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EVALUATION OF RECIPIENT AND EMBRYO FACTORS ON SUCCESS OF INTER-BREED EMBRYO TRANSFER IN SHEEP

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ABSTRACT

Studies on multiple ovulation and embryo transfer (MOET) showed that this technique is widely acknowledged as the best available option to a low cost route of exporting genetic material across international boundaries along with control disease transmission during imports. In addition to that MOET have been used so much to proliferate genes of reputedly superior stock. An appreciation of the potential benefit of MOET was perhaps best demonstrated in dairy cows, however, the application of MOET techniques to sheep has been much more slower. The success of this technique is very unpredictable due to many factors are contributing to the overall results.

Experiments were initiated to determine effects of factors related to recipient and embryo on MOET success in inter-breed embryo transfer in sheep. Three experiments were conducted to compare recipient and embryo genotypes, cryopreservation techniques, and pregnancy rates obtained with fresh and frozen embryo transfers. Further investigations were conducted on such as number of corpus luteum, site of ovulation and transfer in recipients and number of embryos used as single or in pairs, stage of embryo development and quality grades.

In Exp. I, a total of 75 ewes from three breeds (Awassi, Morkaraman and Tuj) were used in twin frozen (ethylene glycol, traditional freezing) thawed embryo transfer. Corpora lutea (CL) at the time of transfer and ovulation site were recorded in recipients. Two embryo genotypes (Romanov and Charollais) were used to evaluate effects of embryo genotypes and the stage of embryo development on MOET success.

Experiment II was conducted over two seasons and across two trials. In the first trial, recipient ewes were grouped two such as Fat tailed (n=48) and Thin tailed (n=47) ewes originated from different regions and were used in single fresh embryo transfer. Second trial was set up to access reproductive success of crossbred prolific recipients (n=36) compared to non prolific native breed of recipients (n=61) in twin fresh embryo transfer. Two trials in the second experiment were conducted to determine the effect season of transfer, genotype or group of recipients and the interaction of season x genotype or group of recipients. Same parameters in exp. I was included in the second experiment for evaluations of factors related to recipient or embryo.

Experiment III was conducted to determine the success of alternative cryopreservation technique, vitrification with open pulled straw, in MOET program. Blastocyst and hatched blastocyst stage of embryos were vitrified and transferred into recipients as single. Intra vaginal sponges were used in estrus synchronization and all recipient ewes received 400 I.U. eCG i.m. at sponge removal at day 12. On d 6.5-7.5 of onset estrus, embryos (fresh/frozen and single/twin) were transferred into recipients after morphological evaluation and quality grades.

Overall MOET success in exp. I was higher in Awassi breed (66.6%) than Morkaraman (56.7%) and Tuj (53.7%) breed of recipients. Although there is no significant difference for pregnancy and embryo survival rates, Awassi breed was determined as better recipients with 10-13% higher MOET overall success. Ovulation rates, site of ovulation and transfer did not significantly affect reproductive success of recipients. Embryo genotype was not found a significant factor in frozen-thawed embryo transfer. Investigation on the effect of stage of

embryo development showed that expanded blastocyst were more favorable for both pregnancy (75%; P>0.05) and embryo survival (100%; P<0.05) rates. Post thaw culture duration of frozen embryos significantly (P<0.05) affected embryo survival rates and 15-20 min culture after thawing resulted with highest (100%) embryo survival while shorter duration of culture favored pregnancy rates.

In experiment II, ovulation rates were significantly (P<0.05) different in breeding (1.7±0.08) and out of season (1.0±0.11). However, pregnancy rates were not differed between two seasons. Fat tailed ewes with restricted breeding season had not significantly but lower pregnancy rates (42.6%) than those observed in Thin tailed (61.5%) recipients originated from warm climate and low latitude region. Second trial conducted in experiment II showed that greater (21.5%; P>0.05) pregnancy rates were achieved with prolific F1 crossbred recipients compared to those obtained with non prolific native breeds (58.3%). Comparison between two trials to evaluate the effect of single versus twin fresh embryo transfer, it was found that similar pregnancy rates were observed in recipients received embryo in pairs (58.3%) or single (52%). Grade of embryos did not significantly affect the pregnancy rates, but embryos less than Grade 2 resulted with 26.5% more pregnancy losses. The number of corpora lutea at the time of transfer was found more meaningful in twin embryo transfer. As it was observed in exp. I, site of ovulation and transfer was not found an important factor in pregnancy outcomes.

Vitrification of later stage of embryos, expanded and hatching blastocyst, resulted with 79.2% and 76.4% pregnancy rates, respectively. In this experiment left uterine horn transfers of vitrified embryos after thawing were found superior to right uterine horns. Recipient ewes

with single ovulation were recorded with not significantly but relatively higher pregnancy results.

In conclusion, recipient “uterine genotype” was one of the main determinant in variability of embryo transfer in inter-breed embryo transfer. Embryo survival rates rather than pregnancy rates were more sensitive to the stage of embryo development in conventional frozen embryo transfers. Number of embryos transferred as single compared to twin were resulted with similar pregnancy rates in fresh embryo transfer. Season of MOET was not found as a limiting factor for pregnancy success in aseasonal recipient breed while native sheep with restricted breeding season was source of variation of overall success. Vitrification as simpler and more cost effective cryopreservation technique is applicable in MOET programs with its high pregnancy success.

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CHAPTER I

INTRODUCTION

Small ruminant production is a very significant component of livestock production throughout the world and more especially in the developing countries (Timon and Hanrahan, 1986). Today, the world's sheep population is around 1000 million, which produce annually approx. 14 million tones of sheep meat (the annual world production of pig meat is approx. 100 million tones and that of beef meat is 65 million tons) (FAO, 2008). International trade in sheep meat is limited (around 7% of the total production), with the bulk of this trade consisting of exports from the southern hemisphere (New Zealand has 47% and Australia has 36% of the total) to the European Union, North Asia, the Middle East and North America.

Sheep give rise to four major products: meat, wool/hair, milk and skins. In many parts of the world, particularly the temperate regions, meat is the major product and the importance of meat production is increasing world-wide. An important attribute of the sheep is that it can live and produce on land unfavorable for other forms of agriculture. Numerous breeds of single or dual purpose types of sheep have been developed with the capacity for high levels of production under appropriate environments and management systems (Owen, 1976). Sheep also have the ability to forage and survive in areas, where cattle would perform poorly.

There has been a universal decline in sheep populations throughout the world over the last five years, especially in New Zealand and Australia, closely linked to economic and climate factors. As production in a number of key countries declines and supply tightens, the current demand in the short-term will not be able to be met by New Zealand and Australia,

which should strengthen prices world-wide. Counter to this, is the world-wide economic slowdown that may have a depressing effect on consumer meat demand and favor a shift towards poultry meat, which remains the cheapest source of meat protein. Current world consumption of sheep meat stands at about 2.5 kg per person annually, out of a total meat consumption of 41.6 kg per person annually (FAO, 2008).

Turkey has been recognized as having an animal production based on potential agriculture since ancient times. Small ruminants have been important components of rural life and still play a substantial role in the livelihood of farmers (Ocak et al., 2010). Turkey has been one of the major sheep and goat producers of Europe and the West Asia and North Africa (WANA) region in the 20th century. Sheep breeding is one of the most important agricultural sectors in Turkey, and is ranked second after cattle for meat production. Breeding of small ruminant in Turkey exhibited a reduction during years. According to the data from Turkish Statistical Institute, the population for sheep and goat were about 34 and 25 million heads in 1960, respectively. In 2007, the numbers dropped into 25 and 7 millions heads for sheep and goat respectively (TUIK, 2009). The main reasons for the decreases in sheep population were expressed by Ocak et al. (2010) as low market place, inadequate support policy by the state, migration of the farmers to big cities for new jobs, decrease in consumer demands, high costs of inputs, etc.

The growth rate of the population in Turkey is 2% per annum and the generations need protein of animal origin which has to be supplied by animal production. Nearly 90% of sheep are fat-tailed and Akkaraman and Morkaraman sheep are the most numerous and make up nearly 65% of the total sheep population (Yalcın, 1986). The Daglıc¸ sheep is the third largest

population known by its hardiness and adaptation to marginal conditions that are reflected in its small size, low reproductive efficiency, and low milk and wool production. The indigenous thin tailed breeds of Turkey are the Kıvırcık and Karayaka. The Gokceada (Imroz) and Sakız (Chios) are native to the Eagean Islands. The Turkish Merino is a synthetic breed derived from two different crossbreeding programmes namely of Kıvırcık × German Wool and Meat Merino and Akkaraman × German Wool and Meat Merino (Gürsoy, 2006). To maximize the sheep production, intensive breeding and feeding methods should be introduced to sheep breeding. Likewise by the widening of the lamb-fattening programs in Turkey the carcass yield in sheep breeding has increased from 13 kg to 19 kg since 1980s (Kutlu et al., 2003).

Interest in sheep meat production has increased over the last few years, particularly lamb meat with lower fat content, which reflects with consumer preference (Woodward and Wheelock, 1990; Momani Shaker et al., 1996). Many authors reported that it is relatively easy and quick to increase fecundity and growth ability of lambs to an optimum level by means of crossing domestic breeds with prolific and mutton breeds (Romanov, Finnish sheep, Charollais, Texel, etc.), as well as forming synthetic breeds or lines (Momani Shaker et al., 1994,1995). Breed substitution has been shown to be a rapid, cost effective means of improving lamb carcass quality (Carson et al., 1999). Stratified crossbreeding program have been used in the sheep industry in Turkey. Texel, Ile de France, Dorset, Hampshire, Lincoln, B. Leciester and German Black face sires have been shown to improve lamb growth rate in the western region of the country (Kaymakci et al., 1999). Production characteristics of sheep breeds in Turkey are relatively low, especially reproductive efficiency. The most important factor determining the success of sheep production is reproductive efficiency, which is the net biological accomplishment of all reproductive activities i.e. puberty, oestrus, ovulation,

fertilization, implantation, gestation and successful lambing as well as survival and growth after birth. For any trait affecting efficiency of meat production, there is useful genetic variation among sheep breeds worldwide.

The production traits of Turkish native sheep are far below the economic production targets and this fact caused a dramatic decline of sheep population in Turkey. The industry needs to produce uniform, nutritious, lean lamb that satisfies the eating preferences of consumers and to improve reproductive efficiency and reduce labor requirements so that seed stock and commercial flocks are both practical and profitable under a range of production environments. This challenge can be met by taking into account of advantage of breed diversity which is important aspect in any livestock breeding program, but is extremely critical in the sheep industry. When establishing a commercial sheep flock, producers are advised to select maternal-type sheep for their ewe flock and a terminal sire to produce large numbers of lambs with desirable carcass traits. This breeding program brings the question of acceptable way of introducing new genetic. Embryo transfer offers new opportunities for genetic improvement in sheep breeding. While this technique is still expensive and its use will mainly be for breed improvement, rather than in commercial production.

The technique of multiple ovulation and embryo transfer (MOET) in sheep has existed from the begging of the last century (Ishwar and Memon 1996). However, commercial application to the sheep industry did not occur until late 80's (Ishwar and Memon 1996, Naqvi et al 2001). MOET can be used as the main component to a low cost route of exporting genetic material across international boundaries (Cognie 1999, McKelvey 1999). This way is widely acknowledged as the best available option to control disease transmission during

imports of genetic material into a particular country and hence to ensure the health status of the national stock (Singh et al 1997, Parker et al 1998, Thibier and Guerin 2000). MOET has the added advantage of allowing imported stocks to develop in recipients well adapted to local conditions and can be used as a means for disease resistance in the breeding objectives and breeding strategies in either indigenous or other susceptible sheep (Saberivand and Outteridge 1996). An appreciation of the potential benefit of MOET was perhaps best demonstrated in dairy cows, however, the application of MOET techniques to sheep has been much more slower mainly due to the lower economic value of these animals because they are not the main livestock for red meat production in nonmuslim countries (McKelvy et al.,1986, Ishwar and Memon 1996, Loi et al 1998). The success of this technology is a main determinant for suitability and feasibility of breeding program in developing countries. The success of embryo transfer depends on factors associated with the embryo, the recipient or an interaction among factors of the embryo and recipient. Many studies have focused on these factors in cattle. However, scarce studies have been conducted in sheep.

The term “maternal effect” indicates an influence of the dam on its offspring other than through the genes transmitted to it. The genotype of the dam therefore affects the phenotype of the young through a sample of her direct, additive genes for growth as well as through her genotype for maternal effects on growth (Meyer, 1992). Variation between females in maternal performance may arise from either genetic or environmental causes. Maternal effects are important in sheep because of the dependence of lambs on their mother`s milk until the time of marketing or weaning (Bradford, 1972).

Multiple ovulation and embryo transfer (MOET) has the potential to increase the rate of genetic gain through the female line. However, the full realization of this potential depends upon maximizing the number of progeny born from high merit females. While influenced by superovulatory responses and fertilization rates of donors in any MOET program, the number of progeny per donor is also directly related to the survival rate of transferred embryos. Factors specific to both embryos and recipients have been suggested to affect the survival of transferred embryos in cattle, sheep and goats. Among them, stage of embryo development, embryo quality, number of corpora lutea, and age and parity of the recipients have been reported to be of significance (Donaldson, 1985; Hasler et al., 1987; Looney et al., 1984; Alabart et al., 1995; Thompson et al., 1995; Armstrong and Evans, 1983; Armstrong et al., 1983).

Progesterone plays a vital role in early embryo development, implantation and the establishment of pregnancy. Plasma progesterone concentrations in recipient animals are related to the number of ovulations or corpora lutea in sheep (Ashworth et al., 1989, Trounson and Moore, 1974). While embryo survival has been reported to increase with an increase in plasma progesterone concentrations in cattle (Hasler et al., 1980 and Remsen at al., 1982) and the number of corpora lutea in goats (Armstrong et al., 1983), little information of this type exists for sheep. Since there is evidence for considerable variation in progesterone secretion over successive pregnancies in sheep (Ashworth et al., 1989). Embryo transfer experiments have demonstrated the critical importance of the state of the uterine environment for embryo viability and that the embryo must be present in the uterus by Day 12.5 post estrus in the ewe to prevent corpus luteum regression (Moore, 1985). Though it is known that an embryo must be present in the uterine lumen by Day 13 postestrus for luteolysis to be prevented (Moor and

Rowson, 1966a,b), the way in which the embryo overcomes luteolysis remains unknown. Rowson and Moor (1967) demonstrated that daily intrauterine injections of homogenates of 14-15-day-old sheep embryos into nonpregnant sheep extended the length of the estrous cycle.

The first success after transfer of a frozen-thawed embryo was obtained 23 yr ago (sheep:88; goat:7). Embryos were first stored in media containing dimethylsulphoxide (DMSO) as cryoprotectant, but ethylene-glycol has emerged as a superior cryoprotectant with higher survival rates approaching those achieved with fresh embryos (Brebion et al., 1992, Tervit and Goold,1984) and with the possibility to transfer directly the embryo after thawing. Sheep and goat embryos are able to survive vitrification procedures and with further research this method may provide an economical alternative to the current freezing methods requiring gradual dehydration of embryonic cells.

Therefore, the purpose of this study is to complete a large scale experiment to characterize the effects of the embryo (genotype, fresh or frozen (traditional method or vitrified), stage of development, quality grade, no of embryos transferred (single versus twin) and the effects of the recipient (genotype, no of the CL at time of transfer, CL and transfer site, season of transfer) on the success of interbreed embryo transfer.

There was a potential 300 recipient to be synchronized and at embryo transfer, ovaries were examined by semi laparoscopy. Recipients presented for embryo transfer received frozen-thawed embryos in pairs while fresh embryos were transferred single or twin. Frozen imported embryos were from two genotypes such as Charolais (n=60) and Romanov (n=60) while fresh embryos were obtained from two donor genotypes such as purebred Romanov

(n=20) and F1 Romanov crossbred (n=15). Recipient genotypes were Turkish native sheep which three of them was fat tailed raised in eastern Anatolia, such as Morkaraman, Kangal, Akkaraman and the other two breeds were thin tailed raised in western Anatolia such as Kivircik and Dagliç sheep and last group of recipient genotype was F1 Romanov crossbred prolific ewes.

BACKGROUND AND STATEMENT OF THE PROBLEM

Turkey spread over an area of 780.000 sq. km. its topography, climate, water and land resources are extremely variable, and there are 9 different agricultural regions with great diversity in the advantage they offer. Eastern, southeastern and central regions of Turkey are greatly arid or semi-arid; the vast natural grazing lands and steppes in these regions are more suitable for sheep and goat raising than crop and dairy cattle production. Sheep and goats are the major source of livelihood for the rural inhabitants of these regions. Even in the more productive western regions of the country, where agriculture is increasingly intensified, sheep production continues to be a profitable business for sheep raisers as well as for feedlot operators, and it is often incorporated in a system of mixed farming.

Turkey sheep population declined from 40 million to 26 million during last 20 years. The reason for this decline is explained by several reason such as the efforts to decrease production cost is not enough, sheep breeding is not attractive for young generation, decline in pasture land in country wide and unpredictable price for mutton and lamb. Total meat production per capita seems on the verge of a continued decline. Food production is barely keeping up with population growth. Per capita cereal production is leveling off and will likely

decline until farm prices increase drastically. Then less and less meat will be produced from grain and less meat will be consumed at higher cost. Sheep is the most efficient convertors of low quality feed materials to high quality food, increase in numbers, productivity and efficiency to provide an adequate supply of low cost meat for everyone in the country and neighborhood countries.

In the region where experiment was conducted, there are 1 million hectares of practically used potential pasture land which would support about one sheep to the 0.4 hectares. Sheep numbers in this region, Erzurum, with high altitude and highest quality of rangeland has been declined from 800.000 to 300.000 in last 5 years. The Morkaraman is a predominant dual purpose, fat-tailed sheep breed of the eastern region of Turkey and its population is 22% in total sheep population. The age at first lambing in Morkaraman sheep is around 24 months and produce about 1.05 lambs per ewe and 40–60 kg of milk per 150-day lactation under semi intensive conditions. The other fat tailed breeds, Awassi and Tuj, are also well adapted to high latitude and cold climate. These breeds have similar reproductive performance with Morkaraman breed of sheep.

Yet to this day sheep breeders are often situated at a relatively low level in the social hierarchy of a village or region. In many countries they are among those who are not benefited from the state aid programs and development services. The breeding of sheep evolved only slowly in the past and has been relatively insensitive to external influences, but in recent years there have been substantial upheavals and some rapid developments. Faced with many external factors such as: globalization of state economies, world-wide and regional market regulations (GATT, CAP, etc.), substantial displacements of populations,

ever greater urbanization, powerful means of communication, new constraints of environmental protection, animal health concerns, and changes in consumer needs, it is obvious that breeding of sheep have to progress.

It is a high priority of the state and national Governments of Turkey to increase production of sheep meat and other livestock products to meet the growing demand for meat by the Turkish population. Another priority is to increase supply of meat at reasonable prices in the rural, less affluent sections of society. The projects conducted to increase productivity of native sheep by selection did not fit targeted production levels. Native breeds with low reproductive performance are advised to crossbred with prolific breeds and the crosses were found satisfactory for maternal characteristics. The present need in the region in terms of breeding program is that selecting ideal terminal sires for proper mate of improved dam line obtained by crossbreeding. While crossbreeding with native and exotic breeds is more favorable than replacing native breeds with exotic maternal breeds, terminal sires are required to be kept as purebred.

Introducing new genetic as adult live animal has been found not practical and effective due to adaptation problems. The transport of embryos between countries has been accomplished in sheep by in vitro methods. Embryos were recovered in one country, stored in an appropriate culture medium, shipped by air to a second country, and transferred to suitable recipients (Baker and Dziuk 1970, Wrathal et al. 1970).

The MOET (multiple ovulation and embryo transfer) has been started to be used as the main component to a low cost route of exporting genetic material across international

boundaries (Cognie 1999, McKelvey 1999). This way is widely acknowledged as the best available option to control disease transmission during imports of genetic material into a particular country and hence to ensure the health status of the national stock (Singh 1987, Foote et al 1993, Singh et al 1997, Parker et al 1998, Thibier and Guerin 2000). MOET has the added advantage of allowing imported stocks to develop in recipients well adapted to local conditions and can be used as a means for disease resistance in the breeding objectives and breeding strategies in either indigenous or other susceptible sheep (Saberivand and Outteridge 1996). MOET have been used so much to proliferate genes of reputedly superior stock, imported either from overseas or elsewhere in the world. Particularly true of MOET, where the incentive to use it is commonly a short term cash gain made from proliferating breeding stock of a particularly valuable and usually novel strain or breed (Evans, 1991). The embryos can be transferred 'fresh' or can be frozen for transfer at a later date (Ishwar and Memon 1996). However, commercial application to the sheep industry did not occur until late 80's (Ishwar and Memon 1996, Naqvi et al 2001). In principal, MOET is a tool for genetic improvement aiming to identify genetically superior female animals and enabling them to have more offspring than would be possible naturally (Ishwar and Memon 1996, Loi et al 1999, Cognie 1999, Bari et al 2000). McKelvey (1999) stated that MOET has the potential to double the rate of genetic improvement in a given population through increasing selection intensities and decreasing generation intervals. On the other hand, embryo transfer offers an effective tool for research on maternal-fetal and fetal-fetal interactions, and in this way can make important indirect contributions to more efficient breeding programs. An appreciation of the potential benefit of MOET, briefly reviewed above, was perhaps best demonstrated in dairy cows where it has been applied with considerable success (Lohuis 1995, Callesen 1996). However, the application of MOET techniques to small ruminants has been much slower

mainly due to the lower economic value of these animals but also due to difficulties as a result of the reproductive anatomy of such animals (Armstrong and Evans G 1983, McKelvey et al 1985, McKelvey 1986, Ishwar and Memon 1996, Loi et al 1999). Under the correct financial management, ET is an effective tool for creating and marketing breeding stock. Several factors affect the value of ET: 1) economic superiority of the embryos transferred; 2) the percentage of pregnancies maintained after ET; 3) the number of live lambs produced; and 4) the minimum return on the investment. For the sheep industry to remain competitive with the cattle or hog industries, it is imperative that these types of reproductive technologies become available. However, these technologies must be implemented in a profitable manner. Returns on a single animal must exceed the cost of producing that animal (Wulster, 1997).

Therefore, overcoming difficulties related to this technology is important not only as a method of increasing the number of elite animals in a flock but also from an economical and practical point of view provided that limitations are understood. The first successful report on the freezing of mammalian embryos was that of Whittingham in 1971. A few years later in 1976, the first birth from a frozen sheep embryo was reported (Willadsen et al., 1976). The sheep has been used as a model for studies on freezing the embryos of other domestic species, especially cattle (Willadsen et al., 1976, 1977; Bilton and Moore, 1976). Since 1995, numerous studies have reported production of live offspring following cryopreservation of sheep embryos (Songasasen et al., 1995; Cocero et al., 1996; Loi et.al. 1998). Cryopreservation of embryos plays a key role in commercial embryo technology and has become an integral part of methods to control animal reproduction. The growth of clinical services for IVF has been a major stimulus for cryotechnology (Leibo et al., 1989). Although, cryopreservation has become an integral part of the commercial embryo transfer industry its application in sheep embryos is based on comparatively few studies (Boundy et al 1985,

Ishwar and Memon 1996) and the process continues to be improved and simplified (McGinnis et al 1993, Naqvi et al 2001). To date the information on the success rate of such cryopreservation techniques is scarce, particularly when applied to the large-scale movement of sheep embryos. Vitrification does not require an expensive freezer, is less time-consuming, and has little influence on the developmental abilities of embryos after warming (Vajta, 2000). Mermillod et al. (2000) reported a high lambing rate (75%) and embryo survival (62%) by direct transfer of vitrified ovine embryos, which were similar to the results by ET following stepwise dilution (67 and 56% respectively).

To make use of the stored embryos, they must be recovered, handled, stored, and transferred to a suitable recipient. All these steps are integral parts of the embryo transfer technique. The embryo could be held in waiting or shipped great distances and then transferred to the recipient on the appropriate day of the estrous cycle. The control of the time of ovulation simply and precisely would be helpful. Perhaps programming the endocrine levels of the recipient by exogenous steroids to produce at will a uterus amenable to implantation and gestation would be advantageous. Many practitioners consider MOET to be the most frustrating of all ART, since the results can vary from complete failure to total success without any variation in the standard operating procedure. The main factors contributing to the unpredictability of this technique are the variability of the superovulatory response, the poor fertilization associated with high ovulatory responses, and early regression of corpora lutea (reviewed by Cognié, 1999; Cognié et al., 2003). These unpredictable results, combined with high costs and the use of surgical procedures for collecting and transferring embryos, have prevented large-scale use of MOET in sheep improvement programs.

As indicated above, MOET program should be examined by investigating the factors related to the success to realize the potential of this program to be used as a tool in introducing new exotic breeds for sheep improvement program and establishing the nuclei flock of high genetic value animals.

CHAPTER II.

2. REVIEW OF LITERATURE

2.1. Factors related to recipient

2.1.1 Recipient *"uterine"* genotype

The procedures that are collectively referred to as embryo transfer (ET) have many uses. They were first used as research tools to study fetal-maternal physiology. Since the first successful mammalian embryo transfer in 1890, ET has been utilized for enhancement of genetic selection; diagnosis and treatment of infertility; control of infectious disease transmission; screening for genetic defects; propagation of rare and endangered species; and the study of developmental biology (Kraemer 2005).

Maternal effects can be classified into prenatal (ovum cytoplasm and uterine components) and postnatal (lactation plus other postnatal maternal components). Prenatal influences using embryo transfer in mammals have been estimated by Moore et al., (1970), Bradford et al., (1972,1974), Humes et al., (1987). and Rogers et al., (1988). Large

differences in maternal effects have been demonstrated in reciprocal crosses for growth traits of beef cattle, lactation in dairy cattle, and metabolic characteristics of swine (Gregory et al., 1978a,b,c; Robison et al., 1981; Dzapo and Wassmuth, 1983). Litter size at birth is determined by ovulation rate, fertilization rate and prenatal survival. The first two of these are characteristics of the parents and only the third is a characteristic of the offspring. However, it too is subject to maternal influence, and in fact the evidence from inter-breed egg transfer experiments reviewed by Bradford (1972) indicates that it is the genotype of the dam rather than of the offspring which is responsible for genetic variation in prenatal survival. However, Pomp (1989) claimed that survival and prenatal growth of mammalian embryos are influenced by genotype of the embryo, genotype of the uterus providing the developmental environment and their interactions. Embryo transfer technology enables experimental manipulation of genotypic combinations of embryos (donors) and uteri (recipients), affording unique opportunities to study genetic control of embryonic survival and growth.

Dixon et al., (2007) reported that breed-type differences were apparent in the percentages of embryos or fetuses lost during several extended stages of pregnancy and were not accounted for by differences in numbers of embryos or fetuses present at the beginning of the stage. They found that Black-faced ewes, mainly of Suffolk breeding, experienced the greatest loss, regardless of stage of pregnancy. White and mottled-faced ewes lost similar proportions of embryos and fetuses from d 25 or 45 to parturition. White-faced ewes had very little fetal loss from d 85 to parturition compared with black-faced ewes, and losses were intermediate in mottled-faced ewes. Cumming et al. (1975) noted that embryonic survival from breeding to d 26 to 30 was greater in crossbred than in Merino twin-ovulating ewes, but did not differ with breed in single-ovulating ewes. Foote et al. (1959) found that Columbia

ewes lost fewer embryos up to d 18, 25, or 140 than Hampshire ewes. De Bacca et al., (1954) emphasized the relationships between the lower concentrations of progesterone and greater percentages of loss observed in black-faced ewes might be related to a shorter breeding season and deeper anestrus in Suffolk than in white or mottled-faced ewes.

The global spread of prolific sheep breeds from their country of origin has been possible through embryo transfer (Fahmy, 1996). Embryo transfer can play an important role for faster multiplication and propagation of prolific sheep breeds. Embryo transfer technology allows for controlling the number of growing fetuses during pregnancy (Ishwar and Memon, 1996). Several studies have shown that fetuses from multiple pregnancies have lower birth weight and rectal temperature at birth, increased neonatal mortality, reduced growth rate, poor performance in subsequent stages of life and are behaviorally slow (Alexander, 1974; Huffman et al., 1985; Reynolds and Redmer, 1995; Brown and Radziewic, 1998; Dwyer et al., 2005). However, not enough report is available following transfer of multiple embryos derived from small size prolific donor sheep into large size non-prolific recipient sheep. It is also not known whether the group survival of genetically distinct embryos of prolific sheep will be favorable in the maternal uterine environment of non-prolific large size recipient ewes in which the limitation associated with uterine size is compensated. Naqvi et al., (2007) investigated the developmental competence, birth and survival of Garole (small size) lambs following transfer of twin or triple embryos in proximity to its average litter size into large size non-prolific recipient ewes. They questioned that if same number of embryos derived from prolific sheep can also develop to term in the uterine environment of approximately 2.65 times higher body size non-prolific sheep, which provided more space for embryo development than small size garole ewes. They concluded that the monotocus character of

recipients since all had single ovulation point was not a limiting factor for pregnancy success of three transferred embryos (57%) rather than embryos in pairs (42.9%).

There several reports which have investigated relative effects of embryonic and uterine genotypes on survival and prenatal growth in other species such as mice, utilizing factorial embryo transfers among inbred strains (Fekete, 1947; Baunack et al, 1986) or genetically selected lines (Brumby, 1960; Moore et al, 1970a, b; Aitken et al, 1977; Al-Murrani & Roberts, 1978; Moler et al, 1981), or cross-nursing and sib analysis studies (Cox et al, 1959; El-Oksh et al, 1967). In terms of embryo survival, these studies generally indicated a strong maternal uterine genotype effect, while in terms of prenatal growth the relative influences of embryonic and maternal genotypes appear to vary according to the genotypes under study. Several authors have studied the role of maternal and embryonic genotype on fetal growth by performing embryo transfers among inbred Mouse lines (Pomp et al., 1989), genetically selected Mouse lines (Al-Murrani and Roberts, 1978; Rhees et al., 1999; Ernst et al., 2000), and different pig breeds (Asworth et al., 1990; Wilson et al., 1998; Biensen et al., 1998). Results obtained did not show a clear pattern because they depend on the genotypes used to perform in those studies. For example, Pomp et al. (1989), using two inbred lines differing in adult body size, reported that prenatal growth was mainly determined by the genotype of the recipient female, but was not affected by the genotype of the donor line or the donor × recipient interaction. Conversely, Al-Murrani and Roberts (1978), working with two lines of mice selected for high and low 6-wk weight, reported that the donor effect was more important than the maternal effect.

"Uterine genotype" in this sense refers to all components of prenatal maternal effects, and it is defined as the genotype of the female in which the embryo develops. Prenatal uterine effects are mediated through such factors as the age and condition of the mother as well as prenatal fraternity size, body size of the female and quality of the environment which the mother experiences during gestation. Knowledge of these latter prenatal effects has special significance for biotechnologies such as embryo manipulation and cryopreservation which rely on embryo transfer for their ultimate success (Cowley, 1989). Reciprocal embryo transfers between lines enable the differentiation of maternal and fetal genetic factors, but results depend on genetic origin and day of gestation. The experiment regarding donor and recipient effects on prenatal survival were reported by Mocé et al. (2004). They observed that fetal survival was mainly affected by the recipient genotype.

The *in utero* environment and(or) the genotype of the dam (maternal effects) have long been known to have an influence on fetal and postnatal development. These effects were documented by Walton and Hammond (1938), who described the growth of Shetland pony-Shire horse reciprocal crosses. Klindt and Maurer (1988) reported that the influences of uterine or prenatal environment (recipient breed) and the interactions of prenatal environment and genotype (sire breed-dam breed combination) and sex accounted for the majority of the significant effects observed. Genotype differences alone accounted for little of the variation in the phenotypic characteristics studied. The differences in maternal genetic contributions between the Angus and Red Poll breeds had little or no effect on most of the parameters estimated. Also, they indicated that recipient breed (the environmental influence) represents the nutrient availability to the fetus and the maternally determined endocrine environment to which the fetus is exposed. Genotype effects and sex represent genetic influences which

determine nutrient requirements of the fetus. Factors specific to both embryos and recipients have been suggested to affect the survival of transferred embryos in cattle, sheep and goats. There have been several reports published over the last 30 years describing embryo transfer programs in sheep. Knowledge of the effects of the embryo and the uterine genotype on fetal and placental development is helpful in better controlling those factors that are limiting both litter size and success of new reproductive technologies. A number of these reports have evaluated the factors associated with the variation observed in embryo survival (eg Moore *et al.* 1960), and the possibility that genetic factors may contribute to variation in embryo survival has frequently been suggested.

In sheep, embryo transfer procedures have long been demonstrated to leverage increases in selection intensity (Rathie, 1982) and genetic gains (Smith, 1988). The uses of MOET for multiplying genetic resources for commercial sale (Lang *et al*., 1982), cryopreservation (Sakul *et al*., 1993), wool trait improvement (Wuliji *et al*., 1995) and improvement of multiple rearing ability (Cloete *et al*., 1998) have also been reported. Wool traits were studied in a MOET program and it was reported that using of crossbred recipients, may improve adult fleece production in Merino lambs. Gimenez and Emsen (2011) also reported that prenatal survival of frozen-thawed embryos were more favorable in native sheep breeds of the region while mothering ability related to production type of recipient genotype is a strong determinant for overall success of MOET program.

2.1.2 Ovulation rates and P4 values of recipient

Improving embryo survival rate following transfer is a key objective in increasing the success of MOET program. Early embryo mortality is a major contributor to reproductive failure (Sreenan et al., 2001) with the majority of this loss occurring in the first 16 d of gestation (Dune et al., 2000). This period covers the cascade of events that occurs from ovulation up to maternal recognition of pregnancy. It is well documented that in domestic animals, lifespan of the corpus luteum (CL) must be extended to permit continued secretion of progesterone for maintenance of pregnancy. Duration of and necessity for CL function throughout pregnancy vary among species (Bazer and First,1983). However, all require an initial maintenance of the CL. The process by which the periattachment conceptus signals its presence to the maternal unit, as reflected by CL maintenance, has been referred to as "Maternal Recognition of Pregnancy." Maternal recognition of pregnancy involves biochemical communication between the conceptus and its mother to provide uninterrupted synthesis and release of progesterone. Extension of luteal function beyond the length of a normal estrous cycle is the first evidence that maternal recognition of pregnancy has occurred (Hodgen and Istkovitz, 1988, Sawyer et al., 1990). Although the mechanisms by which progesterone synthesis is maintained vary between species, only a few general types of signals are used, and timing of the signal is critical. In general, the conceptus secretes factors that either prevent the secretion or luteolytic actions of PGF2a or that are directly luteotropic.

In domestic ruminants, the period of time that the pregnant female is dependent on luteal progesterone for maintenance of pregnancy varies. In sheep, the shift from dependence on luteal to placental progesterone occurs after 45 days of gestation (Casida and

Warwick,1945; Denmaur and Martinet, 1955). Early observations that prevention of luteolysis is extended only to the corpus luteum, which is ipsilateral to the gravid uterine horn (Moor and Rowson, 1966a; Moor and Moor and Rowson, 1966a; Niswender et al., 1970), provided strong evidence that the embryonic signal acts locally rather than systematically. In sheep, the signal must be present at adequate concentrations on *days 12–15* (Godkin et al., 1984; Vallet et al., 1988) . The conceptus produces a unique signal, IFN-g, that extends luteal function beyond the length of the estrous cycle by blocking the synthesis of PGF2a and preventing luteolysis. Interferon-g is produced in pregnant ewes from *days 11* to *23* (Anthony et al., 1988; Farin et al., 1989; Godkin et al., 1982), with peak production occurring between *days 14* and *16* (Godkin et al., 1988; Hansen et al., 1985) after estrus.

Recently, a number of studies have indicated that the probability of a conception occurring is related to the size of the ovulatory follicle (Vasconcelos et al., 1999; Perry et al., 2007; Lopes et al., 2007). It has been suggested that ovulatory follicle size influences subsequent luteal size and function during the early luteal phase (Vasconcelos et al., 2001). Lynch et al., (2010) reported that heifers that ovulated large follicles had reduced embryo survival, whereas those ovulating small and medium-sized follicles maintained high levels of fertility. On the other hand, Diskin and Moris (2008) indicated that low peripheral concentrations of progesterone (P4) during the early luteal phase has been shown to be associated with low embryo survival rate in cattle.

The rate of increase in P4 in the early luteal phase has been shown to have a positive relationship with fertility (Stronge et al., 2005). Lynch et al., (2008) reported that there was a positive association between higher concentrations of P4 on Day 7 and the likelihood of

embryo survival, which was in agreement with previous work conducted with beef heifers (Diskin et al., 2002) and dairy cows (Stronge et al., 2005).

In sheep, a number of environmental factors, including nutrition and stress, influence prenatal survival and affect progesterone concentration. Pregnancy depends upon a sequence of different concentrations of progesterone and loss may be caused by an excess of progesterone or an inadequate amount. During the periovulatory period an excess of progesterone would initiate changes in uterine function prematurely, while an inadequate amount during the luteal phase prejudices survival (Wilmut et al, 1985a).

Chagas e Silvia et al., (2003) reported that plasma P4 concentrations correlated significantly with ovulation rate and ewes that lambed following embryo transfer had significantly higher mean plasma P4 concentrations at Day 18 than ewes that did not lamb. Although the relationship between ovulation rate and P4 concentrations is not linear in either unstimulated (Quirke et al., 1979) or stimulated (Smartzi et al.,1995) sheep, a significant correlation between these two parameters were repotted by other several researchers (Kelly et al., 1984, Smartzi et al.,1995 and Chagas e Silvia et al., 2003).

In mated ewes, Ashworth et al. (1989) observed a significant positive effect of the endogenous concentrations of P4 in the periovulatory period, and a negative effect of the timing of the increase of P4 concentrations to luteal values on embryo survival. These authors also reported a linear decline of embryo survival with increasing CL number. However, Chagas e Silva et al., (2003) repotted that season had a significant effect on the proportion of ewes (donors and recipients) that were acyclic at the start of treatment, but had no effect on

the subsequent ovulation rate and P4 concentrations. Also, they indicated that there was evidence of a positive association between embryo survival and concentrations of progesterone on Day 7.

Jacqueline et al., (1985) studied whether the increase in ovulation rate was reflected by a greater number of embryos surviving at Day 30 of gestation. They reported that embryo survival was affected by ovulation rate as 91% of these ewes had anovulation rate of 3 or 4 and underlined that as the optimum ovulation rate for maximum survival was found to be 3 ova in their study. White et al. (1981) concluded that embryo survival was greater in single-ovulating than in bilateral twin-ovulating ewes, which in turn were superior to unilateral twin-ovulating ewes. In a recent study in a Merino flock, 20.4% of potential lambs were lost when number of live offspring was compared with number of CL at midgestation (Kleemann and Walker, 2005). This value is quite similar to the 22.8% estimated by Dixon et al., (2007). Meyer (1985) described the marginal response in litter size due to ovulation of an additional egg as uterine efficiency, and showed that it decreased as ovulation rate increased.

Armstrong et al., (1983) reported that the highly significant relationship observed between ovulation rate in recipients and embryo survival raises the possibility that survival may be enhanced by higher circulating progesterone levels in recipient does. Therefore they underlined that it might be possible to improve survival by administration of exogenous progesterone to recipients, or by increasing their ovulation rate by administration of a small dose of gonadotrophin to recipients a treatment which would be readily compatible with hormonal methods of synchronization of recipients with donors.

Alabart et al., (2003) reported that recipient ewes having two lambs tended to have lower progesterone concentration at transfer than those with only one lamb. Besides, Land and Wilmut (1977) conducted a research where large numbers of embryos transferred to sheep of breeds with an approximately two-fold difference in ovulation rate. They found that when the same number of embryos was transferred to sheep breeds with low and high ovulation rates, survival rates of embryos was greater in recipient ewes with high ovulation rates.

In view of the complex relationships between the effects of ewe, season, stress, nutrition, number of ovulations, embryo survival and progesterone production and clearance it is not surprising that associations between peripheral plasma concentrations of progesterone and embryo survival are not always apparent.

2.1.3. Ovulation and transfer site

Inequality of ovarian function occurs in various species. Although the occurrence of ovulation has been shown to be random with respect to which ovary contained the previous CL in primates (Hartman, 1932; Morse and van Wagenen, 1936) and many other mammals (Brambell, 1956), in some mammals (e.g. fur seal: Enders, Pearson and Pearson, 1946) ovulation consistently alternates between the ovaries, and in others (e.g. whales: Harrison, 1969; mountain viscacha: Pearson, 1949) ovulation may predominate from one ovary (see Harrison and Weir, 1977, for review).

McKenzie and Terrill (1937) and Henning (1939) reported that the right ovary in sheep produces more corpora lutea than the left ovary. Whether or not similar differences in ovarian function occur in sheep with single ovulations and in those with multiple ovulations has not been shown. Inequality of ovarian function may or may not lead to inequality of function between the right and left horns of the uterus, depending upon differences in embryo mortality between the two sides and differences in embryo migration between the two horns.

The incidence of migration of embryos in the sheep observed by Curson (1934), Cloete (1939) and Casida *et al.* (1945) appears to be 10 to 20%. Boyd *et al.* (1944) suggested that transuterine migration is of frequent occurrence when double pregnancy results from two ovulations in a single ovary, but occurs rarely with single ovulations. Casida et al., (1966) examined the relative activity of the right and left ovaries of sheep showing single and multiple ovulations. They also estimated the percent loss of embryos arising from single and twin ovulations in the two ovaries and the percent of apparently migrating embryos originating from the two sides in single and twin ovulations. They reported that single ovulations occurred in 61.8% in the right ovary while multiple ovulations located in the right ovary was 6.3% lower than in the ewes showing single ovulations. Thus, it can be concluded that the left ovary appears to play a significantly greater role in ewes showing multiple ovulations than in those showing single ovulations. Interestingly, among the single ovulators 21.7% of those which showed a CL in the right ovary did not have an embryo, whereas 26.4% of those that had a CL in the left ovary had no embryo and embryo losses was greater for the left ovary than for the right by 4.7%. They pointed that embryonic loss in the twin ovulators was greater in all instances than for the single ovulators. When the two CL were present in the right ovary, the loss was 35.5%,, whereas, if the two CL were in the left ovary, it was 40.6%

greater by 5.1% when the eggs came from the left ovary. If a CL was present in each ovary, the embryo loss was 31.0% less by 4.5% units than for double ovulations in the right and by 9.6% units than for double ovulations in the left. Embryo loss, however, was still greater by 4.6 and 9.3% than for single CL in the left or in the right ovary. Casida et al., (1966) underlined that the survival of embryos originating on the left side appears to be slightly less than for those originating from the right side.

In most species, the pregnant female quickly recognizes that a conceptus is present (embryo and associated membranes) (Meyer et a.,1995, Morton 1984, O'Neill1985). Interferon-g is produced by the trophectoderm of the conceptus (Bazer et al., 1997) and extends the life span of the corpus luteum by indirect mechanisms. Interferon-g attenuates secretion of uterine PGF2a in sheep (Salamonsen et a., 1991) by suppressing the transcription of genes that encode receptors for estradiol and oxytocin (Spancer and Bazer, 1996). The reduction of estradiol receptor numbers further suppresses oxytocin receptor gene expression by blocking estradiol-mediated upregulation of this gene. Progesterone provides an additional negative effect on estradiol and oxytocin receptors. It has been demonstrated that the primary site of action for IFN-g is the luminal and superficial glandular epithelium (Spencer et al., 1996), whereas the primary site of action of progesterone on oxytocin and estradiol receptors is the deep glandular epithelium during pregnancy (Spencer et al., 1996). A combination of IFN-g and progesterone prevents oxytocinmediated PGF2a release from the uterus and subsequent luteolysis. The ruminant conceptus may also utilize additional mechanisms for extension of the life span of corpus luteum. A second mechanism involves the secretion, by the *day 15* sheep conceptus, of a protein that blocks the effects of PGF2a on the LLC by unknown mechanisms (Wiltbank et al., 1996). This protein does not bind or metabolize

PGF2a, but rather blocks the effect via some intracellular mechanism. Thus the ruminant conceptus ensures that its requirement for luteal progesterone is met by preventing uterine secretion of PGF2a or preventing the actions of PGF2a at the luteal cell level. Because luteal progesterone is essential for embryonic survival and development, it is not surprising that redundant protective pathways are present to ensure survival of the corpus luteum.

The relationship that exists between the unattached embryo and the corpus luteum has been investigated by transferring embryos to various 'isolated' portions of the uteri of non-pregnant recipient sheep (Moor and Rowson, 1966). The term 'isolated uterine horn' refers to that section of the uterus which was ligated and transected at the junction of the horn and the body of the uterus. The 'isolated' uterine horn was not separated from the broad ligament or from the oviduct. Under the above experimental conditions it has been found by Moor and Rowson (1966, and unpublished observations) that corpora lutea are maintained only in the ovary adjacent to the isolated gravid uterine horn. Embryos confined in the contralateral horn were entirely without effect on the life span of the corpora lutea of any of the 15 recipient ewes used in their experiment. Confirmation of these results has been obtained by placing embryos in one isolated horn of 14 non-pregnant sheep that had corpora lutea in both ovaries. In all cases the corpora lutea in the ovary adjacent to the gravid horn were maintained while those corpora lutea in the ovary adjacent to the sterile horn had regressed. These results show that corpora lutea in the two ovaries of the same pregnant sheep are able to function asymmetrically under certain conditions. A local effect of the unattached embryo on the corpora lutea is therefore indicated.

This unilateral effect of the early embryo is, however, demonstrated clearly only when the embryo is surgically confined to one uterine horn in such a way as to prevent any tissue or fluid from passing directly into the gravid horn. Thus, embryos transferred to control sheep with intact uteri maintained the life span of the corpora lutea in the recipient ewe irrespective of where the embryos were placed in the uterine lumen. A recent experiment by Niswender and Dziuk (1966) has confirmed this observation and has further shown that a single embryo can maintain luteal function in both ovaries of a normal ewe.

It is of importance that when embryos are confined to the contralateral horn corpora lutea can still be maintained during pregnancy if the non-gravid horn adjacent to the corpora lutea is removed at the time of embryo transfer (Moor and Rowson, 1966c). Moreover, embryos confined in the ovarian half of the ipsilateral horn maintain luteal function more effectively than those placed in the cervical half of the same horn, due perhaps to differences in the anatomical relationship between the ovary and the two halves of the adjacent uterine horn. It is evident that the regulation of luteal function in the experimental sheep was largely controlled by the relative positions of the nongravid isolated horn and the ovary bearing the corpus luteum. The position of the gravid horn is apparently not in itself of direct importance. These results are interpreted to signify that the effect of the embryo before attachment is probably basically anti-luteolytic rather than directly luteotrophic in nature.

The mode of action of the anti-luteolytic effect of the unattached embryo has not yet been determined. It could act directly on the endometrium, thus preventing the uterus from becoming lyric, or the embryo could act locally on the ovary thereby protecting the corpus luteum from lyric uterine influences. Some properties of the active principle in early embryos

have been studied by infusing homogenates of frozen and thawed embryonic tissue into various sites in non-pregnant sheep (Rowson and Moor, 1967). Daily intra-uterine infusions of 14-day and 15-day sheep embryo homogenates maintained the corpora lutea of nine test animals until day 23 or 24. A single intra-uterine infusion of the same homogenate was only partially able to block luteal regression in 26 non-pregnant ewes, while infusions of this material into extra-uterine sites was entirely without effect on the corpora lutea. Similarly, homogenates prepared from 25-day sheep conceptuses, from 14-day pig embryos or from serum and white cells had no significant effect on luteal function in a total of 17 sheep even when administered daily into the uterus.

These results suggest that the active principle in the early sheep embryo is retained during long periods of storage at -20ºC, but can prevent regression of the corpora lutea only when infused directly into the uterus. Furthermore, the ability of this factor to prevent luteal regression appears to be related to the age of the embryo and is probably also species specific.

Not only in sheep but also in farm animal species (horses, goats and cattle), ovarian and uterine asymmetries have been observed. Arthur (1958) described ovarian activity in the mare and noted a greater proportion of CL present on the left ovary compared with the right. Henning (1939) and Casida et al. (1966) reported that the right ovary in sheep produced more CL than did the left ovary. Similarly, in the goat, Taneja (1959) and Basu et al. (1961) reported that the right ovary was more active than the left ovary, having a greater number of large follicles. Furthermore, Lyngset (1968) found that in the goat, CL were predominately observed on the right ovary compared with the left ovary in both single and multiple ovulations. Reese and Turner (1937) detected the CL in the right ovary more often than in the

left ovary of heifers, and Rajakoski (1959) reported a significantly higher number of follicles ≥5 mm in the right ovary than in the left ovary in mature cows.

Very little is known about the factors that determine the site of ovulation on the surface of the ovary or of those factors that determine the ovary from which ovulation will take place. For most species it is often assumed that the distribution of ovulation between the ovaries is random and that there is an equal probability of ovulation occurring from either ovary. Functional ovarian asymmetry, although uncommon, does occur in mammals (Pearson, 1949). In 1937, McKenzie and Terrill (1937) reported observations obtained following repeated laparotomies in a group of ewes over a 4 years period; 230 (56.23%) of 409 ovulations occurred from the right ovary in these animals. Several other authors have reported similar findings in the ewe (Henning, 1939; Purse1 and Graham, 1962; Casida et al., 1966; Wheeler, 1978; White et al., 1981). There are a number of factors that affect the site of ovulation from the surface of the ovary. In species such as the mare, the site of ovulation is confined to an ovulation fossa; for others, such as the sheep, the site of ovulation may occur anywhere on the ovarian surface with the exception of the hilus. The presence of a corpus luteum may also affect the site of ovulation in the Rhesus monkey (Wallach et al., 1973), but this does not appear to be the case in sheep (McKenzie and Terrill, 1937). Anecdotal evidence from IVF programmes suggests that ovulation tends to occur from alternate ovaries. The reasons for this pattern of ovulation are not known, but may be related to the presence of the corpus luteum. Scaramuzzi and Downing (1997) studied the pattern of distribution of ovulation from the ovaries of sheep and the effect of this pattern on embryo survival with the data derived from 2806 endoscopic examinations of the ovaries of ewes carried out over a 5-year period. They reported that there were significantly more ovulations on the right ovary

(53.47%) compared to the left ovary (46.67%) and the site of ovulation had no effect on embryo survival and embryos from unilateral ovulations were just as likely to survive as were embryos from bilateral ovulations. However, embryo survival was influenced by ovulation rate, and ewes with ovulation rates of four or more had reduced litter sizes and lower embryo survival.

Waever et al.,(1986) studied factors such as transfer method, uterine horn and recipient factors and season in embryos transfer in field condition. They concluded that the uterine horn to which a transfer was made did not affect embryo transfer success while Remsem et al., (1982) reported that uterine horn of transfer is an influencing factor in embryo transfer. Cattle spontaneously ovulate more often on the right ovary (approximately 55%) than on the left (Reece and Turner, 1938). Studies involving non-surgical transfer, mid-ventral surgical transfer (Hasler et al., 1980) and flank surgical transfer (Remsen and Roussel, 1982) have not detected any difference in pregnancy rate between transfers to the left or right side. Sauvé (unpublished) reported no difference in pregnancy rate following non-surgical transfer of over 12,000 embryos by several practitioners. In a study involving the transfer of more than 12,000 IVP embryos, Lamb (2005) reported no difference in pregnancy rate relative to side of transfer. It should be noted that in that study involved 23 different practitioners. For an individual, however, the side of transfer may affect success rate. In an analysis of approximately 8,000 transfers, Steel (unpublished) achieved a small but not significantly higher pregnancy rate with transfers to the right horn (65.0%) versus the left horn (62.2%).

It has also been reported that the transfer of two embryos to the ipsilateral or both uterine horns does not influence the survival of embryos (Torres and Sovellec, 1987).

Transuterine migration of embryos occurs when two or more ova are shed from a single ovary (Scanlon, 1972) or multiple embryo transfer in single uterine horn (Rowson et al., 1971). Migration of embryo to contralateral horn may occur even when that horn is not associated with a corpus luteum and the chances are greater for the survival of non-migrant embryos (Doney et al., 1973).

2.2. Factors related to embryos

2.2.1 Embryo genotype

A number of reports have evaluated the factors associated with the variation observed in embryo survival (eg Moore *et al.* 1960), and the possibility that genetic factors may contribute to variation in embryo survival has frequently been suggested.

The influence of the developing embryo and of maternal environment upon prenatal viability has been investigated by means of inbreeding, crossbreeding and selection experiments (e.g., Bowman and Falconer, 1960; McCarthy, 1965; Bradford and Nott, 1969; Falconer, 1971). These studies have shown that genotypes of both the dam and the embryo may affect prenatal survival. However, in a study (Moler et al., 1980) conducted in different lines of mouse embryo did not resulted with significant differences in pregnancy rates, while it was reported that genotype of the embryo affected survival rate. Conversely, Al-Murrani and Roberts (1978), working with two lines of mice selected for high and low 6-wk weight, reported that the embryo genotype effect was more important than the maternal effect.

Several reciprocal embryo transfer experiments have been carried out to study prenatal growth in mice (Al-Murrani and Roberts, 1978; Ernst et al., 2000) and in pigs (Asworth et al., 1990; Biensen et al., 1998). Reciprocal embryo transfers between lines enable the differentiation of maternal and fetal genetic factors. Rabbit does are particularly appropriate for studying genetic control of prenatal growth because ovulation is induced by coitus and it is easier to synchronize recipient and donor does. Furthermore, because there is no embryonic migration between uterine horns in rabbits, it is possible to have two different embryo genotypes within the same uterine genotype. Moce et al., (2004) studied with rabbits to determine detrimental factors affecting fetal survival and reported that differences in fetal weight depend on recipient genotype, whereas differences on fetal placental weight depend on donor genotype.

In gilts, embryos have been transferred between Meishan and Landrace x Large White (control) gilts on Day 4 or 5 to establish approximately equal numbers of all four possible combinations of donor breed and recipient breed (Ashworth et al., 1990). The breed of the donor gilt was reported that it significantly affected embryo survival with 44.5% of transferred Meishan embryos and 69.9 % of transferred control embryos surviving to Day 30. In this study, there was no influence of the breed of the recipient gilt on the proportion of embryos which survived.

In Cattle, the economical benefits of crossbreeding have been reported in a large study in the USA, (VanRaden & Sanders 2003) and interestingly, a recent *in vitro* study (Bowdoin *et al*. 2003) demonstrated that crossbred embryos develop faster and at higher rate than purebred embryos, suggesting that genotype can influence developmental competence.

Lazarri et al., (2011) investigated the effect of genotype on embryo development, metabolic activity and gene expression in cattle. They observed that crossbred embryos derived from oocytes of slaughtered donors had a tendency to cleave faster than purebred embryos. Several studies performed on different species have demonstrated that the time of first cleavage is positively correlated with the developmental competence of the embryos (van Soom *et al*. 1997, Fenwick *et al*. 2002) and it was demonstrated that bovine fast-cleaving embryos have a different gene expression that reflects their higher quality compared with late-cleaving embryos (Gutierrez-Adan *et al*. 2004). It was further determined by the same researchers that crossbred embryos have a significantly higher rate of compaction compared with purebred and inbred embryos, strongly indicating a greater competence/quality of that group.

Detrimental effect of inbreeding on embryo development reminds, to some extent, the fact that in the mouse, most outbred stocks and some inbred strains display a compromised *in vitro* embryo development that is normally not observed in hybrids (Scott & Whittingham 1996). All together, these findings further confirm that embryo genotype can play a significant role during early embryo development and also highlight the importance of the preservation of rare and endangered breeds to maintain the species gene pool as wide as possible.

MNSOD is a mitochondrial Mn-superoxide dismutase that indicates mitochondrial activity and also has a role in detoxification of reactive oxygen species, cellular differentiation and embryo compaction. Lonergan et al. (2003) demonstrated that the level of *MNSOD* expression is higher in *in vivo*-produced embryos in respect to those produced *in vitro*. The higher level of expression have been found in the crossbred bovine embryos suggests greater

mitochondrial activity and improved quality compared with the purebred bovine embryos (Lazzari et al., 2011). There are other several genes associated with embryo quality such as GP130, FGF4 and PED (Fry et al. 1992; Daniels et al. 2000; Feldman et al. 1995; Fair et al. 2004). Lazzari et al., (2011) reported that high expression of these genes related to higher quality and greater developmental potential was observed in crossbred embryos than purebred embryos. In literature search, it was noted that there are limited observations of conventional (*in vivo*) interbreed embryo transfer procedures in ovine.

2.2.2 Number of embryos transferred

There is an economic incentive on transferring multiple embryos in recipient to reduce the number of recipient ewes and additional burden on their upkeep. However, Anderson et al., (1979) uterine crowding can cause the increased frequency of pregnancy loss in nulliparous recipients receiving more than one embryo. It is evident, however, that herds of cattle vary in their uterine capacity. Thus, it was reported that there were no differences in pregnancy success between recipients of twin embryos placed unilaterally or bilaterally for heifers (Sreenan and Diskin, 1989; Reichenbach et al., 1992) or cows (Sreenan and Diskin, 1989). It was also stated that within the same species, the breed type can effect the embryo survival rates. For example, embryonic survival rate for beef cows selected for twinning was similar for those having unilateral or bilateral multiple ovulations (Echternkamp et al., 1990). In lactating dairy cows, in contrast, the likelihood of a twin pregnancy resulting from multiple ovulation going to term was higher if ovulations occurred bilaterally than if unilateral ovulations occurred (Lo´pez-Gatius and Hunter, 2005).

In sheep, embryonic and fetal mortality contribute to a large economic loss. Estimates of embryonic and fetal loss have averaged approximately 30% (Bolet, 1986). Most embryonic loss has been reported to occur before d 18 (Hulet et al., 1956; Moore et al., 1960; Quinlivan, 1966). Complete losses from d 18 to lambing were estimated at 9.4% (Hulet et al., 1956), and late embryonic or fetal losses from d 30 to term were only 1 to 5% (Quinlivan, 1966). Losses increased with increasing ovulation rate (Quinlivan, 1966; Knights et al., 2003; Kleemann and Walker, 2005). In pregnancies with multiple embryos or fetuses, individual potential offspring can be lost without a total loss of the pregnancy (Henning, 1939; Rhind et al., 1980; Schrick and Inskeep, 1993). Quinlivan (1966) reported that of ewes with twin ovulations that did not return to estrus before d 18, 47 to 50% retained 2 live embryos, 43 to 47% had only 1 live embryo, and 2 to 6% had no live embryos when slaughtered at 18, 30, or 140 d of pregnancy. Survival to term was estimated at 0.95 for single embryos, 0.85 for 2 and 0.70 for 3 embryos by Geisler et al. (1977). Many factors play a role in embryonic and fetal loss in the ewe. Bolet (1986) suggested that these losses were due to 1 of 3 components: a) the male by the quality of semen, b) the female by the quality of the ova and uterine environment, or c) the embryo itself. The greater losses of 1 embryo or fetus in a multiple pregnancy in a study conducted by Dixon et al.,(2007) indicated that the conceptus played a larger role than the dam. In the case of embryo transfer in cattle, McMillan (1998) compared a binomial model and an embryo and recipient model and found that the survival data on single or double transfers fit the embryo and recipient model in most cases. McMillan estimated that embryo and recipient played nearly equal roles in outcome of pregnancy. In earlier work in sheep, Restall et al. (1976) found that fertilization rate fit an all or none model, that is fertilization occurred on a ewe basis, not an oocyte basis. In that study, early embryonic survival (to d 25 to 30) for 4 flocks fit a binomial model, each embryo having an independent fate. In a second report, Restall and

Griffiths (1976) examined 4 other possible models of embryonic survival but reached no firm conclusions because fertilization rate could influence whether distribution of losses deviated from the binomial distribution. Geisler et al. (1977) utilized data from the literature and concluded that the embryonic survival in sheep generally fit the binomial model.

There are conflicting reports available on embryo survival in sheep as it remained unaffected (Armstrong and Evans, 1983), increased (Quirke and Hanrahan, 1977; Cseh and Seregi, 1993) or decreased (Mutiga, 1991). Nancarrow (1994) has reported that there is a possibility of an interaction between embryos that lead to each other's survival. Naqvi et al., (2007) indicated that incidence of embryonic mortality up to 40 day of gestation was reduced when the number of transferred embryos was increased. They recorded that embryo survival up to 40 days of gestation and also up to term was 38.1% when 3 embryos were transferred per ewe and was relatively higher as compared to transfer of two embryos per ewe where it was 28.6%. In the same study, all the embryos were transferred to the ipsilateral uterine horn. It has also been reported that the transfer of two embryos to the ipsilateral or both uterine horns does not influence the survival of embryos (Torres and Sovellec, 1987). Higher pregnancy rate of 55.2% has also been reported in Hungarian Merino ewes following transfer of two embryos per recipient, compared to 45.6% in case of single embryo transfer (Cseh and Seregi, 1993).

Mutiga (1991) also reported that multiple transfer of embryos in tropical sheep increased the number of lambs born per pregnant ewes. Brown and Radziewic (1998) reported that the pregnancy rate was significantly higher after transfer of embryos pairs (64%) than single (39%) embryos in invitro produced embryos. Contrary to this result, data from embryo

transfer studies (Land & Wilmut, 1977) have shown that doubling the number of embryos transferred resulted in a decrease in the number of lambs born.

Armstrong et al., (1983) indicated that the improved embryo survival following twin transfers and the better survival of twin transfers when both embryos were placed in the same oviduct suggest that there is some type of synergism between embryos in influencing each other's survival upon transfer in goat. Possible explanations for such co-operation include enhanced luteotrophic or anti-luteolytic actions resulting in improved luteal maintenance in recipients, or enhanced signals to the endometrium involved in the process of implantation (placental attachment). Whatever the explanation, the finding has important economic implications in enabling the embryo-carrying capacity of the recipient pool of goats to be doubled.

Franco et al., (2006) suggested that one method that might be useful for increasing pregnancy rates in dairy cattle recipients that receive an IVP embryo is to transfer two embryos into the uterine horn ipsilateral to the CL. Such a treatment might increase pregnancy rate, because the likelihood is increased that the cow receives at least one embryo competent for sustained development. In addition, the transfer of two embryos into the ipsilateral uterine horn is likely to increase the amounts of interferon-t and other embryonic signaling molecules in the uterus needed to maintain pregnancy and prevent luteolysis. They reported that the transfer of two embryos into recipients led to pregnancy loss this loss occurred earlier for heifers than for cows. As early as Day 64 of gestation, there was a distinct difference in pregnancy rate between heifers that received one or two embryos. Among cows, in contrast,

there were no differences in pregnancy rate at this stage of gestation between recipients that received one or two embryos.

Co-transfer of embryonic vesicles to increase trophoblastic signals has been reported to increase pregnancy rates in embryo transfer recipients (Heyman et al., 1982). It was reported by researchers (Del Campo et al., 1977; Del Campo et al., 1983) that, two embryos must be transferred into the uterine horn ipsilateral to the CL because of the requirement for the antiluteolytic signal in cattle to be locally administered. In a recent study with a small number of transfers (n = 10–28 recipients), there was a tendency for higher calving rate for recipients that received two embryos in the uterinehorn ipsilateral to the CL as compared to recipients that received one embryo (Bertoloni et al.,2002). Anderson et al. (1979) found a tendency for pregnancy rates to be higher in cows that received two embryos in the same uterine horn (unilateral transfer) than for cows that received two embryos distributed in both uterine horns (bilateral transfer); the opposite was true for heifers. In other studies, transfer of embryos to create two pregnancies in the uterine horn ipsilateral to the CL has produced a similar pregnancy rate as bilateral twins and single pregnancies (Sreenan and Diskin, 1989; Reichenbach et al., 1992).

2.2.3. Development stage and grade of embryos

Embryo transfer procedures in sheep have been successfully used by numerous researchers (Ishwar and Memon, 1996). Pregnancy rates after ET reported by others for sheep varied from 29 to 65% and were affected by the stage of transferred embryo, synchronization protocol, age of oocyte/embryo donor, embryo storage, method of embryo production (e.g.,

in vivo versus in vitro), and culture conditions for in vitro produced embryos (Thompson et al., 1995; Holm et al., 1996; Ptak et al., 1999; Dattena et al., 2000).

The three most important criteria related to embryo morphology are age, stage and quality. Embryo stage and quality are usually based on the descriptions published by the International Embryo Transfer Society (IETS) (Stringfellow and Seidel, 1998). Based on a large number of transfers, no differences were noted in the pregnancy rate of embryos recovered between days 5 and 8 following standing estrus (Hasler, 2001; Hasler et al., 1987). Since embryo age corresponds rather closely to stage of development, it can be stated rather conclusively, based on a large number of fresh in vivo derived embryo transfers, that embryo stages ranging from late morula (stage 4) to expanded blastocysts (stage 7) result in comparable pregnancy rates, whereas following hatching (stage 8), lower pregnancy rates can be expected (Hasler et al., 1987). In contrast, conception rates of IVP embryos are more stage sensitive than are in vivo-derived embryos. Higher conception rates were achieved following transfer of expanded blastocysts compared to morula and earlier stage blastocysts (Hasler, 1998; Lamb, 2005).

Within each embryo stage, morphological quality is also closely associated with pregnancy rate, as reported in a number of studies (Donaldson, 1985b; Hasler, 2001; Hasler, et al., 1987; Lindner and Wright, 1983; Putney et al., 1988; Putney al., 1989). Farin, et al. (1995) showed that agreement among 6 experienced embryo evaluators was higher for in vivo- compared to in vitro derived embryos. In addition, there was a relatively high degree of agreement on evaluating excellent and degenerated (poor) embryos, but a lower degree relative to good and fair embryos.

Block et al., (2009) reported pregnancy rates among recipients that received expanded blastocyst stage embryos were higher than for recipients that received morula and blastocyst stage embryos. Shirazi et al., (2010) indicated that survival following vitrification of IVP ovine embryos progressively increased as the developmental stage proceeded. Among early stage embryos produced in vitro, those cultured to the blastocyst stage before cryopreservation had the highest rate of survival rate after warming. However, they didn't found significant difference in hatching rate of blastocysts derived from cryopreserved early stage and more advanced stage embryos as well as their corresponding controls. However, Garcia-Garcia et al., (2005) worked with in vivo produced ovine embryos and reported that embryos cultured to the blastocyst stage and frozen thereafter had a significantly higher viability than their counterparts frozen at earlier cleavage stages, (66.1% versus 23.1%). They further indicated that cryopreserved early stage in vivo derived sheep embryos were able to reach the blastocyst stage during the in vitro culture, but failed to hatch from the zona pellucida. Especially for crypreserved embryos, it was emphasized that developmental stage influences the cryotolerance of in vivo produced sheep and cattle embryos; early developing stages would have a high sensitivity to cooling (Cocero et a., 1996; Liebermann and Tucker, 2002).

In superovulated ewes it is common to recover embryos at different stages of development due to differences in time of ovulation or fertilization. When recovering embryos on day 5, early morulae that coexist with normal blastocycst often show signs of cellular degenaration, which is evident that they should be rejected. The variations in the developmental stage of in vivo embryos probably reflect biological variation rather than

viability differences (Shea, 1981; Lindner and Wright, 1983; Hasler et al., 1987; Bousquet et al., 1993). Embryos in later stages of development had a better average quality grade than those less developed, just as embryos of the poorest quality grade tended to produce lower pregnancy rates. This illustrates the intention of the grade evaluation, that is, to express the viability of the embryo, because reduced viability in terms of poorer quality grade results in slower rates of development (Walker, 1989) and reduced pregnancy rates after transfer (Greve, 1981; Shea, 1981; Lindner and Wright, 1983; Hasler et al., 1987). Although many comprehensive morphological descriptions have been published (Greve, 1981; Shea, 1981; Lindner and Wright, 1983; Lehn-Jensen, 1986), person-to-person variation in embryo grade and quality ratings is still pronounced (Lindner and Wright, 1983). So, it is not surprising that the embryologist was found to account for significant variation in the embryo's quality grading. Conversely, the embryologist had less influence on the developmental scores, suggesting that this trait is easier to describe. The quality evaluation is further hampered by loose or degenerate cells in the embryo that are often more difficult to see in blastocyst than in morula stages. No practical method to replace the visual morphological scoring method has been found so far (Betteridge and Rieger, 1993).

A large proportion of the variability in embryo development and quality was attributed to the donor animal. The background for this variation was, however, not fully explained, as indicated by the relatively low repeatability for both embryo stage and quality grade (Callesen et al., 1995). Although factors causing donor variability were only briefly examined in a study by Callenesen et al., (1995) (i.e., gonadotropin preparation used), it is likely that donor hormone levels during the preovulatory period greatly affect fertilization and early embryonic development. This is especially relevant for superovulated cattle whose hormonal and

structural changes in follicular fluids and oocytes, respectively, are quite variable during the preovulatory period (Callesen et al., 1986; Dieleman et al., 1987).

Callesen et al., (1995) indicated that the causes of variation between donors examined (i.e., donor breed and parity, insemination bull, year and season) were insufficient to answer the question. This aspect deserves further investigation, one way being in larger studies including other possible factors. As an example of this, the importance of the donor's genetic background on its response to superovulation were examined (Liboriussen et al., 1995). It was speculated that the genetic background of the embryo may interact with the donor in various ways, further complicating the problem and calling for a thorough analysis taking all genetic and environmental relationships into account.

The developmental stage of embryos recovered at d 7 from superovulated cattle, when evaluated by simple morphological criteria, is correlated with the embryo's quality and is affected by the donor animal (Cellesen et al., 1995). The same researchers indicated that the embryologist was of importance in grading the embryo's quality.

Alabart et al., (2003) reported that higher fertility rates obtained when the transferred embryos were in a more advanced development stage and their findings was agreed with previous work conducted by Moore and Shelton (1961) where an increase embryonic survival was observed with an increase in the age of transferred embryos. In terms of age of embryo, the literature is conflicting regarding this point. Moore and Shelton (1962), reported higher survival rate after transfer of embryos of eight cells or more cells to uterus than after transfer of embryos less than eight cells to the oviduct while others found tubal transfers are

more successful than uterine transfers whatever the age of embryos (from embryos recovered 48-84 h after estrus) (Moore and Shelton, 1964).

Hasler (2011) analyzed the results from 5 commercial ET programs in cattle for which pregnancy data relative to embryo stage at freezing were made available. Embryos representing 4 stages of development, as defined by the IETS (4 = late morula, 5 = early blastocyst, 6 = mid blastocyst and 7 = expanded blastocyst) were included in the data. Pregnancy rates of frozen embryos (in either 1.5 M EG or 1.5 M EG + 0.1 M sucrose) were for stage 6 embryos was only 2.6 and 3.2 percentage points lower than stages 4 and 5, respectively, these differences were highly significant and pregnancy rates for stage 6 embryos were lower than those for stages 4 and 5 in 4 of the 5 ET programs. The pregnancy rate of stage 7 embryos was lower than all other stages for the combined dataset as well as in all 5 ET programs, with the difference between stages 5 and 7 ranging from 6.5 to 16.4 percentage points. Clearly, stage 7 embryos survived freezing at a significantly lower rate than stages 4, 5 and 6. It was noted that factors contributing to the decreased survival of stage 7 embryos should be investigated.

In human, the development of stage-specific sequential media has been claimed to allow 36-66% of embryos to develop to blastocysts with a high viability of up to 50% implantation rate (Jones et al., 1998; Gargder et al., 1998). There are two central reasons why an alternative to cleavage-stage embryo transfer was proposed. Firstly, it has long been recognized that it is physiologically premature to expose early-stage embryos to the uterine environment. In vivo, embryos travel through the fallopian tubes and do not reach the uterus before the morula stage (Croxatto et al., 1972), which equates to at least day 4 of in-vitro

culture. The uterus provides a different nutritional milieu from the fallopian tube; it has been postulated that this may cause homeostatic stress on the embryo, resulting in a reduced implantation potential (Gardner et al., 1996). Secondly, there are widely acknowledged shortcomings of the morphological criteria used for selection of cleavage-stage embryos for transfer on day 2 or 3. There is substantial debate over the correlation of embryo morphological features with pregnancy rates (Steer et al., 1992; Palmstierna et al., 1998). Prior to day 3 of culture, when genomic activation and compaction begins, embryonic development is primarily controlled by transcripts and stored RNA messages of maternal origin (Braude et al.,1998). Only after this transitional stage does development proceed under the control of an activated embryonic genome, resulting in the expression of numerous growth factors and receptors. Furthermore, it is suspected that a large proportion of morphologically normal day 3 embryos are chromosomally abnormal, thus contributing to the 80-90% rate of implantation failure observed in cleavage stage embryos (Magli et al., 1998). Extending embryo culture until the blastocyst stage might provide advantages over traditional protocols by allowing transfer of embryos into a synchronized uterine environment, and selection of only those embryos that have demonstrated the potential for continued development under embryonic genomic control (Johnson et al., 2007). Regarding transfer of fair and poor quality embryos at the blastocyst stage was reported that is feasible and is associated with higher implantation rates as compared to transfer of similar quality embryos on day 3 (Balaban et al., 2001).

Zhau et al., (2005) cryopreserved mouse embryos at different stages and reported that the blastocyst rates of the vitrified one-cell (52.5 to 66.7%) and the two-cell (63.3 to 68.9%) embryos were significantly lower than those of the vitrified four-cell embryos (81.7 to

86.4%), the eight-cell embryos (90.0 to 93.3%), morulae (96.7 to 100%), and the expanded blastocysts rate (98.3 to 100.0%) of the vitrified early blastocysts. The highest survival rate in vivo of vitrified embryos were from the early blastocysts (40.4%), which was similar to that of fresh embryos (48.6%). Their study demonstrated that the optimal protocol for the cryopreservation of morulae was suitable for the four-cell embryos to early blastocyst stages and that the early blastocyst stage is the most feasible stage for mouse embryo cryopreservation. In another study with mouse embryos vitrified by the 1-step method, Han et al., (2003) reported that very high proportions (94–100%) of 4-cell embryos, 8-cell embryos and morulae retained the ability to develop to the blastocyst stage. Embryo transfer showed that a high proportion of vitrified morulae (61%) could develop to term. In mouse embryos, the morula is the stage that can survive vitrification in EFS40 quite well (Miyake et al., 1993). However, very few 1-cell (6%) and 2-cell rat embryos (8%) developed to blastocysts in culture. In these embryos, development was more likely to be arrested at the 2-cell stage. They concluded that in vivo survival rate of vitrified morulae (61%) was high, as it was not significantly different from that of fresh embryos (70%).

2.3. Cryopreservation of *in vivo* produced embryos

From the practical viewpoint, use of embryo cryopreservation has many advantages. Freezing of embryos obtained from females with high genetic value, facilitates dispersion of superior genetics from dams, therefore the rate of genetic improvement can be advanced. Embryo cryopreservation facilitates international transport of breeding animals in the form of embryos, which is a financially reasonable and safer (reduced health risk and no death of animals during transport) technique compared to live animal movements.

Embryo freezing also allows establishment of gene banks with frozen materials/embryos as a back-up. Mammalian embryos were successfully frozen and thawed first in mice in 1972 (Whittingham et al., 1972). Then the development of embryo transfer technology pushed freezing technology in domestic species (Wilmut and Rowson, 1973; Leibo and Loskutoff, 1993). In this case, freezing was performed at the blastocyst stage, on embryos collected in vivo and then after IVM/IVF. Transfer is mandatory at the blastocyst stage: transfer at cleaving stages is unsuccessful. In the human, the first baby, after the replacement of a frozen– thawed embryo (Trounson and Mohr, 1983) was born in Australia in 1984.

Controlled (traditional) slow freezing and vitrification (ultra-rapid freezing) have been the two major techniques used for embryo cryopreservation (Fahy and Rall, 2007). Controlled slow freezing was introduced firstly and remains the mostly common technique use, both with domestic animal and human embryos (Vajta and Kuwayama, 2006). However, slow freezing requires a biological freezer and needs longer time to be completed. The ultra-rapid technique, such as vitrification, has reduced time and cost of the procedure since it does not require any special equipment and is, therefore, well adapted to routine field use (Baril, 2001). However, a well-trained person is needed for successful application, since vitrification involves the addition of a greater concentration of cryoprotectants and a very rapid cooling rate (Rall and Fahy, 1985; Rall, 1987).

The open pulled straw (OPS) method was later developed (Vajta et al., 1997) as a modification of controlled slow freezing and OPS have been used for the cryopreservation of

ovine morulae and blastocysts produced in vivo (Baril, 2001; Cocero et al., 1996; Martinez and Matkovic, 1998; Dattena et al., 2004; Martinez et al., 2006) and in vitro (Dattena et al., 2004; Martinez et al., 2006). The loss of viability associated with freezing can be influenced by the type and concentration of the cryoprotectant used, the freezing protocol, the species and genotype of the animal, the developmental stage of the embryos and the type (in vivo vs in vitro) of embryo production (Guinot, 2005; Dobrinsky, 2002; Vajta and Kuwayama, 2006). Comparisons between the different techniques have relied on lambing rates after embryo transfer. However, selection of embryos for transfer is based on the stereomicroscopical evaluation of embryo morphology after thawing (Abe et al., 2002) in accordance to the guidelines of the International Embryo Transfer Society (Stringfellow and Seidel, 1998). The subjectivity inherent to this selection step has been demonstrated by ultrastructural studies of vitrified in vitro and in vivo produced bovine blastocysts (Vajta et al., 1997), and of controlled slow frozen in vivo produced ovine morulae and blastocysts (Cocero et al., 2002), which have shown that certain abnormalities remain undetected by stereomicroscopy.

Sheep embryos have been used in freezing experiments focusing on collecting information about the cryotolerance of embryos and on improving the efficiency of freezing/cooling procedures (Willadsen et al., 1976, Willadsen, 1977, Ali and Shelton, 1993 and Shirazi et al., 2010). The first lambs from cryopreserved embryos were born in 1976 (Willadsen et al., 1976) and lambs from vitrified embryos were born in 1990 (Széll et al., 1990; Yusviati and Holtz, 1990).

The principal factors affecting successful cryopreservation of mammalian embryos are the species, the type, and concentration of cryoprotectants (Watson and Kidder, 1988), cooling and warming rates (Arav et al., 2002) and also the developmental stage at which the embryos are cryopreserved. Moreover, the toxicity of cryoprotectants, the composition of the cryoprotectant solution (Berthelot et al., 2000; Dobrinsky, 1997), the length of exposure and the protein composition of the cryoprotectant medium affect embryo survival (McGowan et al., 1993; Ohboshi et al., 1997).

In order to increase the genetic gain resulting from selection, efficient cryopreservation protocols are required to allow delayed transfer of embryos and for incrementing the use of multiple ovulation and embryo transfer techniques (Baril et al., 2001; Guinot, 2005). Cryopreservation of embryos from many mammalian species have seen a widespread use in reproductive research and in animal breeding in recent years (Guinot, 2005; Dobrinsky, 2002; Vajta and Nagy, 2006), but the technique has a much lower utilization in sheep than, for example, in cattle (Thibier, 2006). This fact may be related to the relatively high cost of the technique in sheep when compared to the value of the animals. Reducing costs at any stage of embryo production and cryopreservation, including through an increase in the efficiency of cryopreservation methods, is likely to augment multiple ovulation and embryo transfer techniques utilization in the ovine species (Baril, 2001; Vajta and Nagy, 2006).

Advancements and factors affecting the efficiency of cryopreservation of domestic animal embryos have been recently reported (Massip, 2001; Dobrinsky,2002). Sheep and goat embryos are able to survive vitrification procedures and this method may provide an

economical alternative to the current freezing method requiring gradual dehydration of embryonic cells (Baril et al.;2001). Vitrification does not require any special equipment and, therefore, may be very well adapted to routine field use. Furthermore, this ultra rapid technique may be more adapted to embryos with a higher cryosensitivity such as in vitro produced, biopsied or cloned embryos (Massip et al., 1995 and Vajta, 2000). When fresh or vitrified sheep embryos recovered 7 days after estrus are transferred to synchronized recipients (2 embryos/recipient), the pregnancy rates (72% in both cases) and the numbers of lambs born per embryos transferred are not statistically different (60 and 50%, respectively- Baril et al.;2001). These results with vitrified embryos are similar to those reported previously for embryos preserved by slow freezing (Heyman et al.;1987) or with other vitrification methods (Naitana et al.;1997). However, using the same vitrification and thawing methods with goat embryos, Baril and coworkers have obtained lower kidding and embryonic survival rates after vitrification (48 and 39%, respectively) than after conventional slow freezing (69 and 55%, respectively). Recently, the promising results in sheep (Dattena et al.;2001) and in goat (El-Gayar and Holtz;2001) with the so-called open pulled-straw (OPS)- vitrification technique, showed an embryonic survival rate of 59% and 64% for sheep and goat embryos, respectively. This remains to be confirmed on larger scale experiments.

Additionally, the possibility to transfer the vitrified embryos directly after thawing (2 embryos per straw) has been tested in sheep by comparison with the standard technique of transfer which involves the removal of cryoprotectants and morphological evaluation of embryos after thawing. Beside the fact that with the standard technique 16% of the thawed embryos are eliminated after morphological evaluation, the results in term of pregnancy and embryo survival rates are similar between traditional and direct transfer (67 and 75% for

pregnancy ; 49 and 53% for embryo survival with standard and direct transfer, respectively) (Baril et al., 2001). Morphological evaluation of frozen/thawed embryos is not accurate (Cocero et al., 1996) and could be a source of conflicts during commercial exchange of embryos. The use of direct transfer eliminate the need of post thawing evaluation of embryos and represents a potential gain of 7 to 8% in terms of youngs born.

CHAPTER III

AIM AND OBJECTIVE

Despite agricultural advances, an estimated 826 million people, or about 13% of the world's population, still go hungry. The development of high-performing livestock breeds has greatly contributed to the increase of food production, especially in developed countries. These advances in technology are increasingly being adopted in developing courtiers, but their indiscriminate export into these countries has at times ended in failure with live animal exportation. The animals imported at adult ages cannot stand the heat or cold, they need optimal inputs and more easily develop diseases. To overcome these weaknesses, the ongoing approach is the widespread promotion of embryo transfer. Embryo transfer is used for similar reasons worldwide, and, while there are special problems in less developed countries, the seemingly marked differences between embryo transfer programs in developed and developing countries are primarily a matter of degree. By most definitions, about one-fourth of countries are developed, one-fourth least developed, and the other half developing. Most developing countries have substantial numbers of middle and upper class citizens, and sometimes considerable resources are available. Conversely, there are "less developed" agricultural sectors in some developed countries.

In analyzing the appropriateness of potential embryo transfer programs, the same principles pertain regardless of location: 1) there should be realistic, well defined goals and 2) one should evaluate, for each particular situation, whether there is a reasonable chance that embryo transfer technology can achieve the goals. Therefore, in this study it is aimed to evaluate factors effecting embryo transfer success by categorizing embryo and recipient intrinsic factors to evaluate feasibility and practicality of such a reproductive technology method of choice for sheep improvement program in developing country.

CHAPTER IV

MATERIALS AND METHODS

Experiment I

Frozen Embryo Transfer *(Way of introducing exotic breeds)*

In vivo embryo production

Adult multiparious Romanov (n=5) and Charollais (n=6) (3-6 years of age) ewes raised in Canada and with a mean BCS of 3 (0-extremely thin, 5-obese) were used as embryo donors. None of the ewes utilized in the experiment had been previously subjected to a MOET program. Estrous synchronization was carried out with the aid of intravaginal sponges containing 40mg flurogestone acetate (FGA; Chronogest®, Intervet Laboratories, Boxmeer, Holland) for a period of 14 days. Donors were superovulated using FSH-p (total of 20 mg)

(Folltropin-V; Vetrepharm, Canada) applied in eight decreasing doses of 2.4, 2.4, 1.8, 1.8, 1.6, 1.2, 1,1 mg i.m. at 12 h intervals, starting 60h before sponge withdrawal. Estrous detection was performed at 12, 24, 30, 36 and 48 h following sponge removal, using vasectomized rams. Donors sedated with anesthetic cocktail containing 100 mg Ketamine (Vetalar, Boehringer Ingelheim Vetmedica, Inc) and 0.12mg xylazine (Romphun, Bayer) were undergone intrauterine insemination with fresh diluted semen (a minimum of 50 x 10^6 motile sperm/each uterine horn) 40h after sponge removal.

Feed and water were withheld from the ewes for at least 24 h prior to laparotomy. The abdominal area anterior to the udder was shaved and sprayed with an iodine solution and 70% alcohol. Animals were sedated with 2 mg/kg ketamine (Vetalar, Boehringer Ingelheim Vetmedica, Inc) and 0.12mg xylazine (Romphun, Bayer). A mid-ventral laparotomy (Figure 1.) was performed under intubation with an attached to the anesthetic machine. On the day of surgery and for 4 consecutive days after surgery all ewes were treated with 2.2 mg/kg flunixine-meglumine im (Benamine, Canada) and kept in an enclosed pen and observed twice daily for signs of discomfort and/or inflammation in the incision area. Each uterine horn was flushed with 40 ml flushing media (PBS containing 2% of bovine serum albumin - BSA), through the insertion of a needle attached to a syringe, near the utero-tubal junction. A 10 gauge Foley catheter was inserted in the base of the uterine horns for recovery of the embryos, and the media collected and examined for the presence of oocytes and/or embryos under a stereomicroscope. After location, embryos were immediately placed in a holding medium (PBS supplemented with 10% FCS—or with 4% BSA).

Six-day embryos were recovered surgically and Grade 1 embryos (IETS classification) were frozen by direct transfer method. At the time of collection, embryos were categorized into six different stages of development (very early morula, early morula, morula, blastocyst, expanded blastocyst, hatched/collapsed blastocyst).

Figure 1. Midventral laparotomy under antestasia

Controlled slow freezing

Only embryos in the morulae to expanded blastocyst stages, classified as excellent or good (Grades 1 and 2, respectively) were considered freezable. Embryos were immersed for 5 min in a cryopreservation solution of 0.4% BSA in PBS, with 1.5M ethylene glycol (EG) added in one step. Two embryos were loaded into the middle portion of each straw (PBS, air bubble, PBS, air bubble, cryopreservation solution with embryos, air bubble, 0.3M sucrose). Straws were sealed with plastic plugs (plastic plugs for 0.25 ml French palettes, IMV®) and

placed vertically in the cooling chamber of the programmable freezer (Bio-Cool III, FTS Systems Inc., Stone Ridge, NY) at −6 ◦C for 3 min and then subjected to manual seeding (Figure 2.). After seeding, straws were kept for 5 additional min at −6 ◦C. Embryos were then cooled at 0.5 ◦C/min until reaching −35 ◦C, where they were maintained at this temperature for 15min, and then rapidly immersed into liquid nitrogen.

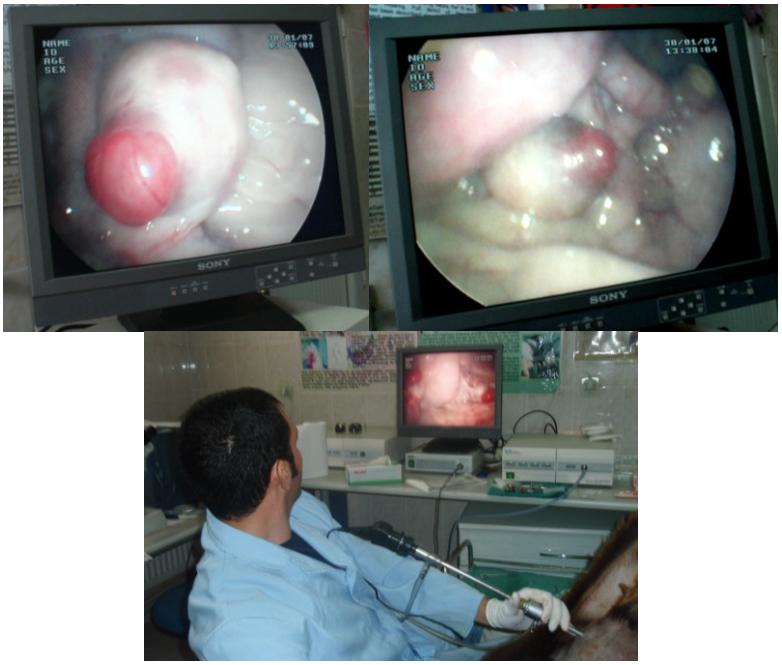
Figure 2. Controlled freezer

Embryo transfer

Recipients from three different fat tailed native breeds such as Morkaraman (n=30), Awassi (n=18) and Tuj (n=27) raised in Turkey were synchronized with vaginal sponges containing 30mg FGA (Chronogest®, Intervet Laboratories, Boxmeer, Holland) for 12 days and ewes received i.m. injection of 400 I.U. eCG (Chronogest®, Intervet Laboratories, Boxmeer, Holand) at sponge removal. Vasectomized rams were introduced to better synchronize ewes and to mark ewes as they came into heat at the rate of 5 rams per 100 ewes. The animals were screened for estrus beginning at 24 h after sponge removal and continuing up to 60 h. Screening was performed every day at 9:00 am and 9:00 pm. Estrus date and time

of onset estrus were recorded. The number of corpora lutea of recipient ewes was assessed laparoscopically at the time of transfer (Figure 3.), and all transfers were made into the uterine horn ipsilateral to the ovary with the greater number of ovulations. A total 100 frozen embryos (Romanov= 46; Charollais: 54) were thawed in 37°C for 30 sec in water bath. After thawing, embryos were randomly assigned to be placed in holding media holding media (ViGro, Holding Plus, AgTech, USA) for 5 min (A; n=34),10 min (B; n=28), 15 min (C; n=20) or 20 min (D=18) prior to transfer. Pregnancy rate of recipient ewes was assessed by ultrasound scanning 30 and 45 days after estrus in two occasions. Embryo survival rates are computed with number of lambs born form twin embryo transfer. The parameters used to assess embryo transfer success were breeds of recipient, embryo genotype, stages of embryo development, corpus luteum number of recipient, site of corpus luteum and transfer site.

Figure 3. Semi laparoscopic ovary examination



Experiment II

2.1 Fresh Embryo Transfer *(out of season and in season)*

In vivo embryo production

All donor ewes (20 Romanov) used to produce fresh embryos were kept under semi-extensive husbandry conditions and fed on a maintenance diet (ARC 1990). The mean live weight and condition score of donor ewes were measured and average live weight 55 kg and body condition score 3 were assigned as a donor. In donor selection attention was paid for also their duration between MOET program and post lambing and, weaning time. Ewes were in at least 8 weeks post lambing and weaned 10 days ago were selected as donors. Thereafter, donor ewes were divided into equally two groups to be superovulated either in breeding season (October) or out of season (May). Donor ewes were synchronized in oestrus using intravaginal progestagen pessaries impregnated with 20 mg of Cronolone (Chronogest: Intervet Laboratories Ltd) inserted on day 0 and then left *in situ* for a period of 12 days. Superovulation were induced by treatment with ovine follicle stimulating hormone, FSH (Ovagen, Immuno-Chemical Products Ltd) that was administered in 8 equal doses at 12-hourly intervals (total dose equivalent to 9 mg NIADDKoFSH- 17) commencing 60 hours prior to the end of progestagen treatment. At the last FSH injection all sponges were removed. All donors were also given prostaglandin F2alpha (0.5 ml Estrumate: Coopers Animal Health Ltd) at the time of the first injection of FSH.

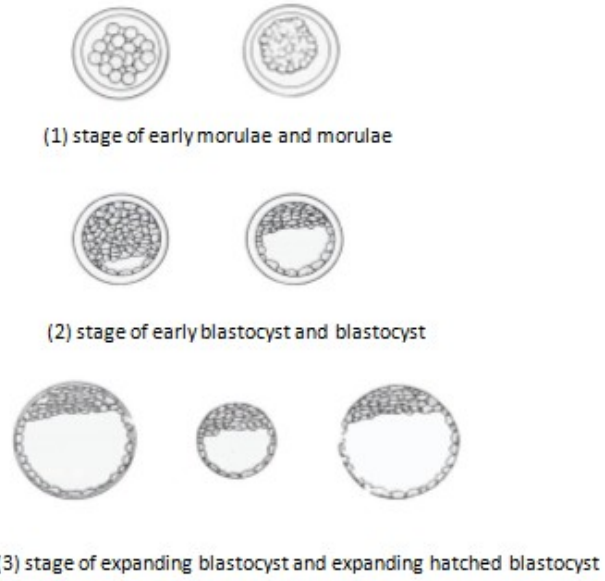
Semen collection and artificial Insemination

Fresh semen were collected on insemination days from two 4-year-old Romanov rams (average live weight 90 kg). Semen was collected using an artificial vagina and was diluted with phosphate buffered saline (PBS) to give a minimum concentration of 100 x 10^6 spermatozoa per 0.4 ml. Intrauterine insemination was carried out at 48 hours after the end of progestagen treatment with a dose of 0.2 ml diluted fresh semen for each uterine horn, containing approximately 50 x 10^6 sperm.

Embryo recovery and evaluation

Embryos were recovered by the surgical procedure on day 6 following insemination as indicated in Experiment I. All viable embryos were graded according to quality. The major criteria for evaluation included: (i) regularity of the shape of the embryo, (ii) compactness of the blastomers, (iii) differences in the cell size, (iv) colour and texture of the cytoplasm, and (v) overall diameter of the embryo. According to their stage of development the embryos were evaluated as follows: (1) stage of early morulae and morulae (2) stage of early blastocyst and blastocyst (3) stage of expanding blastocyst and expanding hatched blastocyst (Figure 4.). Specific attention was paid to minimize any post-operative adhesions in the uterus; its external wall were carefully washed with heparinised saline. Subsequently all donor ewes was injected with 5 ml of long acting oxytetracycline (Tenaline, CEVA) and were kept indoors over the next few days in order to monitor their health status before being allowed to return to grazing.

Figure 4. Embryo evaluation and scoring



Embryo transfer

2.1.1. Single fresh embryo transfer in native non-prolific recipient breeds in different season

Recipients from two groups of native breeds such as Fat tailed (breeding season; n=42 and out of season; n=20) and Thin Tailed (breeding season; n=28 and out of season; n=36) were synchronized with vaginal sponges containing 30mg FGA (Chronogest®, Intervet Laboratories, Boxmeer, Holand) for 12 days and ewes received i.m. injection of 400 I.U. eCG (Chronogest®, Intervet Laboratories, Boxmeer, Holand) at sponge removal. Recipient ewes were observed for signs of behavioral estrus at least four times daily (06:00, 10:00, 15:00, 19:00) for a minimum of 45 min at each session. Estrus detection begun immediately after eCG injection and the date and time of the first estrus for each recipient female were recorded. Based on estrus detection, 48 fat tailed native breeds of recipients (breeding season; n=34 and

out of season; n=14) and 47 thin tailed native breed of recipients (breeding season; n= 22 and out of season; n= 25) were presented to be evaluated for embryo transfer.

At the scheduled time of embryo transfer one technician performed laparoscopic examinations of the ovarian structures. The location and number of CL were recorded. In addition to laparoscopic measurements of CL in recipients, a quality score were applied to the CL of each recipient. A single experienced embryo transfer veterinarian were used the following criteria to assign each CL a quality score of the CL has a diameter of>10 mm and firm or moderately firm consistency (excellent/good); or the CL has diameter of<10 mm, or the CL has a soft texture (poor). Under normal field conditions recipients with a CL characterized as poor were rejected. For the purposes of this experiment all recipients with a solid corpus luteum > 13 mm in diameter or a cavernous corpus luteum > 13 mm in diameter with at least 3 mm of luteal tissue homogeneously distributed around the central cavity as determined by laparoscopy were designated to receive embryo as single. Fresh embryos were transferred randomly to recipients within 4 h of collection with regard to donor-recipient synchrony.

Blood samples were collected from all recipients presented for embryo transfer at 12d after transfer. Blood was collected and allowed to coagulate for 1 to 4 h at ambient temperature. Blood was then centrifuged and plasma were harvested and stored at -20°C until assayed for concentrations of progesterone using a ELISA test.

Each recipient deemed suitable for embryo transfer based on laparoscopic evaluation received single fresh embryo using a standard embryo transfer technique in accord with the International Embryo Transfer Society (Savoy, IL).

Pregnancy rate of recipient ewes was assessed by ultrasound scanning 50 days after transfer. Embryo survival rates are computed with P4 values and confirmated with lambs born form single embryo transfer. The parameters used to assess embryo transfer success were season of MOET, breeds of recipient, embryo stages, corpus luteum number of recipient and site of corpus luteum.

2.1.2 Twin fresh embryo transfer in native non-prolific and prolific recipient breeds in different seasons

Recipients from two groups of recipient breeds such as non-prolific native breed (breeding season; n=34 and out of season; n=27) and Prolific Romanov F1 (breeding season; n=15 and out of season; n=21) were synchronized with vaginal sponges containing 30mg FGA (Chronogest®, Intervet Laboratories, Boxmeer, Holand) for 12 days and ewes received i.m. injection of 400 I.U. eCG (Chronogest®, Intervet Laboratories, Boxmeer, Holand) at sponge removal. Recipient ewes were observed for signs of behavioral estrus at least four times daily (06:00, 10:00, 15:00, 19:00) for a minimum of 45 min at each session. Estrus detection begun immediately after eCG injection and the date and time of the first estrus for each recipient female were recorded. Based on estrus detection, 36 non-prolific native breeds of recipients (breeding season; n=24 and out of season; n=12) and 21 prolific breed of

recipients (breeding season; n= 13 and out of season; n= 8) were presented to be evaluated for embryo transfer.

At the scheduled time of embryo transfer one technician performed laparoscopic examinations of the ovarian structures. The location and number of CL were recorded. All recipients with a solid corpus luteum > 13 mm in diameter or a cavernous corpus luteum > 13 mm in diameter with at least 3 mm of luteal tissue homogeneously distributed around the central cavity as determined by laparoscopy were designated to receive embryo in pairs. Fresh embryos were transferred randomly to recipients within 4 h of collection with regard to donor-recipient synchrony.

Pregnancy rate of recipient ewes was assessed by ultrasound scanning 50 days after transfer. Embryo survival rates are computed with lambs born form twin embryo transfer. The parameters used to assess embryo transfer success were season of MOET, reproductive characteristic of recipient, corpus luteum number of recipient and site of corpus luteum.

Experiment III

OPS vitrification

In vivo embryo production

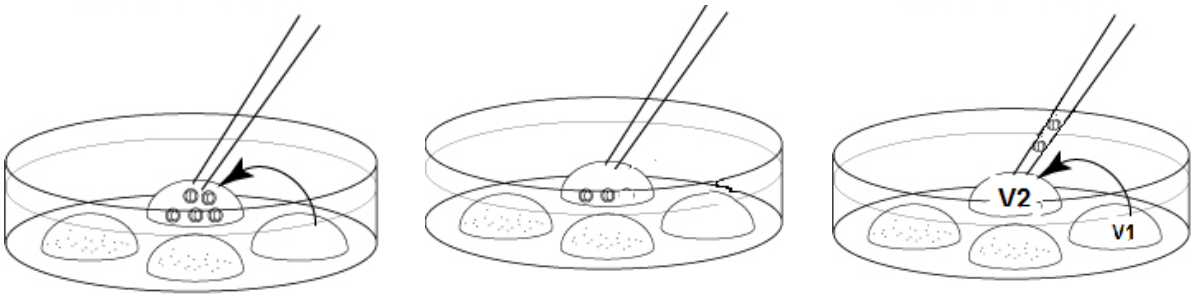
A total of 15 F1 Romanov crossbred multiparipus ewes were used as donors. Estrus was synchronized using intravaginal sponges that contained 60 mg medroxyprogesterone (Espojevet, HIPRA, Spain), which were inserted for 8 d. To induce superovulation, ewes received 6 mL (210 IU) of pFSH (Folltropin®, Bioniche Animal Health, Ireland) and 500 IU eCG (Gonaser, HIPRA, Spain) in a single i.m. administration 24 h before the intravaginal sponge was removed.

At 40 hours after sponge removal, the ewes in estrus were inseminated with fresh diluted semen ($100X10^6$ spermatozoa) using the laparoscopic technique. Six days after the insemination uterine horns were flushed from the donors via mid-ventral laparotomy under general anesthesia and ovarian responses and embryos were recorded. Each uterine horn was flushed with Dulbecco's phosphate buffer (PBS, Sigma Chemical Co., St. Louis, MO, USA) supplemented with 5 mg.mL–1
bovine serum albumin (Fraction V, Sigma) and antibiotics (penicillin and streptomycin). After washing in fresh flushing medium, all embryos were examined and classified according to their stage of development and morphology.

Vitrification

The methods previously described (Dattena et al., 2000) were used for vitrification and thawing. Briefly, French ministraws (0.25 mL, Minitube, Landshut, Germany) were softened over a hot plate at 200°C, pulled to approximately half the original diameter and wall thickness, and cut at the thinnest point. Before vitrification, embryos were assigned a developmental stage and quality grade according to standards set forth by the International Embryo Transfer Society (Savoy, IL). Developmental stage codes were: 3 = early morula; 4 = morula; 5 = early blastocyst; 6 = blastocyst; 7= expanded blastocyst; 8= hatching blastocyst, 9= hatched blastocyst. Quality codes are: 1 = symmetrical and spherical embryo mass with individual blastomeres that will uniform in size, color, and density with at least 85% of the cellular material intact (excellent or good); and, 2 = moderate irregularities in overall shape of embryonic mass or in size, color and density of individual cells with at least 50% of the cellular material intact (fair). Embryos receiving stage codes of less than 5 or greater than 6 or poor quality embryos (quality score of 3 or 4) were excluded from the study.

The embryos were first washed with PBS enriched medium (Glucose: 0,09 g/100 ml, Pyruvic Acid: 3,6 mg/100 ml, 10% FCS) then in diluted (V1: PBS + 20% Fetal Bovine Serum with 10% ethylene glycol and 10% dimethyl sulfoxide) and concentrated (V2: PBS + 20%FBS with 20% EG and 20% DMSO) vitrification media. Embryos were drawn up together in another 1–2 mL drop, loaded by capillarity into the narrow end of an OPS and plunged into liquid nitrogen. The time between the contact of the embryos with the concentrated cryoprotectant solution V2 and the liquid nitrogen did not exceed 45 s (Figure 5.)



a. Washing Blastocyst/Hatching Blastocyst with PBS enriched

b. Two embryos placed in a drop of Solution V1 (drop of 150 µl) for 3-5 min

c. Embryos placed in drop of Solution V2 (drop of 150 µl) for 30 sec

Figure 5. Vitrification of embryos

Embryo transfer

Vitrified embryos were used after storage for at least 7 days in liquid nitrogen (LN_2), embryos were warmed for *in vitro* culture. For warming, straws were held in air for 5 s before narrow end was immerged in Hepes-buffered TCM199-10%FCS with 0.5 M sucrose. After thawing, embryos remained suitable for transfer were transferred into recipient ewes. Survival was defined as the re-expansion of the blastocoele.

A total of 48 Fat-Tailed native Awassi were used as recipient. They were synchronized in oestrus by the use of intravaginal pessaries impregnated with 60 mg of medroxyprogesterone acetate (Esponjavet, HIPRA, Spain). The pessaries were inserted on day 0 and then left *in situ* for a period of 12 days. At sponge removal 400 IU eCG (Gonaser, HIPRA, Spain) were administered by intramuscular injection. Estrus were detected and recorded by using vasectomies rams fitted with crayon marks. A total 15 blastocyst and 15 hatching blastocyst vitrified embryos were transferred, as single, into 30 recipient ewes. Return to service was recorded using teaser rams, with marking colors. The confirmation of

pregnancy to the transferred embryos were be done by ultrasound scanning at day 28 after transfer.

Statistical Analysis

The General Linear Model procedure of MINITAB was used to analyze all data involving categorical pregnancy data (Table 1). Data collected for embryo and recipient intrinsic were evaluated. The effects of using fresh versus frozen/vitrified thawed embryos, recipient and embryo genotype, season of fresh embryo transfer, stage of embryo development, number of embryos transferred, CL number, CL and transfer site and all two-way interactions on pregnancy rate were determined using the combined data set. Correlation and regression analyses of progesterone concentrations at the 12 d of embryo transfer on pregnancy rate and embryo survival rates were performed using the CORR and REG procedures of SAS, respectively.

Table 1. MOET program and factors studied.

BREED OF RECIPIENT	NO	EMBRYO GENOTYPE	NO	EMBRYO FRESH/FROZEN	NO OF EMBRYOS TRANSFERRED	SEASON OF TRANSFER	FACTORS STUDIED
EXPERIMENT I.							
AWASSI	18	CHAROLLAIS	14	FROZEN	TWIN	BREEDING SEASON	RECIPIENT BREED
		ROMANOV	10				EMBRYO GENOTYPE
MORKARAMAN	30	CHAROLLAIS	14				STAGE OF DEVELOPMENT
		ROMANOV	16				CL NUMBER
TUJ	27	CHAROLLAIS	26				CL SITE-TRANSFER SITE
		ROMANOV	20				THAWING DURATION
EXPERIMENT II.							
II.A		SEASON OF TRANFSER		EMBRYO	NO OF EMBRYOS	EMBRYO GENOTYPE	FACTORS STUDIED
FAT TAILED	48	BREEDING SEASON	34	FRESH	SINGLE	ROMANOV	RECIPIENT GENOTYPE
		OUT OF SEASON	14				SEASON OF TRANSFER
THIN TAILED	47	BREEDING SEASON	22				STAGE OF DEVELOPMENT
		OUT OF SEASON	25				QUALITY GRADE OF EMBRYOS
II. B		SEASON AND REPRODUCTIVE ABILITY OF RECIPIENT		EMBRYO	NO OF EMBRYOS	EMBRYO GENOTYPE	FACTORS STUDIED
PROLIFIC	36	BREEDING SEASON	26	FRESH	TWIN	ROMANOV	SEASON OF TRANSFER
		OUT OF SEASON	16				BREED OF RECIPIENT
NONPROLIFIC	61	BREEDING SEASON	48				CL NO AND SITE CL and TRANSFER
		OUT OF SEASON	24				
EXPERIMENT III.							
RECIPIENT BREED	N	EMBRYO STAGE OF DEVEOPMENT	N	EMBRYO	NO OF EMBRYOS	EMBRYO GENOTYPE	FACTORS STUDIED
AWASSİ	15	HATCHING BLASTOCYCST	15	OPS VITRIFIED	SINGLE	G1 ROMANOV	EMBRYO STAGE OF DEVELOPMENT
AWASSİ	15	BLASTOCYST	15				CL NUMBER CL SITE

CHAPTER V

RESULTS

3.1. Frozen embryo transfer

A total of 75 recipients (Awassi; n=18; Morkaraman; n=30 and Tuj; n= 27) were presented for embryo transfer. Attention was paid for selecting recipients with CL firm and quality score of the CL has diameter of > 10mm. Ovulation rates in Awassi (1.6±0.16) and Tuj (1.7±0.10) breeds were found significantly ($P<0.01$) different than Morkaraman (1.2±0.09) breed of recipients. Frozen-thawed embryos transferred to Awassi breed were resulted with average 20% higher ($P>0.05$) pregnancy rates than Morkaraman and Tuj breeds in 30d after onset estrus. The pregnancy rates differences were further increased approximately 8% more between Awassi and Tuj breed of recipients. Pregnancy loss during 30 to 45d after estrus within breeds were 1.6%, 4.3% and 9.7% for Awassi, Morkaraman and Tuj breeds, respectively. Tuj breed of recipients were recorded with higher pregnancy loss compared to Awassi and Morkaraman. Awassi breed was found superior with less reproductive loss between days 30 and 45 of pregnancy.

However, Morkaraman and Tuj ewes were recorded with 18.1% and 21.1% higher embryo survival rates compared to Awassi breed in twin frozen-thawed embryo transfer (Table 2.). Contrary to higher pregnancy loss observed in Tuj breed by day 45, embryo survival rates were found 9.1% and 27.2% higher than Morkaraman and Awassi breeds. Tuj breed can be pronounced as better recipient for embryo survival rates. Overall MOET success

(no. of lambs born/no of. embryos transferred) in frozen embryo transfer was 24/36 (66.6%), 34/60 (56.7%) and 29/54 (53.7%) for Awassi, Morkaraman and Tuj, respectively. Thus, even though differences is not significantly different, Awassi breed can be pronounced as better recipient with 10-13% higher overall MOET success compared to other two native breeds (Figure 6.).

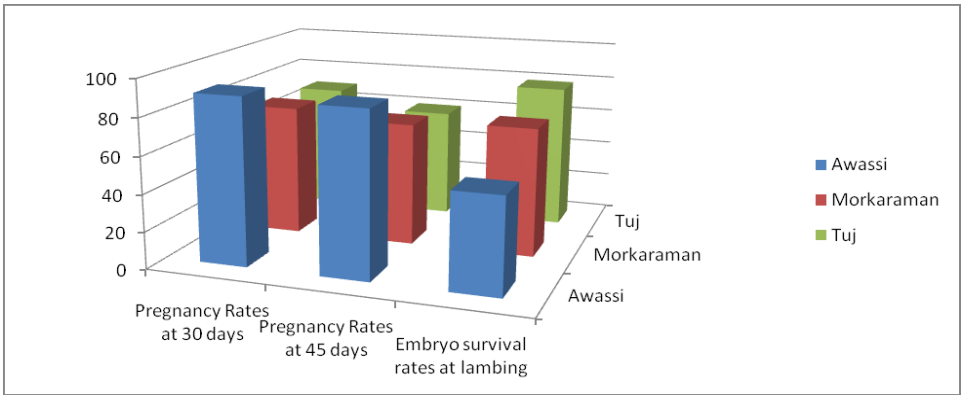


Figure 6. Reproductive response of recipient breeds in frozen-thawed embryo transfer

The other factors related to embryo transfer success such as CL site and transfer site did not significantly affect pregnancy rates and embryo survival rates in this study. However, pregnancy rates at 30 and 45 days were higher when CLs are placed in either left or both ovaries compared to recipients ovulated in right ovary. Embryo survival rates were found 88.9% in recipient ewes with ovulation occurred in left ovary and it was higher ($P>0.05$) than recipients with CL located in right or both ovaries (Figure 7.).

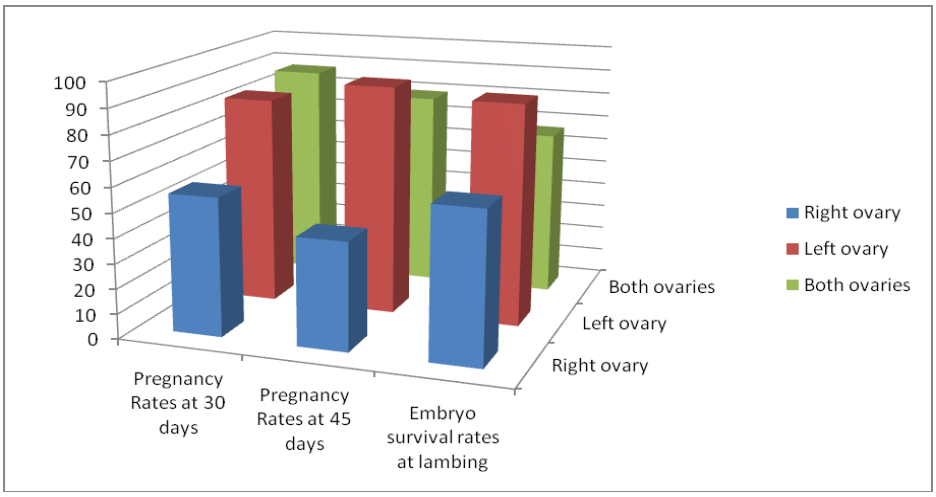


Figure 7. Reproductive response in ovulation sides.

There was a controversy increase in pregnancy rates and survivability of embryos transferred to right horn compared to those transferred left horn. The differences were higher (41.7%) in day 30 compared to pregnancy rates (25%) obtained in day 45. Embryo survival rates were 17.9% higher in right uterine horn transferred embryos.

The total CL number did not resulted with significance differences in pregnancy rates at day 30 and 45. However, pregnancy loss in single and multiple ovulated recipients were two times in day 45 compared to day 30. There was a 12.5% pregnancy loss in recipients with multiple ovulations at day 45. Moreover, embryo survival rates were 15% less in recipients with multiple ovulations (Table 2.).

Table 2. Pregnancy rates (30 and 45 days) and embryo survival rates in recipient related factors in frozen embryo transfer program

Factors	Pregnancy Rates at 30 days (%)	Pregnancy Rates at 45 days (%)	Embryo survival rates at lambing (%)
Breed of Recipient			
Awassi	90.5±15.41	88.9±16.20	51.8±9.99
Morkaraman	71.3±9.91	67.0±10.41	69.9±7.13
Tuj	69.9±10.1	60.2±10.58	79.0±6.70
P value	0.527	0.357	0.096
CL site			
Right ovary	55.5±13.69	43.2±14.41	60.3±9.74
Left ovary	83.9±19.67	92.3±20.71	88.9±13.05
Both ovaries	87.5±10.47	79.2±11.03	66.7±6.89
P value	0.243	0.173	0.276
Transfer side			
Right horn	92.4±14.15	88.1±13.44	80.7±8.61
Left horn	50.7±12.55	63.1±11.92	62.8±8.23
P value	0.239	0.065	0.206
CL number			
Single	74.07±8.43	70.4±9.29	75.0±6.04
Multiple	68.42±10.54	57.9±11.07	60.0±7.63
P value	0.683	0.393	0.136

Embryo genotype was not source of variance in frozen-thawed embryo transfer in this study (Table 3.). However, embryos from Romanov breed, known with high prenatal survival rates, had higher pregnancy rates in 30 (19.3%) and 45 (14.4%) days of pregnancy compared to those recorded for Charollais embryos. Embryo survival rates, computed as number of lambs born from twin embryo transfer, were similar between two embryo genotypes.

Stage of embryo development was differed for embryo survival rates but not for pregnancy rates at day 30 and 45. Even though, morula stage of embryos were recorded with higher pregnancy rates at day 30 of pregnancy, the result was changed contrary at day 45. In general, embryos in expanded blastocyst were found higher both in pregnancy and embryo survival rates. Moreover, embryos in expanded blastocyst stage had 100% of survival rates compared to early blastocyst stages (55.6%) and difference was significantly important ($P<0.05$).

Embryos frozen with slow freezing technique were kept in holding media in different duration to access pregnancy rates and survivability after transfer. When embryos were kept longer than 10 minutes in holding media, pregnancy rates were tended to decline, while survivability of embryos were significantly increased ($P<0.05$) with increasing the time of holding (Table 3.). As pregnancy rate does not discriminate between implantation of one or more embryos, and is still an intermediate outcome, live birth rate *per* transferred embryo, although more demanding, are clearly a more informative means of comparison between groups regarding embryo developmental potential. As so, although pregnancy rates were not statistically different between groups, live birth rates *per* embryo were significantly higher

either duration between 1-5min (77.3%) or 10-20min (average 88%) than the 5-10min culture group (50%), emphasizing the biological impact of the four culture periods.

Table 3. Pregnancy rates (30 and 45 days) and embryo survival rates in embryo related factors in frozen embryo transfer program

Factors	Pregnancy Rates at 30 days (%)	Pregnancy Rates at 45 days (%)	Embryo survival rates at lambing (%)
Embryo genotype			
Romanov	81.8±9.59	72.7±10.26	70.6±6.10
Charollais	62.5±9.17	58.3±9.83	67.9±6.72
P value	0.153	0.317	0.766
Stage of embryo development			
Morula	80.0±11.68	66.7±12.46	75.0±7.93[a]
Early Blastocyst	71.4±12.09	71.4±12.90	55.6±7.48[b]
Blastocyst	72.2±10.66	66.7±11.37	68.2±6.76[ab]
Expanded Blastocyst	75.0±22.62	75.0±24.12	100.0±7.93[a]
P value	0.953	0.982	0.04
Post-thaw culture duration			
1-5 min	70.6±11.10	70.6±11.60	77.3±6.21[a]
5-10 min	85.7±12.23	78.6±12.78	50.0±6.86[b]
10-15 min	55.6±15.26	44.4±15.94	75.0±10.29[a]
15-20 min	66.7±18.69	50.0±19.53	100.0±14.55[a]
P value	0.481	0.320	0.01

3.2. Fresh embryo transfer in different season (single and twin transfer)

Approximately 87% of Turkey's sheep population (20.6 million heads) is fat-tailed breeds. Akkaraman and Morkaraman sheep are the most numerous and make up nearly 65% of the total sheep population. These sheep are thought to have evolved through natural selection under harsh environmental conditions. Awassi is also a fat-tailed breed reared extensively in southern part of Turkey and it is estimated to be about one million Heads.

These sheep are known for their hardiness and adaptability to the local environment but prolificacy is low, with ewes usually producing single lambs. In addition to that, they are highly seasonal due to the geographic origin of these breeds, with those originating high latitude and cold climate having a more restricted season. One of the other fat tailed breed, Daglıc is grown on the mountainous terrain of the region Ege and Marmara and is estimated to be about 3.9 million heads. Thin-tailed Kivircik is the main native sheep breed of Thrace and Marmara regions of Turkey and there are about 1.7 million heads (Turk Stat 2008).

In the Experiment II, Morkaraman, Akkaraman, Awassi and Dağlıç is categorized as fat tailed and Kıvırcık (including its crosses) was grouped as thin tailed recipient breeds. Then, two group of recipient ewes were subdivided and the estrus of the ewes were synchronized in out of season and breeding season. Within the Experiment II, a second set of trial was conducted to access reproductive performance of prolific F1 Romanov compared to native fat tailed counterparts in different season in which fresh embryos were transferred in pairs. Reproductive success of two sets of recipients are given Table 4 and Table 5.

Contrary to widely documented that estrus response were higher (P>0.05) in out of season than breeding season in Fat (FT) and Thin Tailed (TT) native breed of recipients. However, ovulation rates were significantly higher (P<0.001) in breeding season compared to out of season. There was a 0.7 increase in ovulation rates when FT and TT recipients received same dose of eCG in both seasons. Even though recipient categorized as fat and thin tailed, estrus rates were not different, whereas ovulation rates of thin tailed recipient were relatively higher than fat tailed counterparts (Table 4.). Pregnancy rates were found highest (64.7%) in TT recipients used in breeding season. Overall pregnancy rates were found 52% in single fresh embryo transfer in which embryos with less than Grade 3 were not excluded from the experiment.

The overall MOET success were estimated from number of lambs born out of number of embryos transferred into recipients in 6 day after onset estrus. Thin tailed recipients in breeding season were recorded with the highest (65%) MOET success. Same group recipients used in out of season had 58% for MOET success and fat tailed breeds of recipients included in the study in out of season (30%) and breeding season (56%) followed, respectively (Figure 8.). In general, overall MOET success was similar in out of season and breeding season and 57-47% of MOET success was achieved. However, within recipient group comparison, it was found that TT group showed 19% better reproductive performance than FT group.

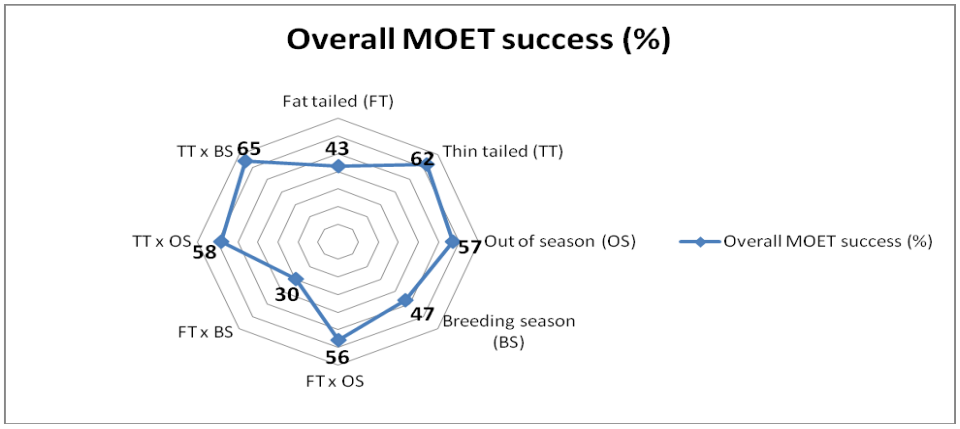


Figure 8. Overall MOET success in different recipient groups used in two seasons.

Table 4. Fat and Thin Tailed recipient's reproductive performance in different season.

Factors	**Estrus rate (%)**	**Ovulation rates**	**Pregnancy rates**
Group of Recipient			
Fat tailed (FT)	89.7±7.22	1.2±0.09	42.6±9.43
Thin tailed (TT)	81.5±6.58	1.5±0.09	61.5±9.23
P value	0.404	0.073	0.157
Season			
Out of season (OS)	92.9±8.23	1.0±0.11	56.9±10.80
Breeding season (BS)	78.3±5.27	1.7±0.08	47.2±7.58
P value	0.142	0.000	0.462
Interaction			
FT x OS	100.0±12.8	1.1±0.17	55.6±16.33
FT x BS	79.4±6.60	1.4±0.09	29.6±9.43
TT x OS	85.7±10.29	1.0±0.14	58.3±14.14
TT x BS	77.2±8.21	1.9±0.12	64.7±11.89
P value	0.536	0.073	0.226

In the second set of trial, it was aimed to evaluate superiority of prolific F1 Romanov recipient obtained with crossbreeding with native fat tailed breeds. Estrus response and ovulation rates were found similar between prolific and non-prolific group of ewes. However, pregnancy rates were 21.5% higher ($P>0.05$) in prolific breed of recipient than non-prolific breed of recipients (Table5.). Embryo survival rates in two breeds of recipient did not differed significantly and average embryo survival rate was 75% in fresh twin embryo transfer.

Season of MOET resulted with significant differences in estrus and ovulation rates. Recipient ewes treated in out of season showed significantly higher ($P<0.01$) estrus response than those treated in breeding season. Even though higher estrus response was achieved in out of season, ovulation rates were significantly ($P<0.01$) lower in recipient ewes treated in out of season.

In terms of pregnancy rates and embryo survivability, season was not found an important factor. Interaction between recipient breed and season, it was found that the highest pregnancy rates (84.6%) were obtained with prolific breed recipients used breeding season. Prolific breed recipient used in out of season ranked second with 75% pregnancy rates. Non-prolific recipient breeds recorded with 8.3% and 34.6% less pregnancy rates compared to prolific breed recipients in out of season and breeding season, respectively. Pregnancy loss was greater in breeding season when two breeds of recipient are compared and pregnancy rates were similar between prolific and non-prolific breed of recipients.

Even though there is superiority for pregnancy rates in prolific breeds, non-prolific breeds showed similar reproductive success in embryo survival rates with those observed in prolific breed recipients.

The overall MOET success was higher in Profile F1 recipients (70%) than those computed for Non-prolific recipients (51%). The MOET success decline between seasons was acceptable for Prolific recipients with 8%, while Non-prolific recipients performed 18% less MOET success in out of season compared to those in breeding season (Figure 9.).

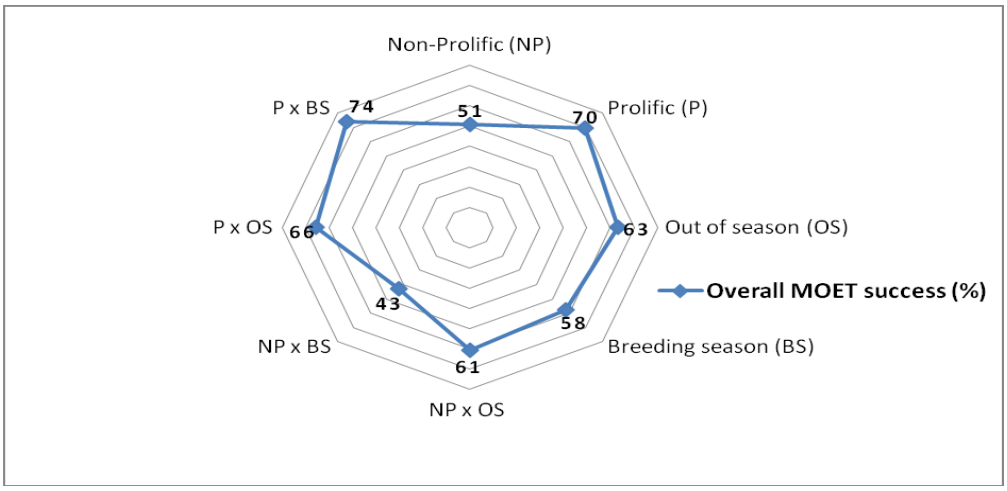


Figure 9. Overall MOET success in Prolific and Non-prolific recipient breeds in two seasons.

In comparison two sets of trial, it was found that overall pregnancy rates were similar between native breeds received single (52%) or twin embryos (58.3%). As it is shown in Figure 10., twin embryo transfer was resulted with slightly higher overall MOET success.

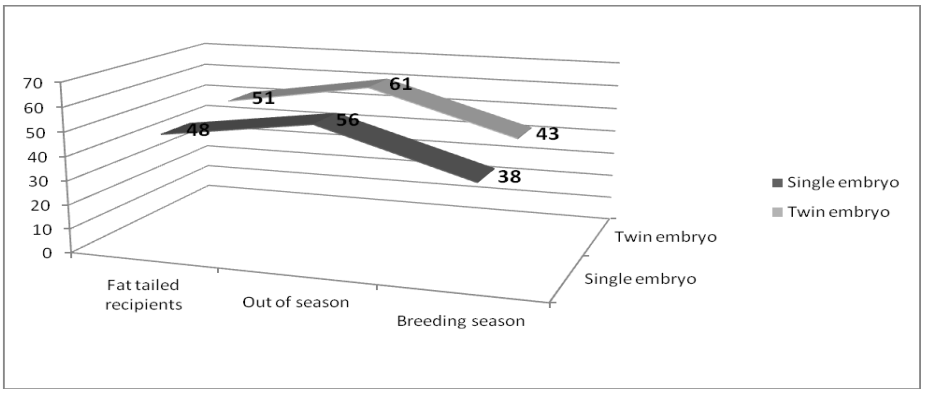


Figure 10. Overall MOET success in single and twin embryo transfer in Fat Tailed breed of recipients.

Table 5. Prolific and Non-prolific recipient's reproductive performance in different season.

Factors	**Estrus rate (%)**	**Ovulation rates**	**Pregnancy rates**	**Embryo survival rates**
Group of Recipient				
Non-Prolific (NP)	69.2±5.87	1.3±0.09	58.3±8.36	76.0±6.42
Prolific (P)	69.5±7.53	1.5±0.12	79.8±10.62	74.3±7.15
P value	0.971	0.293	0.198	0.859
Season				
Out of season (OS)	84.5±6.90[a]	1.2±0.13[a]	70.8±10.80	78.13±7.61
Breeding season (BS)	54.2±6.59[b]	1.7±0.09[b]	67.3±8.14	72.23±5.88
P value	0.002	0.002	0.795	0.544
Interaction				
NP x OS	56.0±8.91	1.3±0.15[a]	66.7±13.66	81.3±9.96
NP x BS	82.4±7.64	1.3±0.11[a]	50.0±9.65	70.8±8.13
P x OS	52.4±9.72	1.0±0.19[a]	75.0±16.72	75.0±11.50
P x BS	86.7±11.51	2.0±0.15[b]	84.6±13.11	73.6±8.49
P value	0.679	0.002	0.336	0.641

In terms of embryo related factors such as, no of embryos transferred, stage of embryo development and quality grade of embryos there was no significant differences (Table 6.). However, embryo grade less than Grade 2 resulted with 26.5% lower pregnancy rates than Grade 1-2 embryos. In addition to Grade of embryos, within the stage of embryo development, expanded blastocyst showed a relatively higher pregnancy rates than earlier stages of embryos.

Table 6. Pregnancy rates in embryo related factors in single embryo transfer.

Factors	**Pregnancy rates (%)**
Stage of embryo development	
Morula	46.7±9.23
Blastocyst	38.9±11.92
Expanded Blastocyst	53.1±8.94
P value	0.631
Quality grade of embryos	
Grade 1-2	73.21±19.82
<Grade 2	46.7±8.73
P value	0.227

Pregnancy rates were compared between two set of trials by considering CL number, CL site and transfer site of embryos (Table 7). There was a higher (76.2%) pregnancy rates in twin embryo transferred recipients with higher ovulation rates compared to recipients (51.3%) received single embryo. In both trials, pregnancy rate was tended to increase when CL number is higher. However, this increase was more dramatic in twin embryo transferred

recipients (17.9% higher) compared to single embryo transferred recipients (5.3% higher). Corpus luteum location in uterine horns was not source variation in pregnancy rates in single embryo transfer, while CL located in both uterine horns in recipient received embryos in pairs resulted with higher pregnancy rates than left horn located (41.7%) and right horn located (32.9%). Transfer site of embryos resulted in similar pattern in single and twin embryo transfer. Left site embryo transfers were resulted with higher pregnancy rates in single (17.3%) and twin (10%) embryo transfer (Table 7.).

Table 7. Pregnancy rates of factors related to CL number, CL and embryo transfer site in two sets of fresh embryo transfer

Factors	**Single embryo transfer**	**Twin embryo transfer**
CL number		
Single	46.0±8.60	58.3±7.96
Multiple	51.3±13.04	76.2±10.42
P value	0.752	0.179
CL site		
Right horn	54.2±17.18	62.1±21.04
Left Horn	41.4±18.02	53.3±20.72
Both horn	50.3±13.19	95.0±18.33
P value	0.897	0.106
Transfer site		
Right	40.0±15.19	65.2±15.69
Left	57.3±13.93	75.2±23.35
P value	0.501	0.786

Pregnancy rates were determined with progesterone (P4) level at day 12 after embryo transfer in single embryo transferred embryo recipients. The P4 levels were grouped four categories such as, A: 0.21ng/mg-1ng/mg, B: 1ng/mg-2ng/mg, C: 2ng/mg-3ng/mg and D: >3ng/mg. Recipient ewes in A, B, C and D groups were recorded with 15.4%, 50.0%, 66,7% and 77.8% pregnancy rates, respectively. There was a significant ($P<0.05$) increase in pregnancy rates when recipient P4 levels are higher than 2ng/mg. Thus, it can be concluded that P4 levels can be strong determinant in early pregnancy diagnosis in embryo transfer.

3.3. OPS vitrified embryo transfer (blastocyst versus hatching blastocyst)

In the experiment III, embryos vitrified with open pulled straws were in two stage different (expanded blastocyst and hatching blastocyst) of development to evaluate pregnancy success. Awassi breed of sheep (n=30) was selected as recipient genotype owing to its better reproductive response in frozen-thawed embryo transfer in experiment I. The first reproductive performance was measured as percentage of animals return to estrus days between 10 and 20 after onset estrus and pregnancy diagnosis were made at day 28 after onset estrus to confirmation of return rates. Recipient received single embryos in both development stages had 50% higher pregnancy rates when embryos were transferred into left uterine horn. Similarly, recipient ewes with more than one ovulation had less pregnancy rates than those only have single ovulation (Table 8. and 9.).

Result of this experiment showed that reproductive success was increased with single ovulated recipient ewes and embryos transfered into left site of uterine horn. Stage of embryo

did not differed for pregnancy success. Hatching blasctocyst stage of embryos were successfully frozen by two step of vitrification as well as blastocyst stage of embryos. Overall pregnancy results were 79.2% for expanded blastocyst and 76.4% for hatching blastocyst.

An additional trial were conducted to compare single frozen embryo transfer success with expanded blastocysts stages. The results showed that conventional freezing method was resulted with (4/6) 66.6% of pregnancy rates which was 12.6% lower than those obtained with vitrification method. The diagram 1. was given for overall evaluation of the effect of transfer site on pregnancy rates at day 45 after onset estrus.

Table 8. Pregnancy rates after single ipsilateral transfer of D 6.5-7.5 ovine vitrified embryos of different stages according to transfer site

Stage of development	Transfer site	Embryos transferred	Pregnancies	
		n	N	%
Expanded blastocyst	Right	9	4	50
	Left	6	6	100
Hatching blastocyst	Right	9	5	55.5
	Left	6	6	100

Table 9. Pregnancy rates after single ispsilateral transfer of D 6.5-7.5 ovine vitrified embryos of different stages according to CL number

Stage of development	CL number	Embryos transferred	Pregnancies	
		N	N	%
Expanded blastocyst	Single	4	4	100
	Multiple	11	7	66.6
Hatching blastocyst	Single	3	3	100
	Multiple	12	6	50

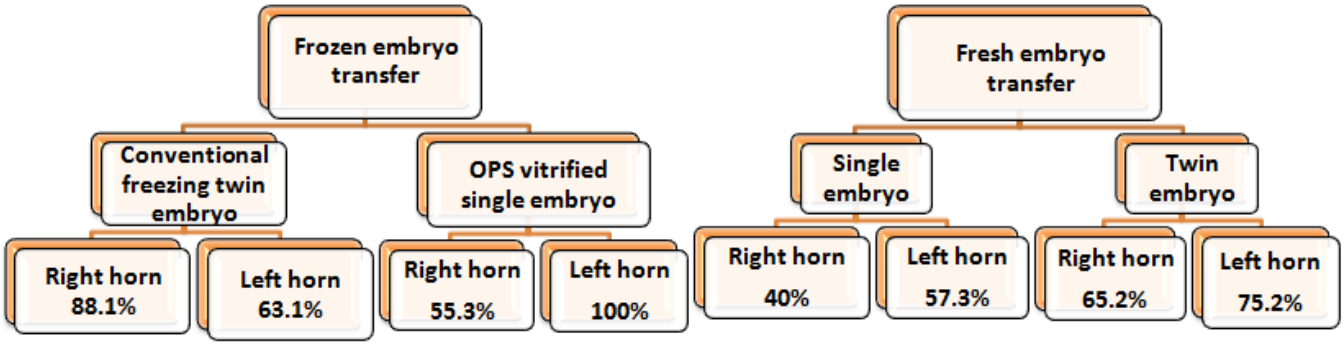


Diagram 1. The effect of transfer site on pregnancy rates in frozen and fresh embryo transfers

CHAPTER VI.

DISCUSSION

Frozen-thawed embryo transfer experiment demonstrates that the genotype of the mother providing the uterine developmental environment influences pregnancy and embryo survival rates of her progeny. Dairy type, Awassi breed of recipient was superior to multiple purpose native fat tailed breeds. Pregnancy losses between day 30 and 45 were 2.6 and 6.1 times lower in Awassi than those determined for Morkaraman and Tuj breeds.

Individual sheep have their own temperament/emotional reactivity. Some sheep are more at ease with isolation, novelty and close contact with humans (calm) whereas others display a more "nervous" disposition and have difficulties coping with these same situations. Previous studies have shown that calm ewes are better mothers. It is understandable that a reduction of fear and emotional reactivity affects reproduction because the reproductive endocrine axis can be profoundly influenced by stress, as has been clearly demonstrated for farm animals (Von Borell et al., 2007).

In fact, the domestication of mammals illustrates that temperament, or emotional reactivity or tameness, affects reproductive capacity and reproductive physiology, either directly or indirectly (Price, 2002). In sheep, so far, the selection for temperament have been demonstrated to affect the behavior of the females during the mating period and in the early stages of gestation and on the survival of newborn lambs.

Recently, Blache and Bickell (2010) have shown that temperament can affect ovulation where ewes of a calm temperament have a greater ovulation rate than nervous ewes (Hart et al., 2008). Calm ewes have been reported with a higher spontaneous ovulation rate compared to nervous ewes (1.63 v 1.26, P=0.003) and a higher ovulation rate than nervous ewes (1.83 v 1.57, P=0.03) in response to synchronization using intravaginal progestagen and an injection of eCG. Also, more multiple gestations were observed in calm ewes than nervous ewes on Day 74 (Calm 1.60 v Nervous 1.35, P=0.03) but the pregnancy rate was similar in both lines (Hart et al., 2008). Blache and Bickell (2010) reported that ewes of calm temperament also carried more twin embryos than nervous ewes (1.39 embryos, n = 472: 1.29 embryos, n = 302; $p < 0.001$). The biological mechanisms behind the differences in ovulation rate and twin bearing percentage have not been truly investigated.

In this study, Tuj breed is recognized with less calm temperament compared to Awassi and Morkaraman. Nervous temperament of Tuj breeds can be a source of greater pregnancy loss compared to other two breed of recipients which are less nervous. Even though, Tuj breed had similar ovulation rates with Awassi breed in synchronized estrus, it could be due to relatively small size of Tuj which responded eCG treatment with higher ovulation rates. The higher embryo survival rates in Tuj breeds can be explained by high ovulation rates induced with eCG treatment.

Recipient breed difference observed in frozen-thawed embryo transfer experiment was in accordance with findings of Bradford (1972) that the genotype of the dam rather than of the offspring which is responsible for genetic variation in prenatal survival. Additional support of the current study result is from the experiment in which black faced ewes experienced the

greater pregnancy losses than those occurred in white faced ewes (Dixon et al.,2007). Similar results also have been obtained with Columbia and Hampshire ewes. In that study, Columbia ewes were superior to Hampshire breed with fewer embryos lost up to 25 days of gestation (Foote et al., 1959). General conclusion of these studies emphasized that the shorter breeding season and deeper anestrus of specific breeds were main reason of greater reproductive losses.

Since 1995, numerous studies have reported production of live offspring following cryopreservation of sheep embryos (Songasasen et al., 1995; Cocero et al., 1996; Loi et.al. 1998). This technique brings numerous advantages such as frozen embryos can be easily transported from one place to another (Leibo, 1989; Ali et al., 1993; Martinez e al., 1997). In this study, one of the main objective was to evaluate feasibility of introducing new genetics through the frozen embryo transfer. Youngs (2011) reported that under optimal conditions, pregnancy rates obtained after transfer of frozen-thawed *in vivo* derived embryos is typically 65-75% in sheep. The pregnancy rates obtained after the transfer of cryopreserved embryos by controlled slow freezing, compared favorably with those reported elsewhere (Ishida et al., 1999, Baril et al., 2001a, Baril et al., 2001b).

In the current study, overall pregnancy results of in vivo derived frozen embryos was 72% and it was higher than 58.3%, 39.5% and 58.3% reported by Heyman et al., (1987), Shelton (1992) and Bettencourt et al., (2009), respectively. The pregnancy rates and embryo survival rates observed in this study with cryopreserved (72% and 66.9%, respectively) embryos were comparable to those reported for the other breeds of sheep. Martinez and Matkovic, (1998) obtained 51.3% pregnancy rates with embryo frozen with ethylene glycol. In another study conducted by Martinez et al., (2006), ovine embryos were cryopreserved in ethylene glycol using 3 steps and pregnancy result was reported as 40% which was lower than

current study findings. Higher pregnancy rates obtained in this study may be attributed to the fact that in this trial only Grade 1 and 2 and morula to blastocyst stage of embryos were frozen and transferred after thawing.

Embryo survival rates observed in this study for embryos frozen with controlled slow freezing was higher in Tuj (79%) and Morkaraman (69.9%) than those observed in Awassi (51.8%). The rates of embryo survival for Awassi recipients following transfer of frozen embryos are in accordance with those reported in Portuguese Black Merino ewes (Bettencourt et al., 2009) and Suffolk ewes (Ishida et al., 1999), but higher than those reported for Saloia breed (Chagas e Silva et al. (2003). The higher embryo survival rates for frozen-thawed embryos in Morkaman and Tuj ewes compared to other studies could be related to the use of different cryoprotectants and advantage of recipients being local breeds of the region.

In recipient selection, monotocous character of recipients used in this experiment was not a limiting factor for the acceptable pregnancy rates of twin frozen-thawed embryo transfer which is in agreement with results reported by Naqvi et al., (2007). Ovulation rates of recipient ewes was not more than 2 and either pregnancy rates or embryo survival rates were not under the influence of CL numbers. The present study results for the effect of ovulation rates on embryo transfer success was in agreement with results obtained by Scaramuzzi and Downing (1997) reported that embryo survival was influenced by ovulation rate if ovulation rates of ewes are four or more.

In ewes with multiple ovulation induced by eCG treatment or prolific characteristic of breed, left ovary plays a significant greater role. It was emphasized that embryo losses are

greater in ewes with single ovulation and CL in the right ovary (Casida et al., 1966). In the current study, CL site did not affect the pregnancy and embryo survival rates which was in agreement by report where site of ovulation had no effect on embryo survival (Scaramuzzi and Downing, 1997). However, lower pregnancy (27-32%) and embryo survival (29-7%) rates were observed in ewes ovulated in right ovary compared to those CL observed in left or both sides of ovaries. There are conflict reports for multiple ovulation either occurs mostly in right or left ovary (McKenzie and Terrill, 1937; Casida et al., 1966; Taneja 1959 and Basu et al., 1961). Some of the researchers claimed that pregnancy losses are greater in right ovary than left ovary in single or multiple ovulated ewes whereas others reported contrary results. In general, differences in pregnancy losses between recipients with ovulation in right and left ovaries were greater (30% versus 5%) than those reported by Casida et al., (1966). The differences could be explained by the origin of embryo which was frozen-thawed embryos used in the current study while reports for lower pregnancy losses was from in vivo embryos produced by natural mating.

It was concluded that the uterine horn to which a transfer was made slightly affected embryo transfer success which is in agreement with Remsem et al., (1982) reported that uterine horn of transfer is an influencing factor in embryo transfer. Whereas, Waever et al.,(1986) indicated that the uterine horn to which a transfer was made is not source of variation on embryo transfer success. The results of different horn embryo transfer in cattle was also supporting that there was no any difference in pregnancy rate between transfers to the left or right side (Lamb, 2005).

Two different embryo genotypes were used in inter-breed frozen embryo transfer in a way of introducing exotic breed of sheep. Embryo genotype was not found as a important factor in pregnancy success of frozen-thawed embryo transfer in this study. This finding is similar to the previous study (Moler et al., 1980) conducted in different mouse lines. However, embryos from Romanov breed, known with high prenatal survival rates, had relatively higher pregnancy rates in 30 (19.3%) and 45 (14.4%) days of pregnancy compared to those recorded for Charollais embryos. Similarly, Boediono et al., (2003) demonstrated that crossbred embryos were superior for faster and higher development rates than purebred embryos. Lazarri et al., (2011) worked with cattle in vitro embryos by investigating gene expression and concluded that genes related to developmental competence of the embryos were expressed differently in various embryo genotypes. Inter-breed embryo transfer results of the current study suggests that efforts toward genetic improvement of prenatal survival should focus on the dam, since differences due to the maternal component were much greater than those due to the offspring.

Stage of embryo development was studied by numerous researchers as a possible source of variation in pregnancy rates. In the present study, pregnancy rates were not affected by stage of development, while embryo survival rates were more stage sensitive. In general, embryo in later stages resulted with higher overall success. This result was agreed with previous works conducted by Hasler (1998), Lamb (2005) and Block et al., (2009). The higher reproductive success with embryos in later stages of development could be due to quality grade which is better than those less developed embryos. Many authors prefer to freeze embryos at early stages: they consider that conditions of in vitro culture are still suboptimal and the benefits of prolonging in vitro culture do not compensate for selecting the

best embryos. However, the experiment conducted by Garcia et al., (2005) was focused on in-vitro culture of early stages of embryos to the blastocysts stages before to be frozen. They found that embryos cultured to the blastocyst stage and frozen thereafter had a significantly higher viability than their counterparts frozen at earlier cleavage stages, (66.1% versus 23.1%; $P < 0.0001$). Probability for reaching the hatching blastocyst stage after thawing of 2- to 4-cell embryos frozen just after recovery was reported as 6.2 folds lower than of embryos obtained in the same stage but cultured to the blastocyst stage before to be frozen.

Although the cryopreservation of surplus embryos is an established technique, success rates after frozen embryo transfers are lower than after the transfer of fresh embryos. Apart from slow-freezing related cryodamage, several factors have been identified as major players in the reduction of post-thaw implantation and developmental potential of frozen-thawed embryos (Rato et al., 2012).

It is accepted that embryo culture systems, although aiming to mimic the physiological environment, do not replace it completely (Lane and Gardner, 2005). This raises the possibility that post-thaw culture could provide insufficient support to the less fit frozen-thawed embryos, diminishing their implantation and developmental potential. Rato et al., (2012) compared a short (2-5h) with a long (18-24h) post-thaw culture period upon the pregnancy rates, implantation rates and development to term in human frozen embryos. They identified a long (18-24h) post-thaw culture period as being associated with a loss of implantation and embryo developmental potential. In contrast, a short (2-5 h) post-thaw culture period was shown to associate with increased implantation and developmental potential of frozen cleavage stage embryos. In this study it was found that a short post-thaw

culture period (1-5 or 10-20 min) was associated with higher live birth rate *per* embryo. Longer duration (> 10 min) of culture had a deleterious effect on pregnancy rates in 30 and 45d after onset estrus, which pregnancy loss was greater with a longer exposure to culture conditions (55.6% vs 85.7% at 30 days and 44.4% vs 78.6% at 45 days). This result is confirming that sheep embryos may have temporal sensitivities to the culture environment, in a similar way to cattle (Lonergan et al., 2003). To my knowledge, this is the first study to identify a culture period of post-thaw associated with embryonic survival.

In experiment II, recipient ewes were grouped according to their tail type which is an indication of origin of these breeds. Thin tailed (TT) breeds are raised mostly in low latitude and warm climate, whereas fat tailed (FT) breeds are raised in high latitude and cold climate. These genotype groups were used in different seasons to access reproductive success in fresh MOET programs.

Recipient groups were not differed in estrus response and ovulation rates. However the highest pregnancy rates after single fresh embryo transfer were obtained with TT recipients used in breeding season. Overall pregnancy rates were not significantly but relatively higher in TT recipients which are less seasonal breeds than FT recipients. Season did not affect estrus rates and pregnancy success but significantly higher ovulation rates were observed in breeding season. Chagas e Silva et al., (2003) reported contrary results where season had no significant effect on the subsequent ovulation rates. In season x recipient groups interactions, TT recipients showed similar pregnancy success while FT recipient were recorded with lower pregnancy rates in breeding season. Higher ovulation rates of FT ewes in breeding season could negatively affect the embryo survival rates and resulted with 26% pregnancy loss.

These results suggest a higher sensitivity to eCG in the breeding than in the out of season, which might be related to the prevalence of ovarian cyclic activity between two seasons. Maternal recognition of pregnancy in FT recipients were somehow lower than TT recipients in single fresh embryo transfer. The results reported here show that TT recipients responded to estrus synchronization treatments as well in the breeding season as in the out of season, which might suggest a breed effect. As previously discussed, ewes with shorter breeding season are poor in reproductive success (Dixon et al., 2007). When single embryo is transferred to uterine horn with higher ovulation rates it could be lack of IFN-g signal produced by conceptus to establish pregnancy by blocking the synthesis of PGF2a. Moreover, Wilmut et al., (1985a) reported that pregnancy loss may be caused by an excess progesterone amount which would initiate changes in uterine function prematurely. The current study result obtained from FT ewes received single fresh embryo in breeding season showed decline of embryo survival due to increased progesterone levels was in agreement with previous reports (Meyer, 1985; Ashworh et al., 1989; Kleemann and Walker, 2005; Dixon et al., 2007).

Overall MOET success varied 30% to 65% in single fresh embryo transfer conducted in different season. Actual success rates within the ET experiments, as measured by the mean number of lambs born from number of embryos transferred have changed very dramatically over the recipient group and season interaction. This differences indicated that season of MOET and selection of recipient profoundly influence the level of success. Variation sources of the success determined in this aspect is under the control of practitioners rather than factors such as selection of specific donor is ultimately chosen by the owner.

In the second part of experiment II, prolific F1 recipient ewes were compared with nonprolific recipients in twin fresh embryo transfer conducted in the breeding and out of season. Prolific F1 Romanov crossbred recipients were found superior with 21.5% higher pregnancy success. The superiority was more notable in comparison between seasons. Out of season pregnancy rates was 50% in nonprolific recipients, whereas 34.6% more pregnancy success was achieved in prolific F1 recipients. The superiority of crossbred recipient obtained in this study was in agreement by ZhiYuan (2009) used crossbreds Simmental and local cattle as recipients to select the ideal recipients for embryo transfer of dairy cows. It was reported that the estrus synchronization rate, available recipient rate and pregnancy rate of the Simmental hybrids were markedly higher than that of local cattle.

Eventhough it was noted that transferring multiple embryos in recipient can reduce the number of recipient ewes, uterine crowding can cause higher pregnancy loss (Anderson et al, 1979). Ewes are differed in their uterine capacity and prolific ewes have higher uterine capacity than monocotous ewes. In sheep embryonic and fetal loss have been reported approximately 30% (Bolet, 1986) and losses increased with ovulation rate (Kleeamnn and Walker, 2005). Thus in this study, fresh single embryo transfer is preferred to twin embryo transfer mainly due to the monocotous characteristic of recipient breed. Comparison between single and twin fresh embryo transfer in native breeds of recipient within experiment II, it was noticed that pregnancy rates was not differed (52% for single embryo and 58.3% for twin embryos). Survival to term was estimated at 0.95 for single embryos and 0.85 for two embryos in naturally mated ewes (Geisler et al., 1977). In the current study, single embryo transfer resulted with average 52% pregnancy rates which was higher than 45.6% for Hungarian Merino ewes, 33.3% for Greyface ewes and 39% in in-vitro produced embryos

(Brown and Radziewic, 1998). Additionally, single fresh embryo transfer success was found similar to pregnancy success (55.2%) in twin embryo transfer studies by other researchers (Cseh and Seregi, 1993). In contrast to those mentioned above Nancarrow (1994) has reported that there is a possibility of an interaction between embryos that lead to each other's survival. In addition, the transfer of two embryos into the ipsilateral uterine horn is likely to increase the amounts of interferon-t and other embryonic signaling molecules in the uterus needed to maintain pregnancy and prevent luteolysis. However in twin embryo transfer there are two significant factors should be taken into account; one is that embryos should be carefully selected as pairs in similar development stages to avoid development competence which can decrease embryo survivals. The other important factor is that uterine crowding which can cause pregnancy losses. Uterine crowding was reported as a main reason of increased frequency of pregnancy loss in recipients cows receiving two embryos (Anderson et al., 1979, Franco et al., 2006). Especially if recipients are from nulliparous or monocotous breed of sheep, uterine crowding will be a strong limiting factor for twin embryo transfer success.

Quality grade of embryos less than 2 in fresh single embryo transfer was resulted with 26.5% lower pregnancy rates compared to that of Grade 1-2 embryos. The result of quality grade was in agreement with Gutierrez-Adan *et al*. (2004) and Walker (1989). Stage of embryo development did not differed for pregnancy rates in single fresh embryo transfer as it was observed in frozen-thawed twin embryo transfer trial. Hasler (2001) supported this current resulted by based on his study conducted on a large number of transfers in cattle and indicated that no differences were noted in the pregnancy rate of embryos recovered between days 5 and 8 d following standing estrus. As it is underlined by Hasler et al., (1987) embryo stages ranging from late morulae (stage 4) to expanded blastocysts (stage 7) result in

comparable pregnancy rates, whereas following hatching (stage 8), lower pregnancy rates can be expected.

The pregnancy results regarding to ovulation rates and site of ovulation showed different pattern in single and twin fresh embryo transfers. Single versus multiple ovulations in recipient ewes caused 3.3 times pregnancy losses in twin fresh embryo transfer than that of single transfer. Higher ovulation rates in twin embryo transfers are more desirable and should be an selection criteria of recipient. In addition to that, site of ovulation makes appreciable contribution on pregnancy rates if ovulation occurs bilaterally in twin embryo transfer. This result was in disagreement with White et al., (1981) claimed that embryo survival was greater in single ovulating than in bilateral twin ovulating ewes. The effect of transfer site of embryo as pairs or single on pregnancy rates showed similar pattern and left side transfers were more favorable than right sides. Transfer site result was disagree with Casida et al., (1966) reported that survival of embryos originating on the left side was less than right side. However, Remsen et al., (1982) highlighted the fact that uterine horn of transfer is an influencing factor. Further supporting result has been reported by Lamb (2005) that side of transfer may affect success rate.

Use of vitrification has been increased by the development of vitrification in open pulled straws (OPS). The benefits of this procedure include reduction in cryoprotectant volume in the narrow part of the straw down to 0.5 µl, low heat insulation characteristics of the straw wall, and more than tenfold acceleration of freezing when the straws are immersed into liquid nitrogen (Vajta et al., 1997a,c, 1998a, 1999; Lewis et al., 1999; Dattena et al., 2000; Lazar et al., 2000; and others). Rapid freezing inhibits the formation of ice inhibits zona

fracturing; in addition toxic and osmotic effects at thawing are minimized by immersion of the capillary containing the embryo into a thawing solution. Vajta (2003) presumes the future role of OPS vitrification to be in special areas where the other methods have failed. Thus, OPS vitrification was applied in hatching blastocysts which is an especially important stage for the cryopreservation, because they are highly sensitive to low temperature (Wilmut, 1972; Polge, 1977). However, hatching blastocysts begin to acquire tolerance as they develop to peri-hatching stages (Nagashima el al, 1989).

Attention has concentrated on the cryopreservation of zona-intact mammalian embryos. However, the first calf derived from a frozen-thawed embryo was born from a day 10—11 blastocyst frozen after hatching from the zona (Wilmut and Rowson, 1973). The first lambs from frozen embryos were also produced from a group of embryos including hatching blastocysts (Willadsen et al, 1976). The first piglets from frozen embryos were produced from blastocysts frozen after hatching from the zona during culture (Kashiwazaki et al, 1991). In this study hatching zona free embryos were vitrified and transferred after thawing. Pregnancy rates were similar with those obtained with expanded blastocyst stage of embryos. Shaw et al (1995) showed that high proportions of hatching mouse blastocysts can survive after conventional slow freezing. However, when mouse blastocysts partially or fully hatched from the zona were frozen by a rapid method, the survival rate was lower than that of embryos at earlier stages of development (Shaw et al, 1991). It was also stated by Mazur et al., (1976) that the permeability increases as development proceeds, together with the shedding of the zona pellucida, the permeation of ethylene glycol into hatching blastocysts is expected to be more rapid than into zona intact blastocysts are more likely to be injured by the toxicity of ethylene glycol after rapid permeation. In the current study, ethylene glycol was found

successful cryoprotectants for acceptable pregnancy rates with vitrified hatching blastocysts. It was noted that, zona free human blastocycst were transferred for better cell-to cell interactions and anchoring of the embryo to the endometrium might be expected, with improved implantation rates and embryonic losses (Fong et al., 1997; 1998; Urman et al., 2002). However, in this study there was no superiority detected with vitrification of hatching blastocysts.

Differences in cryotolerance between studies may be also related to the cryopreservation method (Massip et al.,1984). In the comparision between conventional freezing with ethylene glycol and vitrification, similar pregnancy rates at day 30 were achieved (average 77.2% and 77.8%, respectively). It should be noted that only blastocysts and hatched blastocysts were used in vitrification procedure which can have led to an increase in pregnancy rates compared to earlier stages (morula) of embryos were included in conventional freezing experiment. However, conservation procedure was reported that it may be less important for divergences between studies, since similar results have been described after transfer of either vitrified or conventional freezing ruminants embryos (Massip , 2001, Massip, 1999, Massip et al., 1995 and Kaidi et al., 2001).

The present study average pregnancy rates for conventional frozen and vitrified embryo transfer were higher than results (frozen: 38.6% and vitrified embryos: 55.8%) obtained by Green et al., (2009). It was reported by Bettencourt et al., (2009) that there was no differences in lambing (55–68%) and embryo survival (36–45%) rates after the transfer of embryos cryopreserved by either controlled slow freezing or OPS vitrfication in native sheep breed, the Portuguese Black Merino. Previous studies in other sheep breeds (Baril et al., 2001,

Martinez and Matkovic, 1998 and Martinez et al., 2006) were also in agreement that both methods resulted with similar pregnacy and embryo survival rates indicating the advantage of ultrarapid techniques, such as vitrification, has reduced part of the costs, since it does not require any special equipment and is, therefore, well adapted to routine field use (Baril et al., 2001).

Survival rates of 30 to 60% have been reported after transfer of embryos frozen-thawed by conventional methods with a variety of cryoprotectants (Willadsen, 1977; Tervit and Goold, 1984; Heyman el al, 1987). The average embryo survival rates reported here, 67% (Awassi: 52%; Morkaraman 69.9% and Tuj: 79%) are higher than those reported by above researchers.

The average pregnancy rate of fresh embryos was considerably lower compared to the result achieved by other groups. It has been reported that pregnancy rate of in vivo ovine embryos range from 70% to 90% after fresh transfer (Dattena et al. 2000; Papadopoulos et al. 2002; Martinez et al. 2006). However, our lower results could be due to the season and recipient effects which represents a disadvantage by their lower reproductive characteristics on MOET success. Within the breed and season interactions it was noted that current pregnancy outcomes are consistent with literature reviews. Another reason of relatively lower pregnancy rates obtained with fresh twin embryo transfer with non prolific breeds in the comparision with earlier studies could be due to the fact that embryos were not strictly classified as excellent or good by morphology and chronology between age and recovery. In other studies, researchers mostly avoided any effect of possible alterations of developmental competence detected by microscopic evaluation.

IMPLACATIONS

This study indicates that introducing exotic breeds via embryo transfer and expanding flock size with MOET programs is useful tools for an establishing nuclei flock. Factors studied for recipients and embryos showed that genotype of the mother rather than of the embryos is responsible for variation of MOET success. In vivo derived frozen embryo transfer success was higher than other results obtained from different researchers. Lambs born from frozen embryo transfer cost three-four times less than live importation. Ovulation rates obtained with local breeds of recipients were within range that does not affect pregnancy success negatively. Neither ovulation or transfer site were an important factors, while left site or bilateral ovulation might be preferred due to relatively higher embryo survivals.

Stages of embryo development were source of variation in embryo survival and embryo in later stages were more favorable. Thus, especially embryos to be crypreserved should be in later development stages or they should be cultured to the blastocyst stages prior to freezing to achieve acceptable pregnancy rates by increasing their cryotolarance. Pregnancy success for number of embryos transferred (single versus twin) was similar and it was concluded that twin transfer can be safely used to save number of recipients used. By the way, it should be noted that higher ovulation rates in recipient ewes are desirable when embryos will be transferred as pair. Season (breeding verus out of season) did not differ for the overall MOET success in which estrus of recipients is induced with exogenous hormone therapy. However eCG dose should be carefully decided to not cause superovulation which has detrimental effect on embryo survivals. Vitrification as simpler and less time consuming

technique can be replaced with traditional freezing method with its similar pregnancy success in sheep.

In conclusion, MOET programs can be successfully used for inter-breed embryo transfer with special attention given for recipient selections.

Chapter VII

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