the other hand, all these observations were disagreements with the results of other researchers were reported that fertilization rate was significantly lower in short insemination versus overnight insemination (human, Lundqvist et al, 2001; Barraud- Lange et al, 2008; bovine, Pavlok, 2000;Saekietal, 1991).

Whereas among the different concentration of BSA and sperm- oocytes co- incubation time effects, the cleavage rate was increased surprisingly in 20% BSA compared with 10, and 15% BSA after 1 and 2 h co-incubation times, while there were no significant differences in further co-incubation times. These results contrast with those reported in first experiment, that using 20% BSA as cryoprotective in sheep semen diluents leads to decreased motility and ATP concentrations compared with 10 and 15% BSA (review; results experiment one). These results confirm two points. First; sperm assessments like sperm motility in vitro have little/ or no correlation with the ability of the sperm to fertilize (Watson, 1979; Maxwell and Watson, 1996; Papadopoules et al., 2005), or for further development of resulting zygotes (Budworth et al., 1988; Eppleston and Maxwell, 1995; Evenson et al., 2002). Second, under in vitro condition it is possible that the sperm concentration usually used for IVF can overcome the differences in the sperm quality obtained by different diluents/ or methods to select spermatozoa. It seems that in this case the three methods, studied can be used with no detrimental effect on blastocysts rate.

Several researchers were reported that long co-incubation times and high numbers of sperm leads to increase the incidence of polyspermic insemination (human, Bungum et al., 2006; Gianaroli et al, 1996a, b; Kattera et al., 2003; bovine, Chian et al., 1992; Sumantri et al., 1997; porcine, Funahashi and Nagai, 2000). Although, Morton et al., (2005) observed that no differences were observed between short and long co-incubation insemination times in sheep polyspermic fertilization (3, 4.6%), and (2, 3.1%), respectively. Conversely, in our experiment we didn't observe any evidence of polyspermic insemination with short and long co-incubation time.

Under the high risk of brief co-incubation time could reduce embryo production. High levels of embryonic development were obtained after brief co-incubation times. Three hours co-incubation time was higher in blastocysts rate compared with 1, 2 h and overnight co-incubation times (54/110, 49.1%), (31/100, 31.0%), (42/101, 41.6%), and (54/125, 43.2%), respectively. In agreement with our results, Morton et al., (2005) reported that reducing gamete co-incubation time increased ($P<0.05$) sheep blastocysts development in short *vs.* long co-incubation time (240/602, 39.9%) and (205/617, 33.2%). Gianaroli et al., (1996) observed that the rates of embryos were significantly higher in short versus long insemination groups. In porcine, Alminana et al., (2007) reported that the blastocysts formation rates tend to be higher in 10 min *vs.* 6h co-incubation time. Conversely to these results, Enright et al., (2000) reported that decrease insemination time leads to decrease formation of bovine blastocysts.

In human studies, several studies reported that reducing the gamete co-incubation length during IVF leads to increase normal fertilization (Gianaroli et al., 1996b; Quinn et al., 1998; Dirnfeld et al., 1999; Hyun et al., 2000), and

embryo development (Gianaroli et al., 1996a, b; Lin Shau et al., 2000). Furthermore, increased embryo morphology (Dirnfeld et al., 1999; Lin et al., 2000; Quinn et al., 1998; Kattera et al., 2003), implantation and pregnancy rates (Dirnfeld et al., 1999; Kattera et al., 2003). Indeed, our data on blastocyst yield contradict the earlier reports which indicated that the duration of the sperm-oocyte interaction has no impact on blastocyst yield (Long et al., 1993; Rehman et al., 1994) or decreased the blastocysts yield (bovine, Enright et al., 2000) and confirm the findings of polyspermic fertilization (Alminana et al., 2007; Morton et al., 2005; Gianaroli et al., 1996).

Under our experiment condition there were no significant differences in embryo quality between brief and standard co-incubation times, and before or after freezing. Although, the percentage of A+B grade embryos were higher in brief compared to long co-incubation time. Very similar finding was reported by Gianaroli et al., (1996a, b) that observed an equivalent or better embryo quality in the shortened sperm-oocyte co-incubation group, in regards to the morphological evaluation compared to overnight co-incubation. In human, similar results were observed that no differences in embryo quality between short and long spermatozoa- oocytes co-incubation (Gianaroli et al., 1996 a; b; Free et al., 1998; Bungum et al., 2006; Anvari et al., 2009). While, Koifman et al., (1995) and Quinn et al., (1998) observed some of the statistical differences. The inferior embryo quality obtained from the overnight exposure of oocytes to large numbers of spermatozoa is most likely due to the suboptimal culture conditions created by the excessive generation of ROS and other deleterious products from sperm metabolism (Gianaroli et al., 1996a, b). Therefore, the shortening of egg- spermatozoa exposure may improve embryo morphology by preventing the negative effect caused by oxygen free radicals

produced by spermatozoa during prolonged exposure. It may be contributes to the zona hardening effect, through the release of zona hardening factors, such as tissue type plasminogen activators (Wasserman, 1988). Conversely, other researchers were reported that the quality of embryos was significantly improved with short exposure time (bulls, Smantri et al, 1997; Quinn et al, 1998, Kattera et al., 2003, Dirnfeld et al., 1999). Lundqvist et al., (2001) reported that the embryo quality was significantly higher in long insemination than short insemination. Moreover, Anvari et al., (2009) reported that embryo generated from short insemination IVF could tolerate the cryo-environment better than embryos from long insemination protocol.

In conclusion, the effect of the short duration of gamete co-incubation tested in the present study may be attributable to the potential for causing a minimum level of cellular damage and the consequent harmful metabolites. Therefore, short co-incubation of oocytes and spermatozoa produced better or equivalent fertilization, and embryos rate than the generally accepted overnight protocol. Finally, the time of co-incubation is also an important aspect to be considered when one wants to increase IVF results. **5.3 Third Experiment:**

STUDIES ON THE EFFECT OF SUPPLEMENTING SHEEP SEMEN DILUENTS WITH DIFFERENT TYPES OF EGG YOLK ON THE QUALITY OF FROZEN- THAWED SHEEP SEMEN AND IVP EMBRYOS

The main finding from this study is the replacement of hens egg yolk in sheep semen cryopreservation medium with partridge, and ostrich egg yolk improve the post- thaw sperm quality, and in vitro production embryos. To our knowledge there are no studies that examine the effect of used partridge, ostrich, and tortoise EY as cryoprotective in sheep semen diluents, and there effects on production embryos *in vitro.*

Several authors were reported that the different composition of the yolk types, particularly the amount of cholesterol, fatty acids and phospholipids, were thought to potentially afford a greater level of protection to sperm against damage during freezing- thawing procedure, plus egg yolks triacylglycerols and phospholipids constitute the main source of energy and structural lipids for the developing embryo. These differences in the proximate composition in EY could be attributed to several factors, including different in species, bird growth stage and feed habits.

In 1988 Pasard and co-authors reported that the duck egg yolk had more protein, lipid, cholesterol (Maurice et al, 1994), monounsaturated fatty acids (MUFA), phosphotidylinositol (PI) than hens EY. Indeed, Turk and Barnett, (1971) reported that duck egg was much richer in cholesterol than chicken eggs. In front, Kazmierska et al., (2005) reported that the highest amount of cholesterol was observed in ostrich compared to duck, and hen EY (16.29), (10.81), and (13.91) mg/g of yolk, respectively. Similar results were observed by Sinanoglou et al., (2011) that ostrich and turkey EY were contain a significantly ($P<0.05$) higher cholesterol content compared to duck EY (17.9± 0.55), (16.5± 0.73), and (13.84± 0.7) mg/g of yolk, respectively. In agreement, Bathgate et al., (2006) reported that hen EY contained more cholesterol than duck EY (22.9± 0.02), (10.6± 0.01), mg/g of yolk, respectively. Whereas,

other researchers were reported that the mean cholesterol content of yolk 15-19 mg/g for chicken and ostrich (13 mg/g) (Reiner et al., 1995; Horbanczuk et al., 1999). In addition, Sinanoglou et al., (2011) reported that the egg yolk total lipid content was decreased in the following order: ostrich, duck, and turkey, and the fat from ostrich eggs was characterized by the highest content of a-linolenic and a low level of linolic acid and palmitoleic acid. In front, duck eggs were characterized by the highest total fat content compared to hen EY (Kazmiersika et al., 2005; Bathgate et al., 2006). The analysis of the content of fatty acids indicates the duck egg yolk characterized by highest concentration of arachidonic and oleic acids. While ostrich EY have higher levels of a-linolenic acid compared to duck EY (7.24), (5.17), respectively (Kazmierska et al., 2005). In front, hen's yolk contained higher proportions of 18C MUFA than duck yolk (Bair and Marion, 1978; Surai et al., 1999; Choi et al., 2001; Bathgate et al., 2006). The eggs from hens were characterized by a significantly higher content of stearic acid (8.54%) than the eggs from other species. The total content of monoenic acids was from 40- 47%. While, the yolks from ostrich eggs had the highest content of palmitoleinic acid (9.61%) (Kazmierska et al., 2005). These chemical differences between yolks may explain the differences in frozen- thawed motility and integrity of sperm when frozen in extender containing the different egg yolks types (Bathgate et al., 2006).

The freeze-thawing process resulted in changes to the sperm membrane, including a loss of fluidity which may be related to the level of cholesterol in the membrane Buhr et al., (1994). Indeed, several authors have observed change in the lipid composition of sperm membranes after cryopreservation, including a loss of cholesterol (Buhr et al., 1994; Cerolini et al., 2001;

Maldjian et al, 2005; Moore et al, 2005). This less cholesterol content in cryopreservation has an impact on cryopreserved sperm longevity. In front, high cholesterol level in sperm membrane most likely benefits sperm cells by eliminating or at least lowering the temperature at which sperm plasma membranes undergo the lipid phase translation from the gel state as the cells are cooled (Ladbrooke et al., 1968). Cholesterol modulates the fluidity of membranes by interacting with the fatty acyl chains of the phospholipids and controls membrane structure by interacting with the phospholipid hydrocarbon chains (Darin-Bennett and White, 1977), and at temperatures below the phase transition, forces the chains apart, making the membrane more stable (Quinn, 1989). In addition, the cholesterol to phospholipid ratio of sperm membranes plays an important determinant of membrane fluidity, and sperm resistance to cold shock damage (Watson, 1981; Parks and Lynch, 1992). Darin- Bennett and White, (1977) reported that cholesterol: phospholipid ratio is very high in sperm from species whose sperm are resistant to *"cold shock"* damage (0.88-0.99, like rabbit and human), and low in sperm from species whose sperm are susceptible to cold shock (0.38 and 0.45, like bull and ram). Moreover, cholesterol efflux from sperm membranes contributes to signaling mechanisms that control sperm capacitation, and the longevity of sperm in this pre-capacitated state is reduced (Travis and Kopf, 2002).

Very similar results were observed in our experiment with species with high level of cholesterol, the frozen- thawed spermatozoa with partridge and ostrich egg yolk showed higher sperm quality compared to other groups, with significant differences compared with hen EY (54.36± 0.24%), (51.90± 0.28%), respectively. The exact mechanism by which cholesterol improves sperm cryosurvival is still not known. But the high level of cholesterol may

broaden the phase transition of the sperm membranes, thereby reducing lipid of the same species from aggregating into specific domains within the membrane (Drobnis et al., 1993), as well as increasing membrane fluidity at lower temperatures (Purdy et al., 2005). By increasing the cholesterol: phospholipid ratio of ram sperm, sperm membranes may exhibit reduced membrane phase separations and therefore reduced leakage of cellular components (such as potassium) from the cell (Drobnis et al., 1993) or it may inhibit calcium entry into the sperm, which is a prerequisite for capacitation and/or senescence (Visconti et al., 1999). Indeed, Purdy and Graham (2004) found the addition of cholesterol to bull sperm before cryopreservation increased the percentage of motile and cryosurvival rate. Similar results were observed also in other species (stallion, Combes et al., 2000; Moore et al., 2005; donkey, Alvarez et al., 2006; pig, Galantino- Homer et al., 2006; ram, Mocea et al., 2010). However, by understanding the mechanism by which cholesterol affects sperm membranes a greater understanding of membrane physiology may be gained during cryopreservation, and develop even better techniques for cryopreserving sperm.

On the other hand, our results with duck EY showed lower sperm motility compared to traditional semen extender with hens EY. Subsequently, the ATP concentration of using duck EY was less than hens EY (28.25± 2.01), (52.14± 8.57) nmol ATP/ 10^6 spermatozoa, respectively. Our results in contrast with those reported by (Prasard et al., 1988; Maurice et al., 1994; Burris and Webb, 2009) that using duck EY improve the protection of sperm during freeze-thaw process resulting in higher sperm motility compared to hen EY. The reason for these differences is unclear, probably related to bird feeding regime.

In agreement with sperm quality results, the positive results of treatment with partridge and ostrich EY were continued with in vitro production embryos. We obtained a higher cleavage rate with partridge and ostrich egg yolk in comparison with hen EY. However, the percentage of cleavage is highly correlated with the intrinsic quality of the sperm and oocytes (Lonergan et al, 1999), as well as culture conditions that are favorable to the metabolic activity of the male and female gametes. In our study, random distributions of oocytes were taken in all groups. The cleavage rates were higher after fertilization with partridge, ostrich, turkey, and duck egg yolk compared with hen EY. There was significant difference ($P< 0.05$) between frozen semen with partridge than hen EY. Some of the cleaved embryos were not able to sustain an embryonic development as they stopped their development at 8-16 cell stage corresponding with the time of embryonic genome activation (Telford et al., 1990). Subsequently, the blastocysts rates were higher in partridge, ostrich, turkey, and duck EY treatment compared to hen EY. There was significantly differences ($P<0.05$) in blastocysts rate between ostrich and hen EY. However, no data are available for ram semen cryopreservation using different egg yolk sources to compare with our results. In conclusion, our results have suggested that supplementation of partridge EY improve the cryo-tolerance of sheep semen, and could be used as an alternative to hen egg yolk in extender. Moreover, the ostrich EY offers advantages over hen EY in production of sheep embryos.

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