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" BIOTECHNOLOGIES OF REPRODUCTION: SOME
ALTERNATIVES PROCEDURES TO APPLY SUPEROVULATION
AND EMBRYO TRANSFER   IN SARDA SHEEP"

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RIASSUNTO

Primo esperimento :

"Trattamento di superovulazione FSH-p senza l'utilizzo di spugne contenenti progesterone"

Lo scopo di questo studio e stato quello di valutare la risposta superovulatoria al trattamento con FSH-p senza l'utilizzo di spugne intravaginali contenenti progesterone per cercare di ridurre l'uso di ormoni e il costo delle procedure in un programma MOET. Venti animali sono stati pressi in considerazione e divisi in due gruppi: trattamento con estro naturale (NT) (n=10) e singola spugna (SS) 40 mg FGA per 12 giorni (n=10) (gruppo controllo).

Il trattamento di superovulazione per ogni pecora consisteva di 350 Unità Internazionali di FSH suino somministrate in otto dosi decrescenti ogni 12 ore a partire dal giorno 4 dopo l'inizio dell'estro (giorno 0) nel gruppo NT e 48 ore prima della rimozione della spugna nel gruppo SS. Una singola dose di 125 µg (i.m) di cloprostenolo e stata iniettata nel giorno 6 dopo il rilevamento dell'estro nel gruppo NT per indurre l'ovulazione. Contemporaneamente al trattamento di FSH lo sviluppo follicare e stato valutato mediante ultrasonografia.

Le pecore sono state accopiate mediante monta naturale 24 ore dopo l'iniezione di cloprostenolo o la rimozione della spugna sia nel gruppo NT che nel gruppo SS rispettivamente.

Sette giorni dopo l'accoppiamento, e stata eseguita una laparatomia inguinale dopo di che il numero dei corpi lutei e stato segnato. Gli embrioni sono stati recuperati chirurgicamente mediante flushing in ogni corno utero. Gli embrioni recuperati sono stati valutati e classificati con un punteggio in scala da 1 a 3.

Gli embrioni con voto 1 sono stati considerati di alta qualità. I dati riguardanti il numero dei corpi lutei (CL), degli embrioni recuperati (ER), degli embrioni fertilizzati (EF), e gli embrioni di alta qualità (EQ_1) per ogni pecora sono stati analizzati con ANOVA. I tassi di recupero (RR), fertilità (FR) e qualità embrionale (Q_1R) per trattamento con un'analisi Chi Square. La classe di risposta ovulatoria (media vs alta) e tassi di qualità embrionale (Q1, Q2 and Q3) per trattamento sono stati analizzati con il test Fisher's Exact.

Per valutare l'effetto della presenza di un largo (presunto dominante) follicolo sono stati applicate per le variabili studiate un Modello Lineare, Chi Square, Correlazione Pearson e procedure di regressione Lineare.

Tra tutte le variabili analizzate i risultati hanno dimostrato differenze statistiche solo nella media del numero dei CL/pecora (10.7±3.4 vs 7±3.2) e FR (100% vs 80%) ($p<0.05$) tra i gruppi NT e SS rispettivamente. In conclusione è possibile superovulare pecore evitando l'utilizzo di spugne intravaginali durante il trattamento di FSH-p e alcune variabili (CL/pecora; FR%) sembrano essere migliorate e anche ridotti i costi e la lunghezza dei trattamenti.

Secondo esperimento:

"Utilizzo dell' open-pulled-straw come catheter (OPS-C) per semplificare la tecnica di trasferimento degli embrioni vitrificati"

Lo scopo di questo studio è stato di testare l'efficienza della tecnica di trasferimento embrionale con l'utilizzo dell' OPS-catheter, come alternativa, per incrementare l'uso del trasferimento embrionale nelle più svariate condizioni aziendale, con un occhio di riguardo a procedure più semplici, veloci ed effettivamente economiche.

Ventuno riceventi sono state selezionate attraverso il rilevamento dell'estro con arieti vasectomizzati e divise in due gruppi: OPS-catheter (OPS-C) (n=11) e Tom Cat (TomCat) (gruppo controllo).

Considerato che, il numero degli animali nell'esperimento era ridotto, solo gli embrioni del gruppo NT (Esp.1) sono stati reclutati per il trasferimento. Tra tutti gli embrioni recuperati (N°72) dopo il flushing del gruppo NT 52, sono stati vitrificati, ma solo 22 sono stati utilizzati per il trasferimento nel gruppo OPS-C, mentre, 20 embrioni freschi sono stati utilizzati per il trasferimento nel gruppo TomCat. Gli embrioni sono stati trasferiti in coppia attraverso una laparatomia inguinale in riceventi sincronizzate al giorno7 (D7: giorno 7 dopo l'inizio dell'estro naturale) direttamente dopo lo scongelamento o dopo valutazione morfologica nei gruppi OPS-C e TomCat rispettivamente.

In seguito all'analisi Chi Square dei tassi di gravidanza (82% vs 70%), tassi di sopravvivenza embrionale (59% vs 60%), tassi di gemellarità (44.4% vs 71.4 %) peso degli agnelli nati (2.8 ±0.8 vs 2.7±0.4), non è stata dimostrata nessuna differenza statistica tra i gruppi OPS-C and TomCat rispettivamente.

I risultati in questo esperimento hanno dimostrato che, il trasferimento embrionale con OPS-catheter è possibile evitando l'uso del microsopio e l'impiego di tecnici esperti. In questo modo diminuendo il costo della procedura è possibile incrementare l'utilizzo dell' embryo transfer anche in aziende in diversi condizioni e scarse possibilità economiche.

ABSTRACTS

First experiment :

"FSH-p superovulatory treatment without progestagen sponges"

The aim of this study was to evaluate the superovulation response to FSH-p treatment without the use of intravaginal progestagen sponges in attemps to reduce the use of hormones and cost of procedures in a MOET programme. Twenty animals were divided into 2 groups: natural oestrus (NT) (n=10) and single sponge (SS) 40 mg FGA for 12 days (n=10) (control group).

Superovulatory treatment per sheep consisted of 350 I.U. of porcine FSH administered in eight decreasing doses at every 12 h starting on day 4 after onset of oestrus (day 0) in the NT group and 48 h before sponge removal in the SS group. A single dose of 125 µg (i.m) cloprostenol was injected on day 6 after oestrus detection in the NT group to induce ovulation. Contemporary to FSH treatment follicular development was assessed by ultrasonography. Ewes were naturally mated 24 h after cloprostenol injection or sponge removal in the NT and SS groups respectively.

Seven days after mating, inguinal laparotomy was performed and the number of corpora lutea (CL) was recorded. Embryos were recovered surgically by flushing each uterine. The recovered embryos were evaluated and scored on a scale of 1 to 3. Embryos with a score of 1 were considered of high quality. Data on number of corpora lutea (CL), embryos recovered (ER), embryos fertilized (EF) and high quality embryos (EQ_1) per ewe were analysed by ANOVA. Recovery (RR), fertility (FR) and high embryo quality (Q_1R) rates per treatment by a Chi Square analysis.

The ovulation response classes (medium vs high) and embryo quality rates (Q_1, Q_2 and Q_3) per treatment were analyzed by Fisher's Exact Test. In order to assess the effect of a presence of a large (presumptive dominant) follicle a Linear Model, Chi Square, Pearson Correlation and Linear regression procedures were applied to the variables in study.

Among all variables analysed results show statistical differences only in mean number of CL/ ewe (10.7 ± 3.4 vs 7 ± 3.2) and FR (100% vs 80%) ($p < 0.05$) between NT and SS groups respectively. In conclusion it is possible to superovulate ewes avoiding the use of intravaginal sponges during the FSH-p treatment and some variables (CL/ewe; FR%) seem to be improved as well as costs and length of treatments are reduced.

Second experiment :

"Using the open-pulled straw as catheter (OPS-C) to symplify the embryo transfer technique with vitrified embryos"

The aim of this study was to test the efficiency of the OPS-catheter embryo transfer technique as an alternative to increase the use embryo transfer program in different farm conditions looking at cost- effective and less time consuming procedures.

Twenty one recipients were selected by oestrus detection with vasectomized rams and divided into 2 groups: OPS-catheter (OPS-C) (n=11) and TomCat (TomCat) (control group).

Due the fact that, the number of experimental animals was reduced, only embryos of NT group (Exp. 1) were considered for transfer. Among all recovered embryos (N°72) after flushing of NT group 52 were vitrified but only 22 were utilised for transfer in the OPS-C group, while, 20 fresh embryos were utilised for transfer in the TomCat group. Embryos were transferred in pairs by inguinal laparotomy into D7 synchronised recipient (D7: day 7 after natural onset of estrus) directly after thawing or after morphological evaluation in the OPS-C and TomCat groups respectively.

After a Chi Square analysis pregnancy rates (82% vs 70%), embryo survival rates (59% vs 60%), twinning rates (44.4% vs 71.4 %) and birth weight at lambing (2.8 ±0.8 vs 2.7±0.4) did not show any statistical differences between OPS-C and TomCat groups respectively.

The results in this experiment demonstred that, embryo transfer with the OPS as catheter is possible avoiding the use of microscope and expertise technicians. Thus, with this technique we decrease the cost of procedures and increase the use of embryo transfer in different farm conditions.

ACKNOWLEDGEMENTS

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CHAPTER I

Introduction

During the last fifty years new biotechnologies have been developed and introduced into animal breeding and husbandry. The aims of these reproductive technologies were initially to speed up the genetic improvements of farm animals by the increase of offspring of selected males and females and the reduction of the generation intervals.

The biotechnologies of reproduction such as MOET imply costly and time consuming hormonal treatments, surgeries and embryo handling so the introduction of this biotechniques into farm animal husbandry suggest that should be accompanied by a study of health and welfare with the help of a comprehensive protocol for the benefit of a sustainable animal production.

Torp-Donner and Juga, (1997) has been stated that *sustainable animal production* has to satisfy a number of ethical, economic and ecological (i.e. environmental and biodiversity-related) conditions. Therefore, we believe that within the context of farm animal biotechnologies, animal sustainable production should receive special attention.

According to Olesen et al. (2000), *"sustainability"* refers to the tenet that environmental concerns, genetic diversity, ethical considerations and social issues should be addressed as well as short term and long-term economic value.

Actually consumers in the developed, food rich world appear to have a real interesting in foods produced in a sustainable way. For this reason farm animal breeders are already looking at sustainability related traits (Gamborg and Sandøe, 2005).

The purpose of sustainable animal production, thas not to be difficult because, as we work towards a better understanding of the physiology and behaviour of our animals, we can improve productivity and profitability and, simultaneously, promote "clean, green and ethical" production, one alternative is, to look for practices in the industry where drugs, chemicals and hormones are used, and try to find ways to reduce the usage and perhaps, eliminate it. (Martin et al., 2006).

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There are considerable national and regional differences in breeding practices and in public attitudes. Each country tries to identify an equilibrium between local needs and global uniformity. Breeders and breeders scientists must remain sensitive to public attitudes, the bioethical debate and economic advice and update society with developments in breeding .(Quédraogo A., 2004).

Not all hormonal treatments are "Clean", they may also not be "green" there is a perceived risk associated with liberation of sex steroids into environment with the disposal of used progesterone intravaginal devices. The are also issues of expense: Such treatments are too costly for small producers in developing countries and the labour cost incurred are too great for producers with large extensive flocks (Martin et al., 2006). On the other hand, currently, the enforcement of the regulation of hormonal residues has discontinued the administration of progestagens in the USA and increased the regulation of Maximun Residue Limits in the E.U.

In the last years MOET in sheeps are been widely used and also remains an efficient and inexpensive technique to maximize the number of lambs borned from females with a high genetic merit (Driancourt M., 2001). So with the MOET technique we can improve the quantity and quality levels production on the farm, but, to complemented a more friendly management and good animal production levels we must find a system, reducing the costs, times of treatments and more ecological breeding practices.

The aim of this thesis is to simplify some steps in a sheep MOET programme, starting with reduction and shortening of hormonal treatment, simplification of surgery and embryo transfer techniques.

CHAPTER II

Literature Review

2.1.Characteristics of oestrus cycle

2.1.1 Seasonality

One of the most important features of ovine reproduction, is seasonality with alternated periods of anoestrus and sexual activity. Normal oestrous cycles occurs in the fall and winter (breeding season) while ovarian cycle ceases in the spring and summer (anoestrus season) Bartlewski et al., (1998). This condition of seasonality ensures that lambs are born in the spring when environmental conditions are favourable for their survival (Gordon 1997).

Photoperiod is one of many environmental variables capable to affect seasonal breeding in the ewe (Legan and Karsch, 1980). They are short-day breeders because they become fertile (i.e. oestrous cycles begins) when day length decreases in the autumn months (Robinson, 1959 and Karsch et al., 1984). Shortening daylight stimulates the output of melatonin by the pineal gland, increasing its blood concentrations during darkness (O'Callaghan 1994). The melatonin increase has a profound effect on the secretion of gonadotrophin-releasing hormone (GnRH) from the hypothalamus modulating the release of pituitary gonadotrophins, which in turn modulate the seasonal reproductive activity. The seasonal activation of the reproductive axis initiates with cyclic elevations of progesterone and oestradiol that triggers the expression of oestrous behaviour in the ewe and stimulating increased production of testosterone by the testis which in turn triggers the expression of libido in the ram (Perkins and Roselli, 2007).

The breeding season in sheep is characterized by the start of sexual activity and the onset of oestrus. However, there are considerable differences in seasonal reproductive patterns among differents breeds (Goodman, 1994).

The length of the breeding season seems to depend on the location of the breed of ewe (Hafez, 1952; Robinson, 1959), for example Dorset, Merinos and Rambouillet are breeds whit a long breeding season while Southdown, Shropshire and Hampshire are breeds whit a shorter breeding season, on the other hand, sheeps living in the equatorial regions shift the occurrence of anoestrous and breeding seasons by 6 months. Despite these

differences in most of the breeds the fertility peak takes place in the late autumn, with the highest lambing rate in spring.

2.1.2 Distribution and incidence of natural oestrus during breeding season

The ewe is considered as a spontaneous ovulator (Robertson 1977) and repeated cycles provide the female repeated opportunities to copulate and become pregnant. According to Evans and Maxwell, (1987) during the breeding season, in natural conditions, daily about 6-8% of females comes in oestrus spontaneously.

In most of breeds the length of the oestrus cycle during the breeding season is 16-17 days, with a range of 14-19 days. The duration of oestrus varies with the age, breed and season ranging, between 18 and 72 hours, with an average of about 36 hours (Evans and Maxwell, 1987). Ovulation is spontaneous and takes place approximately about 20-40 hours after the onset of oestrus (Henderson and Robinson, 2000).

However, short cycles of less than 12 days or ovulations without oestrus behaviour ("silent oestrus") can be observed in the transition period between anoestrus and sexual activity. The onset of this "silent oestrus" is due to an inadequate exposure to high concentrations of progesterone during this transition period.

2.1.3 Sexual behaviour

Sexual behaviour is a very important factor that contributes to the reproductive efficiency. In wild or feral types of the domesticated ruminant species, males and females live most of the year in segregated unisex troupes (Alexander et al., 1980). As the breeding season approaches, males and females come together and then forms a multimale-multifemale group in which reproduction is promiscuous, with females mating with several males during one oestrous period and males mating with several females on the same day.

During breeding season as males congregate they engage in numerous fights to stablish dominance hierarchies. Usually most of oestrus females mate with the dominant male, generally the oldest and the strongest, but some times competitions can be observed among females which often gather around the dominant male and form a sort of harem competing whit other the females for male attention (Fabre-Nys and Gelez, 2007). In contrast to wild sheep population, sexual segregation is enforced in most of the modern

husbandry practices by exclusion of rams from ewes flocks, except during breeding season, when the rams are introduced in the female flock.

2.1.3.1 Female sexual behaviour

In sheep sexual behavior is displayed for a short period during the oestrus cycle. It is preceded by an increase in oestradiol plasma concentration, but progesterone has to be present for several days and then disappear before the increase in oestradiol.

According to Beach (1976) there are 3 components to distinguish in females sexual behavior: attractivity, proceptivity and receptivity. Attractivity refers to the female's value as a sexual stimulus whereas proceptivity consists in a series of appetitive activities shown by females which depends on stimuli passively emitted by females, receptivity is an immovilization reflex.

The attractiveness can differs between females and is one of the reasons why males shows a preference towards certain females rather than others. This condition is relatively stable from one cycle to the next and is independent from the oestradiol dose of used to induce oestrus (Tilbrook and Lindsay, 1987). However, the most important cue for attracting males is the female's proceptive behavior which is demostred by an increase in motor activity, when oestrus starts the females tend to leave the main flock and look around males. This behaviour has been defined as "ram-seeking" (Hart, 1985) and used as a practical method for monitoring oestrus by Ortman (2000).

The ram seeking activity of ewes is frequent: 75% of oestrous ewes display it and older females display it more frequently than nulliparous. In addition to this behavior, females can display specific behavioural patterns which will increase the males' interest such as: a movement of the head toward the male and tail fanning, but the main display is just to stand near the male (Banks, 1964). The expression of this proceptive behaviour is of major importance when males are not highly motivated or when many females are in oestrus at the same time (Lindsay and Fletcher, 1972; Madlafousek et al., 1976).

The receptive behaviour consists of an "active immobilization" during which the female will resist if you try to push her (Alexander et al., 1980). In ewes as in does, inmobilization is a sign both of proceptivity and receptivity.

2.1.3.2. Male sexual behaviour

Rams use olfactory cues to detect ewes on oestrous (Lindsay, 1965). If the ewe is receptive, the ram will approach her within few seconds. There are several stereotyped behaviours that the ram may engage in prior to his initial mount, which are frequently called "courting behaviours". These behaviours include sniffing the genital region of the ewe; pawing at her flank repeatedly with his foreleg while standing behind and at a small angle to her (foreleg kick); nuzzling, licking and nibbling at her flank and at the ano-genital area. Just before to copulation rams will also elevate the head and retract the upper lip in response to the odour or taste of the ewe's urine, a behavior called "flehmen", some males additionally emit low-pitched "gargling" vocalizations before and while pawing the ewe. However, it is important to outline that there is a considerable variation in the frequency and duration of these precopulatory behaviours among males. (Perkins and Roselli, 2007). Subsequently, copulatory behaviours in rams are accompanied by a series of shallow pelvic thrusts and usually they mount several times prior to vaginal penetration and ejaculation, while an experienced ram may ejaculate on the first mount.

2.1.3.3 Monitoring oestrus

Monitoring oestrus is not frequently used in natural mating breeding systems, while it is vital for the success of assisted breeding practices, because only throught an accurate oestrus detection it is possible to identify the optimal moment to apply artificial insemination (AI) and embryo transfer (ET) protocols.

Visual observation of oestrus

To confirm ewes in oestrus by the visual observations, a vasectomized ram is introduced in a pen, the ewes in oestrus will show the behavioural patterns (Fig.1). The proceptive behavior is evidenced by the ewe-ram seeking activity, if the ewe is in oestrus searchs for the ram and movements toward to him (Ortman, 2000; Mayorga et al., 2007 a). Therefore, proceptive behaviour can be considered such as a reliable parameter for monitoring the onset of estrus. After the proceptive behavior appears the receptive behavior, can be evaluated observing the "standing reactions" (active immobilization displayed by ewe in estrus when they are courted by the ram) (Fig.2). Two grades of receptivity can be distinguished: a "weak receptivity" is observed in young animals, at the

end of oestrus or after small doses of oestradiol; while a "strong receptivity" is observed in the middle of oestrus in mature ewes (Fabre-Nys et al., 1993).

Fig.1. A vasectomized ram is introduced in the pen.

Fig.2. Active immobilization displayed by the ewe in estrus.

Electric mucus impedance

Ovarian follicular growth and luteal tissue development are related with the histochemical changes in the mucosal layer of reproductive organs, including vagina. These changes are followed by alterations in electric properties of the mucosal tissues (Adam et al., 1981). It has been suggested that electric conductance of vaginal mucosa is well correlated with the circulating hormone levels allowing an estimation of serum progesterone concentration and oestradiol/progesterone ratio (E_2/P_4) during follicular phase of the sheep oestrous cycle (Canfield and Butler, 1989; Bartlewski et al., 1998; Gupta and Purohit, 2001).

In ewes, it has been demostred that the highest conductivity/lowest impedance of the vaginal mucous membrane occurs just before oestrus, and it remains high/low for 24 to 48 hours (Fi.3.) (Olic et al., 1990; Rezac and Olic, 1990; Masia et al., 2007). Therefore, electric resistance values can be used as a cost effective and functional tool for oestrus detection and to establish the best time for insemination in the field conditions in ewes (Canfield and Butler, 1989, Masia et al., 2007).
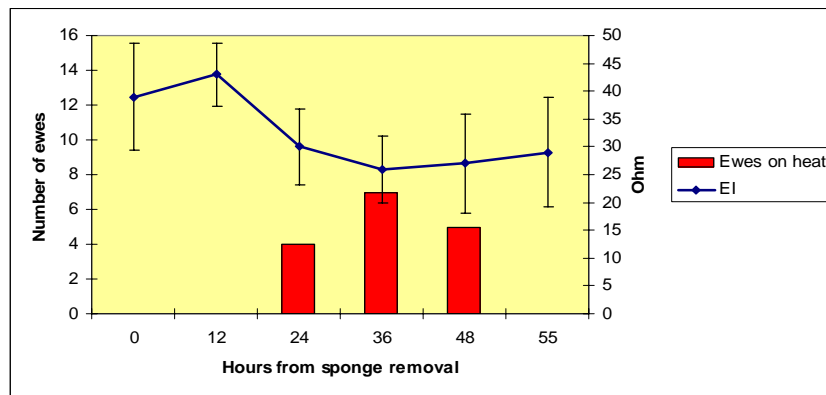
Fig. 3. Mean value (±sd) of electric mucous impedance (EI) and oestrus distribution in synchronized Sarda ewes (Masia et. al., 2007).

Fig. 4. Dramisnki probe ® to assess electric mucous impedance.

Ovarian ultrasonography

During the last decade, the application of ultrasonography has allowed us the study of the reproductive tract in small ruminants, faciliting a rapid increase in knowledge of animal reproductive physiology and its control.

With the use of transrectal ultrasonography the study of ovarian follicles both during their preovulatory growth and their postovulatory transformation has been enormously improved due the fact that transrectal ultrasonic imaging represent s valid tool for repeated, direct and non-invasive monitoring and measuring of follicles larger than 2mm (Fig.5.), regardless of their depth within the ovary. Particularly, throughout this method it has been possible to enhance superovulatory responses in MOET programs (Evans 2003; Rubianes and Menchaca 2003; Martin et al., 2006).

Many athours , showed the effectiveness of ultrasonic imaging to stimate the superovulatory response in sheep scanned during superovulatory FSH treatments, regadless of the ovarian status when the FSH superovulatory treatment is started, the total number of large follicles recruited is achieved around the onset of oestrus (Menchaca and Rubianes., 2001). This findings can have practical implications because it is very important to know the ovulation rate obtained after the treatment and determine if animals have ovulated or if

multiple ovulation have occurred on the day of uterine flushing (Rubianes and Menchaca, 2003; Gonzalez-Bulnes et al., 2004).
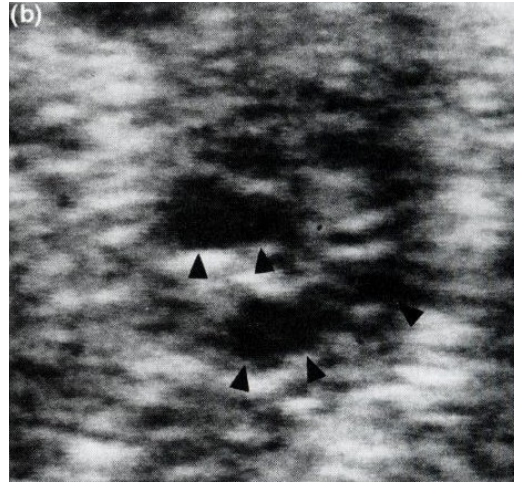
Fig. 5. Ultrasonic image of follicular development in the ovary.

2.1.4 Hormonal oestrus cycle

In physiological conditions, hormones play a fundamental role by controlling the events of the ovarian cycle.

2.1.4.1 Estrogens

In all females mammals, estradiol is released by follicles (Bjiersing et al., 1972; Baird and Scaramuzzi, 1976; Evans et al., 2000) and it plays an essential role in the control of oestrous behaviour (Morali and Beyer, 1979).

The estradiol secretion occurs during all oestrous cycle, but during luteal phase mean concentrations remain low and show no consistent pattern during this period (Baird et al., 1976a). After luteolysis and subsequent progesterone drop, estradiol begins to rise (Baird et al., 1976b), this increment peaks at the start of the preovulatory LH surge to days 2-3 post ovulation and then declines again to basal levels on day 5 (Karsch et al., 1979; De castro el al., 1999). Estradiol rise during this period appears to come largely from the follicle destined to ovulate (Bjersing et al., 1972) which is rapidly growing at this time (Smeaton and Robertson, 1971).

The estradiol increase, always precedes the expression of sexual behavior by 1 to 2 days (Morali and Beyer, 1979). In the most species oestrous behavior can be triggered by estradiol alone, however, in ewes as in rats, estrous behavior is not displayed unless progesterone is present (Fadem et al., 1979). In Ruminants estradiol is the "Trigger" for both the behavioural an the endocrine changes (Fabre-Nys and Gelez, 2007). The preovulatory rise of estradiol generally lasts 12 to 24 hours, but in sheep, 4 h of circulating estradiol is enough to induce both an LH surge and receptivity behaviour in females (Pillon et al., 2003). In sheep, the latency for the occurrence of these events decreases with the increase of estradiol dosage, but also depends on the time of progesterone disappearance (Fabre-Nys and Martin, 1991).

2.1.4.2 Progesterone

It has been well described that progesterone is secreted during luteal phase by the corpus luteum. Its serum concentrations start to increase from day 0 (ovulation) to day 11 of the oestrus cycle, reaching the lowest level   by day 15 (Edgar and Ronalson 1958; Bartlewski et al., 1999).

The fundamental role of progesterone in mammals is the maintenance of pregnancy. However, in many species during the oestrus cycle the progesterone is critical for the initiation of oestrous behaviour. Specifically in ewes progesterone is required before estradiol rise to induce estrous behavior, this action of progesterone is dependent more on the duration than on the dose of progesterone priming (Robinson et al., 1956;Scaramuzzi et al., 1971). For this reason at the start of the breeding season, the first ovulation is not accompanied by estrus behaviour "Silent oestrus" (Wheeler and Land., 1977; Robinson., 1954), because the lack of progesterone priming.

2.1.4.3   Gonadotrophins

Gonadotrophins, represented by the Luteinizing (LH) and Follicle Stimulating (FSH) Hormones, provide the primary mechanisms that control follicular dynamics via negative inhibitory feedback loops with the hypothalamo-pituitary unit.

FSH is the main hormone controlling follicular growth in cattle, sheep and pigs and its secretion is in turn controlled by the main secretory products of a large dominant follicle (s), oestradiol and inhibin A. (Baird et al., 1991; Knight et al., 1998; Hunter et al., 2004). Therefore, changes in peripheral concentrations of FSH relate primarily to ovarian follicular activity , reflecting the output of estradiol (McNeilly 1995).

FSH secretion during the ovine estrous cycle is non-pulsatile, there is a day to day variation in serum FSH concentrations (Baird et al., 1981; Cahill et al 1981). It is well established that in sheep and cattle the emergence of follicular waves is preceded by a transient increase in FSH plasma concentration (Adams, 1999; Webb et al., 2003). The combination of ovary ultrasonography and blood sampling has confirmed that peaks in serum FSH concentrations occurs every 5 days and are associated with the emergence of the follicular waves (Ginther el al., 1995; Bartlewski et al.., 1998; Souza et al., 1997; Evans et al., 2000).

During the oestrus cycle the rise in FSH concentration at the time of luteal regression stimulates the development of a number of antral follicles. It is around the time of follicular selection that granulosa cells acquire LH receptors that are essential for further follicle development (Campbell et al., 1995; Webb et al., 2003). This recruitment phase is followed by a decline in FSH due to the negative feedback exerted by oestradiol and inhibin from recruited follicles to below the threshold for further follicular selection. Consequently to the decline in FSH concentration, only the selected dominant follicles which had LH receptors on their granullosa cells can use LH to support its growth. There is some evidence suggesting that, an increase in FSH secretion is not an absolute prerequisite for follicular recruitment (Foxcroft and Van de Wiel, 1982) there is a switch from FSH-LH dependency as the follicles matures an FSH concentration decline in the ewe (Campbell et al., 2003).

There are two functionally distinct modes of LH secretion in the ewe, the tonic secretion and the preovulatory surge (Goding et al., 1970; Dyer 1985; Arthur et al., 1989).

Tonic o pulsatile LH secretion occurs throughout the cycle (Rawlings and Cook 1993) and is important for ovarian steroidogenesis (Goodman 1994). Rhytmic LH pulses are generated in response to Gonadotrophin-Realising-Hormone (GnRH) released from the hypothalamus, which controls both the synthesis and release of these pituitary gonadotrophins through binding to specific receptors in the plasma membrane of the gonadotrophs (Stojilkovic et al., 1994). Investigations by Baird (1978) demonstrated that the increase in tonic LH secretion during the pro-estrus period is related to an increase in LH pulse frequency, from one pulse every 3 to 4 hours during mid-luteal phase to a maximum of one pulse every 20 to 30 minutes just before the LH surge.

The LH surge occurs around estrus inducing ovulation and formation of the corpus luteum (Goodman 1994). The interval between the LH surge and ovulation (22-26 hr) is remarkably constant, an observation that helped to establish the role of the LH surge in initiating ovulation (Cumming et al., 1973). The preovulatory LH surge is primarily induced and sustained by decreased progesterone and increased by estradiol secretion during the final stage of the oestrus cycle (Scaramuzzi et al.,1970, Kaynard et al., 1988; Moenter et al, 1990). The preovulatory surge release of LH is accompanied by an FSH surge (Rawlings and Cook 1993), moreover according to Bartlewski et al. (1999) a second FSH surge occurs within 20 to 36 hours after preovulatory gonadotrophin surge which has a lower amplitude but is longer in duration (20 to 24 hours) when compared to the preovulatory surge (11 to 12 hours).

Several authors demonstrated that estradiol regulates LH pulse amplitude while progesterone regulates LH frequency (Bjersing et al., 1972; Karsch et al., 1979; Goodman and Karsch 1980; Rawlings et al., 1984). Thus, a decrease in progesterone and an increase in estradiol secretion during the preovulatory period gives rise to maintains the preovulatory LH surge (Kaynar et al., 1988; Moenter et al., 1990; Joseph et al., 1992), while, during the luteal phase progesterone has an inhibitory effect on pulsatile release of LH (Rawlings et al., 1984).

2.1.4.4 Prostaglandins

Prostaglandins were first discovered in seminal plasma of mammalian semen and were believed to originate from the prostate gland, thus they were named prostaglandins. They are considered among the most ubiquitous and physiologically active substances in the body .

There are at least six biochemical prostaglandins and several metabolites with an extremely wide range of physiologic activity. Indeed, these substances lowers blood pressure, stimulate the uterine smooth muscle, influence the lipid metabolism and mediate the inflammation process. Among all prostaglandins one of the most important for reproductive system is PGF $_{2\alpha}$.

The discovery that PGF $_{2\alpha}$ is an uterine hormone able to cause luteolysis (destruction of corpus luteum) in the female, opened a new world for its application in the control of the oestrus cycle and becomes widely used in the small ruminant reproduction field (McCracken et al., 1972).

The PGF $_{2\alpha}$ is produced by endometrial glands of the uterine endometrium (Knickerbocker et al., 1988) they travels to the ovary through uterine venous and the uterine lymph vessels. The communication between the corpus luteum and the uterine endometrium of contralateral uterine horn is very important due that PGF $_{2\alpha}$ is transferred across the wall of the uterine vein into the blood of the ovarian artery by passive diffusion without dilution in the systemic circulation and bring successful luteolysis.

In ewes, as luteal phase progresses and corpus luteum is matured, ovarian estradiol, progesterone and oxytocin became regulators of PGF $_{2\alpha}$ secretion. Thus, at the end of luteal phase progesterone drops and oestradiol level rises, this stimulate the formation of endometrial receptors to oxytocin (Mc Cracken et al., 1984; Fogwell et al., 1985; Vallet et al., 1990). As number of endometrial oxytocin receptors increase, there is greater ability of oxytocin to stimulate the syntehis of PGF $_{2\alpha}$.

It has been described that the injection of PGF $_{2\alpha}$ to randomly cyclic ewes is effective to induce luteal regression in most of them with a consequent return to oestrus (Acritopoulou and Haresing, 1980). The variability of the response to PGF $_{2\alpha}$ is partially attributted to the differences in ovarian status among ewes at the time of the treatment. When PGF $_{2\alpha}$ is given early during the oestrus cycle, the interval between oestrus and ovulation is shorter than when it is given in a later phase of the cycle, because a large time is required to reduce progesterone concentrations to basal levels as the luteal phase progresses and the corpus luteum acquires its full endocrine functionality (Houghton et al.,

1995); on the other hand, the variable response to PGF $_{2\alpha}$ treatment has also been related to the individual follicular status of each ewe at the time of PGF $_{2\alpha}$ administration (Viñoles and Rubianes, 1998). If a growing healthy large follicle is present at the time of treatment, this follicle continues its development and oestrus and ovulation will occur shortly after PGF $_{2\alpha}$ administration. However, if luteolysis is induced when the largest follicle of the wave is regressing, a new follicle needs to emerge and grow, thus oestrus and ovulation will occur later (Menchaga and Rubianes 2004).

Studies indicate that ovine corpus luteum show refractoriness to luteolysis restricted to the first two days after ovulation. Luteolysis, oestrus behaviour ovulation and a newly formed corpus luteum have been observed in ewes treated with PGF $_{2\alpha}$ at Day 3 after ovulation (Rubianes et al., 1997; Rubianes and Menchaca., 2003).

2.1.5 Ovarian dynamic during oestrus cycle

During the oestrus cycle the ovarian activity is characterized by a follicular dynamics, but the dominant structure (follicles or corpora lutea) present on the ovary in the different stages of the cycle determine divisions of the estrus cycle in a follicular phase and a luteal phase.

2.1.5.1 Follicular dynamics

Assisted reproduction programmes often relies on the manipulation of follicle development and is desire to understand the pattern of ovarian follicle development (Evans, 2003).

It has been described that primordial follicles are laid down during foetal development in sheep, with the first follicles being formed during the first 70 days of gestation (Mariana et al., 1991) existing about 40 to 3000 primordial follicles in lambs at birth (Driancourt et al., 1991). The time required for these follicles to growth from the primordial to preantral stages in ewes takes about 6 months (Cahill and Mauleon, 1980) and from initial antrum formation to the preovulatory phase takes an additional 34-43 days (Turnbull et al., 1977; Cahill and Mauleon, 1980).

The growth of follicles from the primordial to preantral stage is termed "early follicular development" (Cahill and Maulon 1980) and it seems to be independent of gonadotrophic hormones (McNatty et al., 1981). As the follicle development progress and the follicles reach the diameter of 0.8 to 2 mm they become gonadotrophin sensitive

(Driancourt et al., 1993), the fact that follicles become responsive to gonadotrophins is a prerequisite to subsequent antral follicular growth and maturation (Campbell et al., 1995).

During the antral follicular dynamics follicles require gonadotrophin support to growth and reach preovulatory stage. This phase involves a sequence of recruitment, selection, dominance and atresia processes (Fig.6.).

The process of recruitment is defined as a synchronised initiation of gonadotropin-dependent folliculogenesis by a cohort of heathy follicles. Recruitment of the cohort containing the future preovulatory follicle occurs during a "recruitment window" which lasts 1, 2, or 3 days in sheep, cattle or horse, respectively (Driancourt, 2001). The link between size at recruitment and the size at which follicles become gonadotrophin-dependent is when they reach at least 2 mm in diameter (Driancourt et al., 1993).

After recruitment, only a limited number of this growing follicles that have not undergone atresia are selected. At selection a dominant follicle is chosen and the remaining follicles of the cohort become subordinate and enter in atresia. This atresia is catacterized by a block in their growth rate followed by a steady decrease in size. Dominance means that one follicle grows significantly larger than the next largest follicle and this follicle contains more oestradiol, inhibin and a higher oestradiol/progesterone ratio than the other follicles in the same wave (Bigelow and Fortune 1998; Austin et al., 1999; Evans et al., 2000; Viñoles et al., 2002). It has been described that oestrogens are mainly produced by the largest follicle of the wave and the other follicles contribute less than 10% of ovarian production of oestradiol (Mann et al., 1992) this play an inhibitory influence promoving suppressed FSH concentrations in the blood. In all species, the dominant selected follicle appears to be the first one developing LH receptors on its granulosa cells, in sheeps follicles develop LH receptors when they reach 4-5 mm in diameter (Driancourt, 2001). This follicular dominance is characterized by the preovulatory follicle growths and maturation, the other antral follicles recruited and selected from the cohort complete regression by atresia, while no recruitement occurs. The magnitude of dominance is usually defined by the size difference between the dominant follicle and the largest subordinate follicle whit a size gap of 2-3 mm in sheep.
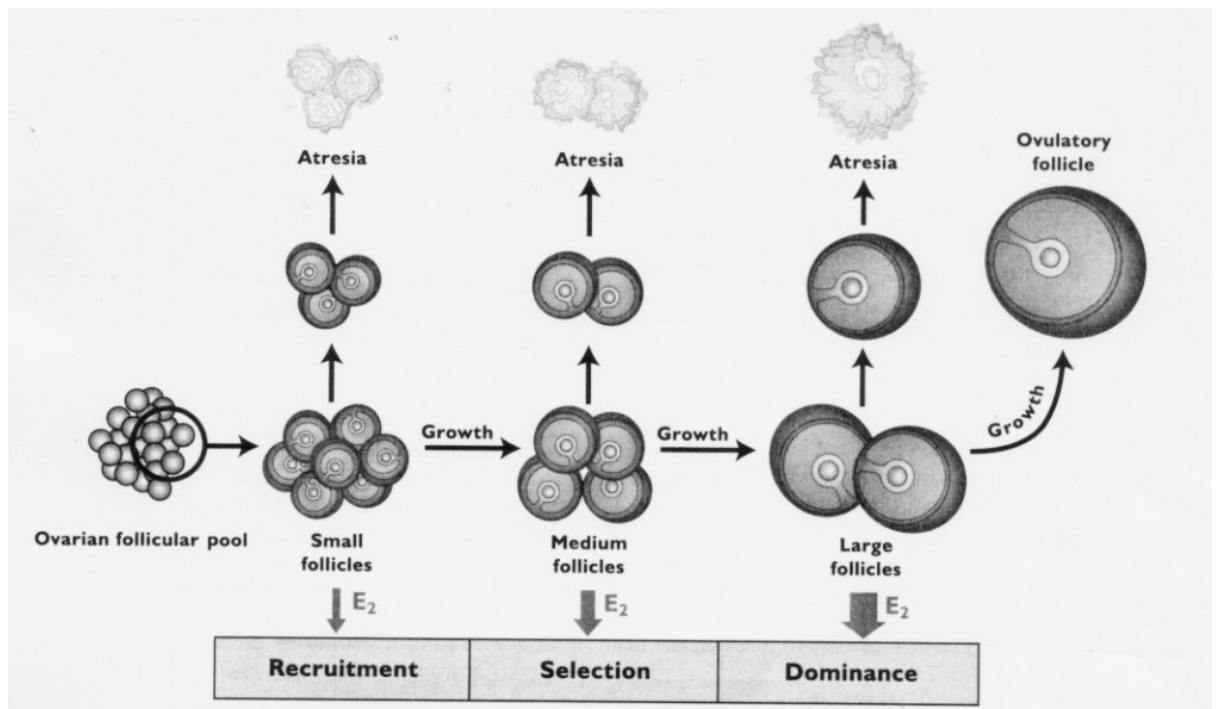
Fig. 6. Main events occurring during a follicular wave: recruitment, selection and dominance (Senger, 2003).

It must be recognized that the majority of a follicle's life time is spent in the preantral stages. Recruitment, selection and dominance are relatively short-term processes the succession of this process shows a wavelike pattern (follicular wave) (Ginther et al., 1995; Evans et al., 2000).

A follicular wave is defined as the synchronous growth of a cohort of 1 to 4 small follicles from 2 to 3 mm in diameter (emergence), one that continues growing to a maximun size of 4 to 12 mm (the dominant follicle) while others regress (subordinate follicles) (Evans et al., 2000; Viñoles et al., 2001).

It was not until the develop of ultrasonography that the follicle development could be monitored and the pattern of follicular waves has been determined (Fig.8.) (Nöel et al., 1993; Schrick et al., 1993; Ravindra et al., 1994; Ginther et al., 1995; Souza et al., 1997).

According to different authors the number of follicular waves detected through the oestrous cycle ranges between two and five, in ewes the most common pattern is three or four follicular waves during a cycle (Ginther et al., 1995; Gibbons et al., 1999; Evans et al., 2000; Viñoles et al., 2000; Duggavathi et al., 2003).

The emergence of each follicular wave takes place whithin 24h and it is preceded by a transient increase in serum FSH concentrations (Ginther et al., 1995; Bartlewski et al., 1998; Souza et al., 1998; Evans et al., 2000; Evans et al., 2002). Only the FSH-dependent follicles are recruited (2mm) for growth, all this follicles are capable of ovulating but generally the largest follicle of the cohort is selected to establishing dominance and the remaining become subordinate follicles and enter atresia. Depite the fact that, the diameter of this large dominant follicle differs between waves all of them are able to ovulate after spontaneous or induced luteolysis.

In cattle, emergence of the next wave of follicles occurs only after the dominant follicle loses its inhibitory effect on the growth of other follicles and after an increase in FSH concentrations (Adams et al., 1992; Sunderland et al., 1994; Evans et al., 1997). In sheep, the new follicular wave emerged only after that the previous biggest follicle stopped growing or is in a static phase of development (Evans et al., 2000). Therefore, there appears to be a relationship between the demise of the follicles of one wave and the emergence of follicles in the subsequent wave, providing further evidence for the phenomenon of dominance in sheep may be less absolute than in cattle (Evans, 2003). Although in presence of a growing dominat follicle the recruitment of a new wave not occurs, it has been described that, a new follicle wave can emerge in the presence of large follicles from a prevoius wave (Johnson et al., 1996; Leyva et al., 1998 , Bartlewski et al., 1999; Flynn et al., 2000).

According to Menchaca and Rubianes (2004) the prediction of the day of the emergence of each follicular wave is difficult, with the exception of the first wave of the cycle which emerges around the day of ovulation (Day 0) of the previous cycle. The day of emergence for each wave is variable and depends on the number of waves in each cycle. In the ewes with a pattern of 3 follicular waves the 1st, 2nd, and 3rd waves emerge at days 2.1, 6.9 and 11.7 respectively (Evans et al., 2000) or at days -1 to 2, 4 to 7 and 8 to 10 for waves 1, 2 and 3 respectively (Ginther et la., 1995). In ewes with 4 waves the pattern of emergence for the1[st], 2nd, 3rd and 4th waves are about (-1 to 2) (3-6) (7-10) and (11-13) respectively (Duggavathi et al., 2003).

The inter-wave interval is the period between the emergence of the largest follicle (3mm in diameter) of two consecutive waves, which is around four to seven days. As luteal phase progresses, follicular turnover increases and the inter-wave intervals are shorter than during early luteal phase (Ginther and Kot, 1994; De Castro et al., 1999).
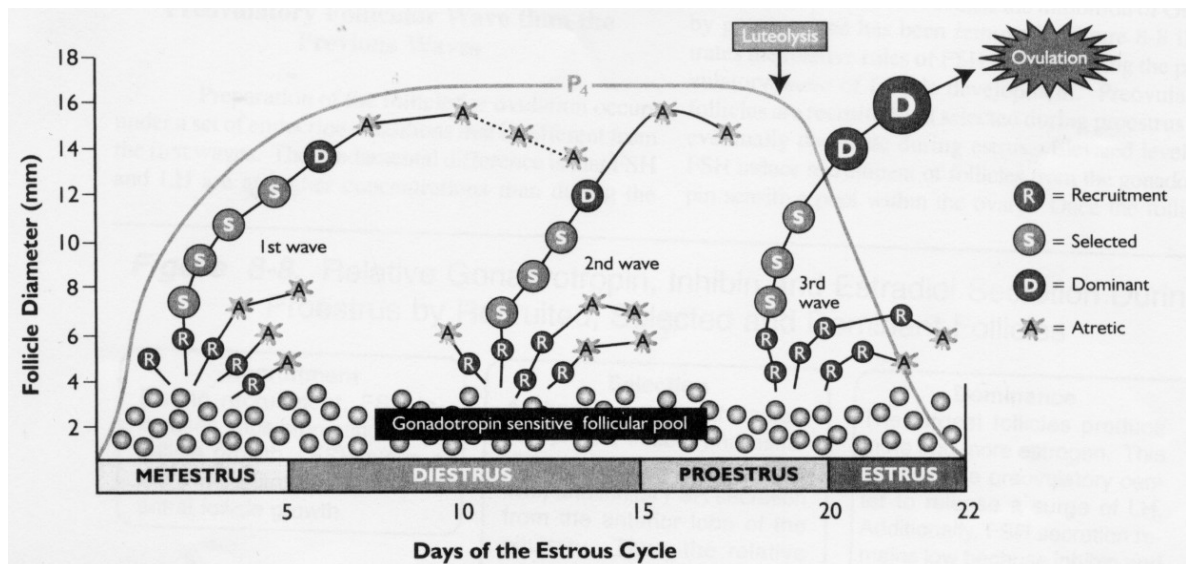
Fig. 7. Follicular waves during the oestrus cycle (Senger, 2003).

2.1.5.2 Follicular phase

The follicular phase comprises the regression of corpora lutea, the final follicular development of the preovulatory follicle from the last follicle wave and the subsequent ovulation. This process takes about 3-4 days.

After luteolysis there is a marked reduction in blood progesterone concentrations. Therefore, the negative feed-back exerted by progesterone on the hypothalamus is removed and the GnRH is released at higher amplitudes and frequencies. This causes that FSH and LH are released at higher concentrations, thus promoting follicular development and the production of estradiol. The dominant preovulatory follicle begin to produce more and more estradiol and inhibin, this declines FSH concentration. When estradiol reaches a threshold level, or peak (during estrus), the preovulatory center is "turned on" and releases large quantities of GnRH that stimulate the anterior lobe of the pituitary gland to secrete a preovulatory surge of LH.

2.1.5.3 Luteal phase

The luteal phase lasts about 13 days of the cycle and is characterised by the maturation of the corpus luteum with the production of high levels of progesterone that reach a peak at about 6 days after ovulation. Under progesterone dominance neither complete follicular development or ovulation can occur because the GnRH is released in low quantities only and consequently FSH and LH concentrations are low. However the

dominant follicle of each wave will ovulate if luteolysis occurs. It should be emphasized that even though follicles in the first two follicular waves become atretic they still produce some estradiol. After luteolysis, a third wave of follicles develops and one of these follicles will reach the preovulatory stage.

It has been reported by some autors that, during the luteal phase a weak form of dominance may exist based on the observation that the largest follicle may delay or prevent the development of the other follicles (Ravindra et al., 1994; Ginther et al., 1995; Rubianes et al., 1997).
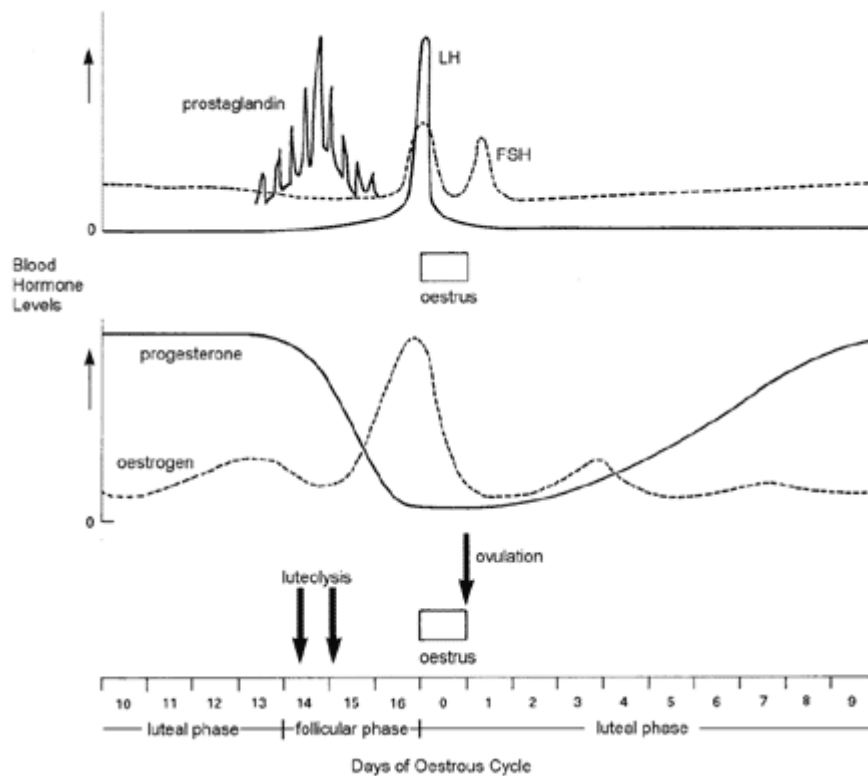
Fig. 8. Hormonal changes during the follicular and luteal phases.

2.2 Superovulation and Embryo Production

Multiple Ovulation and Embryo Transfer (MOET), is today an indispensable tool for the implementation of genetic improvement and conservation programs of endangered livestock (Bettencourt et al., 2008). In sheeps MOET remains an efficient technique to maximize the number of lambs borned from females with a high genetic merit (Driancourt M., 2001; Gonzalez-Bulnes et al., 2004; Bartleswski et al., 2008) .

2.2.1 Factors affecting the success of a MOET programme

Among many variables in a MOET programme, the unpredictable number of the ovulations and transferable embryos in response to a superovulation treatment is the variable that major affects the success of a MOET programme. It has been widely described that this variability can be affected by both intrinsic (Breed, age, nutrition and ovarian dynamics) and extrinsic factors (synchronisation, superovulation protocols and fertilisation).

2.2.1.1 Breed, age and nutrition

Related to intrinsic factors, **Breed** was early identified as a factor that may contribute to the variability of the superovulatory response (Bindon et al., 1986) and as a general rule, prolific breeds show a better superovulatory response (Nuti et al., 1987; Dufour et al., 2000) than non-prolific breeds (Kiessling et al., 1986; Picazzo et al. 1996). According to Ammoun et al. (2006) differences between breeds seems to be related to differences in follicular dynamics in response to a FSH treatment, breeds with a higher ovulation rate and embryos recovered have been associated to a higher follicular recruitment and growth rate leading to a higher number of follicles ≥ 4 mm in diameter at sponge withdrawal; wich was related to the number of preovulatory follicles at oestrus.

The influence of *Age* on superovulatory yields is determinated by the fact that natural spontaneous ovulation rate is affected by age (Theriez et al., 1971). It has been established that, in ewes the best embryo outputs will be found approximately at 6 years old (Torres et al., 1987). Despite that prepuberal females can be induced to superovulate (Driancourt et al., 1990) the ovulation rate is lower than adults (Driancourt and Avdi 1993). It has been suggested that this lower success with juvenile animals may derive from suboptimal progesterone concentrations, which leads to an inadequate preparation of the hypothalamus-pituitary axis for the induction of the superovulation and/or of the uterine enviroment to permit normal fertilization and formation of high quality embryos (Amstrong et al., 1983).

Is has been widely reported that, *Nutrition* affects many aspects of reproductive cycle in animals. Inadequate nutrition may compromise follicle oocyte competence (O'Callaghan et al., 2000), luteal function (Jabbour et al. 1991) embryo development (Robinson, 1990; Abecia et al., 1997) and pregnancy rates (Parr et al., 1987). In sheep, follicle populations are very sensitive to nutritional input and folliculogenesis and ovulation rate can be readily increased by nutritional manipulation (Scaramuzzi et al., 2006). It has been demonstrated that the nutrition during the peri-conception period (-18 days to +6 days relative to Day 0= ovulation) plays an important role on reproductive outcome, particulary in relation to ovulatory performance (Kakar et al., 2005).

Several reports indicate that nutritional conditions that improve ovulation rate can be detrimental to oocyte and embryo quality. Lozano et al. (2003) found lower superovulatory responses and decreased oocyte and embryo quality in animals feeded ad libitum diets when compared with low and control diets. Indeed, Kakar et al. (2005) found an increase in embryo development in sheep feeded with low energy intakes rather than high or control levels. This findings explain that, nutritional conditions required to maximise ovulation rate (i.e. an increasing dietary intake) differ from the conditions required for enhanced embryo quality (i.e. a relatively low intake, at least immediately after ovulation) This results are according whit previous reports (Creed et al., 1994; McEvoy et al., 1995) were the proportion of viable embryos from superovulated ewes was significantly higher in ewes that received diets whit low energy compared with high level energy, even under invitro culture conditions (Papadopolous et al., 2001).

2.2.1.2 Ovarian dynamics during superovulation treatment

The principle of superovulation is to provide to the female an high levels of exogenous gonadotrophins so a greater number of follicles are recruited and selected for ovulation.

It has been described that, during a superovulation treatment the ovulation rate increases through a combination of 5 mechanism, such as: i) a reduction of the size at which follicles are recruited, ii) a wider window of time for follicle recruitment (Driancourt and Jego, 1985), iii) a smaller size of the preovulatory follicles compared with non-stimulated ovulatory follicles, iv) an increase of the follicular growth rate (Driancourt, 2001) and v) a prevention of the atresia process by blocking selection of dominant follicle

and increasing the number of follicles in the ovulatory cohort (Scaramuzzi et al., 1993; Bartlewski et al., 1999; Driancourt, 2001).

The ovarian response to a superovulation treatment seems to be strongly affected by the ovarian dynamics at the beginning of exogenous gonadotrophin treatment. Several studies have shown that, exists a positive correlation between the number of small follicles (2-3 mm) at the first FSH dose and the ovulation rate and embryo output after superovulatory treatment. However this positive association can be negatively affected, by the presence of large ($\geq$ 5 mm) dominant follicle(s) at the onset of gonadotropin stimulation, resulting in lower number of transferable embryos (Rubianes et al., 1995, 1997; Cognie et al., 2003 ; Gonzalez-Bulnes et al., 2004; Bartlewski et al. 2008).

The decrease in ovarian response suggest that this large (presumptive dominant) follicle would affect the developmental competence of smaller follicles or of the enclosed oocytes (Gonzalez-Bulnes et al., 2000, 2002; A. Veiga-Lopez et al., 2005). This large follicles secrete high levels of inhibin and estradiol, decreasing FSH concentrations and determining the atresia of smaller follicles (Campbell et al., 1995). Thus, there is lower follicle recruitment, fewer ovulations, fewer transferable embryos, and the development of functionally subnormal corpora lutea (CL) (Rubianes et al., 1997). More recent studies have demostred that the superovulatory responses in ewes seems to be related to the size and physiological status of two largest follicles detected at the onset of the FSH treatment (F1 and F2) diameters of F1 and F2 are negatively correlated with embryo recovered and viability rates, suggesting the existence of follicular co-dominance during superovulatory treatments in sheep (Veiga-Lopez et al., 2006; Bartlewski et al., 2008).

The dominant follicle avoids its own regression by shifting its dependence from FSH to LH (Campbell et al., 1995;1998), due this fact, they become exquisitely sensitive to LH. This observations show that LH concentration has a key role in the establishment of dominance from large follicles in sheep. Superovulatory yields would be improved by starting FSH treatment in the absence of large follicles or coincidentally with low LH levels.

Some authors suggested that dominance is weak or not operative during the luteal phase (Schrick et al., 1993; Ravindra et al., 1994), maybe due the fact that, follicles produce variable amounts of estradiol according to the physiological status of the female and seem to be limited during the first wave of the luteal phase and high for those developing during the follicular phase (Driancourt, 2001) by other hand, the largest follicles that grow during the mid-late luteal phase do not produce high levels of

oestrogens, because high progesterone concentrations reduce pulsatile LH secretion (Rubianes and Menchaca 2003).

Some strategies have been focused on starting the superovulatory treatments at wave emergence in the absence of a dominat follicle to overcome a deleterious effect on superovulatory yields. (Rubianes and Menchaca 2003). The results indicate that higher superovulatory responses can be obtained when treatment were initiated at the emergence of the follicular wave compared with later treatments (Nasser et al., 1993; Bo et al., 2006).

Menchaca et al. (2007) described that a FSH treatment, soon after ovulation at the expected time of emergence of wave 1 in abscense of a large follicles, provokes better follicular recruitment and greater ovulatory response than later treatment. In addition, this responsive follicles have a similar status of maturation and adequate process of multiovulation follows gonadotropin stimulation otherwise, if the pool of follicles shows a large heterogeneity the response is characterized by an asynchronous process of follicular growth and luteinisation and this could be related to inadequate responses (i.e. inadequate luteal function, luteinised follicular cysts) (Rubianes et al., 1996; 1997).

The better results of superovulatory treatments when recruited follicles have a similar status of maturation is explained because even exogenous FSH administration allows continued growth of all follicles in the wave. Post selection treatment appears to have been associate with competition whitin the cohort of follicles in FSH utilization, where the most successful follicle become dominant and rescue of static or regressing subordinate follicles with a lower ability to ovulate, subnormal luteal gland formation, and lower developmental competence of the oocytes (Adams et al. 1993; Rubianes et al., 1997).

Follicular status is not the only ovarian factor influencing the embryo outcome, the presence of a young corpus luteum at the beginning of the superovulatory treatment exert a protective effect on embryonic viability by decreasing the deleterious effect of the dominant follicles (Nöel et al., 1994; Rubianes et al., 1996; Veiga-Lopez et al., 2005). This reduction of dominance results in a higher number of viable embryos obtained from ewes bearing a corpora lutea at fisrt FSH dose, since the degeneration rate is higher in ewes without corpus luteum (Gonzalez-Bulnes et al, 2003). The presence of a corpora lutea induce higher levels of progesterone and low levels of LH consequently large follicles become critically dependent on FSH without an opportunity to establish their dominance, this effect induced by LH reduction, explains the suppressive effects from the corpora lutea on dominant follicles in sheep (Savio et al., 1993; Adams, 1999; Rubianes

and Menchaca 2003). On the bases of these findings Mayorga et al., (2007 b) described, significant better ovulatory responses and fertility rates when the FSH treatment was started on day 4 after spontaneous ovulation, compared with progestagen treated ewes.

2.2.1.3 Protocol of synchronization

Progestagen sponges treatment

The progestagen treatments had been used as the most traditional method of oestrus synchronization in superovulation and embryo transfer programs. The more commonly used may be either fluorogestone acetate (FGA), or medroxy-progesterone acetate (MAP) for 12 or 14 days by the insertion of an intravaginal sponge. (Thibier and Guérin, 2000).

The principle of this method is to simulate the action of a natural corpus luteum, therefore, during treatment the pituitary output of gonadotrophins is suppressed. Once the sponge is removed, the pituitary gland releases increasing amounts of gonadotrophins which stimulated follicular growth and subsequent ovulation. However, despite the benefits of females synchronisation, progestagen treatments have been identified as causal agents of alterations both at a systemic level, in the patterns of LH release (Scaramuzzi et al., 1988) and at ovarian level, in the patterns of follicular growth and dominance (Nöel et al., 1994; Leyva et al., 1998).

The initial release of progestagen from intravaginal sponges increase during the first 48h of treatment, but decreases with the time, in this way, the progestagen protocol is not able to suppress the LH concentration to the level achieved during the natural luteal phase (Kojima et al., 1992) and leads to inadequate follicular development, with persistent large estrogenic follicles (Johnson et al., 1996; Leyva et al., 1998; Viñoles et al., 1999; Flynn et al., 2000). On the contrary, during natural estrous cycle, progesterone concentration is at first low and then increases until luteolysis (Dattena M., 2006) (Fig.9.).

It has been described that, serum medroxy-progesterone acetate concentrations decrease in about a 63% between the 2nd and 13th days after sponge insertion (Greyling et al., 1994), demonstrating that the progestagen supply from intravaginal sponges decreases with the time. The low progestagen concentrations and increased LH secretion in ewes lead to larger follicles (Viñoles et al., 1999), older larger persistent ovulatory follicles (Leyva et al., 1998, Flynn et al., 2000; Evans et al., 2002) delay of wave emergence (Johnson et al., 1996; Viñoles et al., 1999) and higher oestradiol concentrations (Leyva et

al., 1998; Flynn et al., 2000). The ovulation of these persisted aged follicles in their static or early atretic phase determines a decreased fertility (Johnson et al., 1996; Ungerfeld and Rubianes 1999; Viñoles et al. 2001) thus affecting the ability to ovulate an oocyte able to be fertilized and develop in to a viable embryo (Theodosiadou et al., 2004;Gonzalez-Bulnes et al., 2005 a).

The detrimental effects of low progesterone/progestagen concentration would be exacerbated in cyclic females in which a corpus luteum persists after sponge removal. To overcome this, it has been described that better superovulatory responses in terms of ovulation rates total and viable embryos recovered can be achived including the insertion of a second progestagen sponge from day 7 to 14 to maintain constant concentrations of progesterone during treatment and in combination with a prostaglandin analogue injection to provoke luteolysis before progestagen removal (Dingwall t al., 1994; Gonzalez-Bulnes et al., 2002 b).

Alternatively, to usual 12-14 days progestagen treatment a shorter duration of progestagen treatment (5-9 days) have been demostred to avoid detrimental effects of low progesterone/progestagen concentration at the end of treatment and improve reproductive performace (Viñoles et al 2001; Dixon et al., 2006). According with this results (Mayorga et al., 2007b) using short progestagen treatment in superovulatory protocols on Sarda sheep, observed a higher tendency in terms of ovulation rate, total embryos recovered, number of viable embryos and fertility rates in ewes treated with short progestagen versus long progestagen treatment.
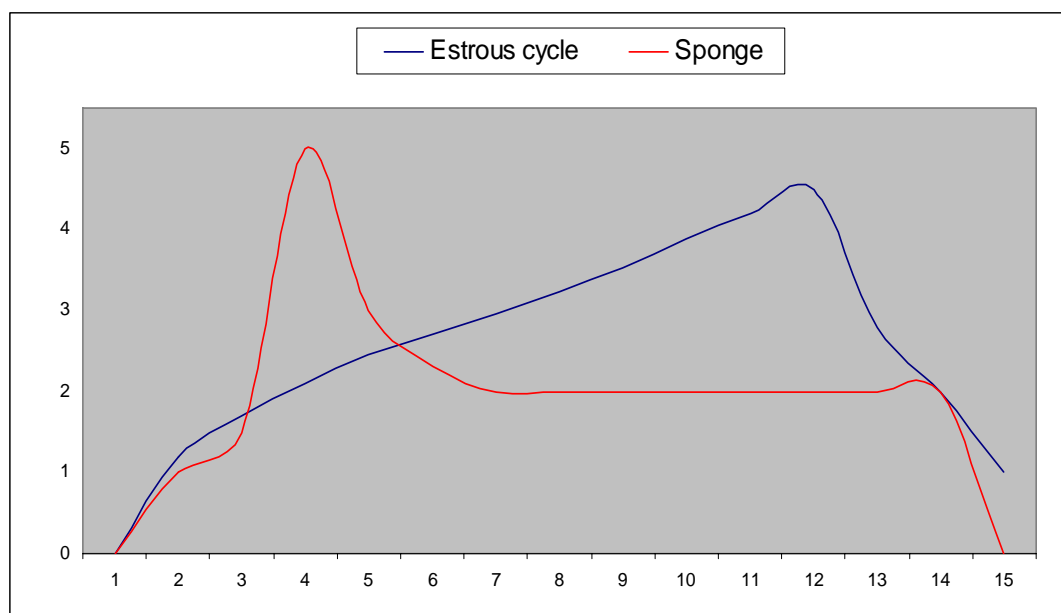
Fig. 9. Progesterone serum profile induced by sponge treatment is the opposite of that observed during a normal estrous cycle (Dattena M., 2006).

Prostaglandins

As alternative to progestagen treatments, the synchronization of estrus cycles with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) can be considered as possible way during breeding season. The administration of exogenous $PGF_{2\alpha}$ or its analogues can be applied to induce a controlled luteolysis, and the use of two doses 9-11 days apart is effective in synchronizing estrus. However some authors (Boland et al., 1978; Godfrey et al., 1997) demonstred a detrimental effect on the fertility of the ewes at first service when compared with those treated with progestagens, due to a disruption of the ovulatory follicular dynamics and the normal luteogenesis and/or a variability in the timing of ovulation after $PGF_{2\alpha}$ induced luteolysis (Barret et al., 2002).

By other hand, studies have been demostred that, the use of prostaglandin analogues such us cloprostenol in superovulatory protocols can achieve similar responses in terms of in vivo embryo production (Gonzalez-Bulnes et al., 2005 a,b). and in vitro blastocysts out put (Berlinguer et al., 2007) compared with progestagen treated ewes. Althought differences founded were not significant the results encourage the use of prostaglandin in superovulation protocols. Indeed, Mayorga et al., (2008) described that the use of cloprostenol injection instead the intravaginal prosgestagens treatments can be useful under

practical field conditions to simplify the application of MOET programs without affect the ovulatory responses.

2.2.1.4 Protocols of superovulation

The ovarian response to the superovulation treatment is closely related to the protocol of gonadotropin administration.

Gonadotrophin administration

The superovulatory treatment is usually given at the end of the synchronization treatment twice daily for a period of 4 days, several superovulation treatments have been tested to reduce the frequency of FSH injections (Dattena et al., 1994) but, due to the short half life of the gonadotrophin molecule they have failed (Thibier and Guérin., 2000).

In a protocol of superovulation a very important factor that influences superovulatory responses is the variable LH content in the commercial FSH products available (Lindsell et al., 1986). Comercial products with high LH content decreases ovulatory response when compared to purified FSH (Chupin et al., 1987; Torres et al., 1987) determining the follicular regression during the treatment and their consequent inability to ovulate (Gonzalez-Bulnes et al., 2000). However, excessively low amounts of LH at the end of treatment also induces a lower ovulation rate (Chupin et al., 1987; Torres et al., 1987). Therefore a basal LH concentration is required for the optimal follicle growth (Mc Neilly et al., 1986; Mc Neilly et al., 1992) and an adequate FSH/LH ratio provides the best superovulatory yield (Amstrong et al., 1989).

It has been reported that, the ovarian response to superovulation protocols in terms of the mean ovulation rate and mean numbers of recovered and viable embryos generally tend to be higher in sheeps treated with decreasing FSH dosages (step-down) than those treated with constant dosages. (Thibier and Guérin, 2000; Gonzalez-Bulnes et al., 2002 b; Gonzalez-Bulnes et al., 2004; D'Alessandro et al., 2005). Similary results have been reported in womens during reproductive assisted programs were the "step-down protocol" with decreased FSH/LH ratio seems to be better than constant doses (Macklon and Fauser, 2002; Loumaye et al., 2003).

There is a general consensus based on the concept that decreasing FSH/LH ratios is closer to the endocrine changes in the pituitary secretion during of a non-stimulated oestrus cycle. FSH shows to decrease from luteal regression to the preovulatory peak (Cahill et al., 1981), due the increase of oestradiol and inhibin from preovulatory follicles (Baird and

McNeilly, 1981), being minimal 1-2 days before oestrus (Miller et al., 1981) On this base, the FSH/LH ratio in a protocol of superovulation probe to be a determinat factor on superovultory response.

2.2.1.5 Fertilisation

Although, the ovarian response to the superovulatory treatments has been notably enhanced through the years, a great difference still persists between the number of corpora lutea and the final number of transferable embryos obtained, one of the causes that has been reported is the low fertilisation rates found in some females, mainly in those showing higher ovulation rates (Amstrong and Evans 1983; Cognie 1999; Gonzalez-Bulnes et al., 2004). Usually the fertilization can be achieved either by natural mating or by artificial insemination (AI). (Thibier and Guérin, 2000).

Natural mating has been widely applied in supeorvulatory programs because represents an easy and practical method (Gonzalez-Bulnes et al., 2002 a; Bartlewski et al., 2008), but by other hand, many studies reported that the synchronization and superovulatory treatments themselves can interfere with sperm transport through the cervix, and this, in turn, compromises the fertilization rate and embryo quality by resulting in either aged sperm cells or ova at the time of fertilization (Mutiga and Baker, 1982; Evans and Amstrong, 1984; Hawk et al., 1987). Breeding should be supervised and repeated every 8 hours until the donor is out of heat. Males used for breeding should be established on the property for 3-4 weeks before to be used in a MOET program and should have no history of recent illness. Stress and elevated body temperatures due to fever commonly cause temporary infertility of males. Alternatively to natural mating *artificial insemination* can be achieved with fresh or frozen semen usually by vaginal or cervical techniques, but the convoluted structure of the ewe cervix makes precise deposition of semen difficult, thus reducing the chances of fertilization (Lightfoot and Salamon, 1970; Boland et al., 1983; Buckrell et al., 1994). Some studies have reported to enhance fertilization rates in superovulatory programs with artificial insemination plus natural mating (Bari et al, 2000; D'Alessandro et al., 2005).

2.3 Embryo recovery and storage

Embryos are collected between the 6th and 7th day after the onset of oestrus, a "window" which arises from various factors such as the timing of the embryos entry into the uterine cavity, the fact that they must not have hatched from their zona pellucida for sanitary reasons and also the necessity for such embryos to be able to withstand deep freezing. They are at this time at the morula or early blastocyst stages.

2.3.1 Embryos recovery technique

The more common techniques have been used for embryo recovery, are the mid-ventral laparotomy and the laparoscopy.

2.3.1.1 Mid-ventral laparotomy

In this procedure all donors are taken of feed for 24 h and water for 12h prior to surgery. After general anaesthesia is induced a midventral line insicion (15-20cm long) is performed cranial to the udder attachment, to alow elevation of each side of the reproductive tract, first ovaries are exposed with the minimal manipulation and corpora lutea are recorded to asses the superovulatory after that, ovaries return into the peritoneal cavity and only uterus is exposed for the flushing of uterine horns. This technique has been described on the paper of Tervit and Havik (1976).

It has been described that mid-ventral laparotomy generally ensure over 80% of embryo recovery (Wallace, 1992), however, the acceptability of this procedure has been questioned due to the reduced fertility as a result of postoperative adhesions when several collections are required from a value donor (Boundy et al., 1985 ; Thibier and Guérin., 2000).

2.3.1.2 Laparoscopy

In this procedure probably just sedation can be enough but usually general anesthesia is required for optimal control over the procedure. As in the laparotomy technique all donors are taken of feed for 24 h and water for 12h prior to surgery.

After restraining the animal on a surgical table in dorsal recumbency, the head is held down at a 45°C angle to the horizontal and the ventral abdomen is clipped free of wool or hair for surgical preparation. Prior to insertion of the laparascope, the peritoneal cavity is insufflated with approximately 4-5 lt of CO_2 gas . The laparoscope is inserted and ovarian response is recorded while viewing the uterus through the laparoscope the tip of the horn is grasped with Allis tissue forceps and exteriorized through a 2.5 cm incision through the linea alba. Both uterine horn tips are canulated with an intravenous catheter (18g) as close to the utero-tubal junction as possible (Schiewe et al., 1984) a Foley catheter is introduced into the uterus and a volume of aprox 50 ml PBS is instilled for a washing of the cavities before aspirating and collecting the fluid which contains the embryos (Thibier and Guérin, 2000).

The laparoscopy technique offers several advantages in terms of animal welfare, when it is done correctly the adverse sequellae on the genital tract seem to be less than after laparotomy technique (McKelvey etal., 1985 a; Vallet et al., 1987). However, it has been described that this technique is associated with a significantly lower embryo recovery rate than full laparotomy (65 to 70%) (Mc Kelvey et al., 1986; Baril et al., 1993). More recently semi-laparoscopic approach was described by (Bari et al., 2000) and demostred to improve until 83% of recovery rate embryos.

2.3.2 Evaluation of embryos

After flushing of embryos, their further developmental capacity has to be evaluated. A correct method of evaluation, is of critical importance for a successful transfer.

The most frequently method used is the ***morphological evaluation*** by microscopic inspection of embryos. The first evaluation criteria is based on the expected developmental stage at the moment of the flushing. If the developmental stages do not correspond with the age of the embryo, the embryo will not be suitable for transfer, cryopreservation or other purposes.

The morphological criteria used to evaluate embryos are shape, colour, number and compactness of cells, size of the perivitelline space, number and size of vesicles and status of the zona pellucida (ZP). The ideal embryo shows and empty perivitelline space and a regular diameter. According to their morphological appearances, embryos can be classified into four groups (Niemann et al., 1981; Linder and Wright, 1983): *Excellent embryos* are embryos in the appropriate developmental stage with perfect morphology, they must be spherical and symmetrical with cells of uniform size, colour and texture. *Good embryos* are those in the appropriate stage of development, but with slight morphological deviations, for example some minor damage of the ZP, and excluded blastomeres or vesicles in the previtelline space. *Degenerated and/or retarded embryos* are embryos in the appropriate developmental stage with major morphological deviations (degenerated embryos, or embryos which are not in the appropriate developmental stage with or without morphological deviations (retarded embryos). *Unfertilized ova* comprise the final category. (Cocero et al., 1996; Cognie et al., 2003).

By other hand, some authors suggest the use of four categories, with grade 1 being excellent and 4 being very poor (Gordon, 1999).

Despite morphological evaluation is a simple, non invasive and a rapid procedure, is a subjective method of analysis and requires experienced technicians to accurately classify or "score" embryos (Overström E. 1996).

2.3.3 Cryopreservation

Embryo cryopreservation has enormously simplified the management of genetic resources in domestic and wild species and it is essential in the commercial embryo transfer technology providing an easier and cheaper embryo transport, reducing health risk and avoiding loss of animals during transport. The purpose of cryopreservation is to hold embryos in a state of hypobiosy (reduction of the biological activity) so that when they are thawed, the normal biochemical and developmental processes will be resumed. Through the years, different techniques of cryopreservation have been developed. Such us controlled **slow freezing** (Whittingam,1971 and Whittingam et al., 1972), **fast or rapid freezing** (Takeda et al., 1984; Trounson et al., 1987; Reichenbach and Rodrigues, 1988) and **ultra rapid freezing techniques/Vitrification** (Fahy, 1986; Rall et al., 1987; Takahashi and Kanagawa, 1990; Shaw et al., 1991; Isachenko et al 2003; Rypka et al., 2006).

Vitrification tecnique

The first to use it for freezing of mammalian embryos were Rall and Fahy (1985), and later Massip et al., (1986; 1989). This procedure uses a higher concentration of cryoprotectant and faster cooling rate (compared with traditional freezing methods, thus preventing ice crystal formation). Massip (2001) defined vitrification as a cryo-preservation method which enabled passage from the liquid to the solid state due to high concentration of cryoprotectans and very rapid cooling, such that the viscosity of the solution increases and forms a glass-like solid which depresses ice crystal formation. This procedure allows the embryo to be plunged directly into LN2, avoiding crystallization during the freezing and warming steps (Kuwayama et al., 1992; Leibo and Loskutoff, 1993; Tachikawa et al., 1993; Mahmoudzadeh et al., 1995; Vajta et al., 1998 a, Van der Zwalmen et al., 2002).

Recently, many different vitrification protocols have been used to cryo-preserve in vivo and in vitro produced embryos. They differ in many ways, including type and concentration of cryo-protectant, number of equilibration steps, type of cryopreservation device used, and time of exposure and number of dilution steps at warming (Massip et al., 1987; Rall, 1987; Szèll et al., 1990; Schiewe et al., 1991; Yang et al., 1992., Ali and Shelton, 1993 ; Vajta et al., 1998a;b; Dattena et al., 2000b).

The components of the cryoprotectant mixture should penetrate embryos rapidly to minimize osmotic schock and also ensure appropriate protection for the whole cell. The cryoprotectants most widely used in vitrification are glycerol, ethylene glycol, propylene glycol and Dimethysulfoxide (DMSO) sometimes supplemented with non permeable solutes such us sucrose, threalose, etc. (Rall and Fahy ,1985; Ali and Shelton, 1993; Martinez and Matkovic, 1998; Vajta et al., 1998 a).

In the 1980s the plastic straw become the container of choice for vitrification and storage of embryos (Rall and Fahy, 1985; Massip et al., 1989; Ishimori et al ., 1993; Riha, 1994; Lane et al., 1998; Hurtt et al., 2000) their advantages are that they have a considerably smaller diameter, thinner walls, and contain a significantly smaller volume of liquid compared with ampoules or tubes.

A recent modification the open pulled straw (OPS) method, this new approach has many benefits including minimizing the volume of vitrification (cryoprotectant) solution down to 0,5 μl, wich allows short and direct contact between the embryos with the cryoprotectants (less than 30 sec over –180°C). Toxic and osmotic effects at thawing are minized by inmersion of the OPS containing the vitrified-embryos into a thawing solution,

thus enhancing the cooling and warming rates (over 20 000°C/min) (Vajta, 1997; Vajta et al ., 1998a;b;1999; Lewis et al., 1999; Choi et al., 2000; Dattena et al., 2000 a; Vieira et al., 2002; Isachenko et al., 2003a).

The advantages of vitrification protocols are that by direct plunging into liquid nitrogen, the embryos are rapidly cooled in the fine portion of the open-pulled straw; warming is easily and rapidly achieved by directly immersing the straws in a tube containing the thawing medium; and embryo transfer is directly performed using the open-pulled-straw as a catheter, without the need optical microscope this increase potential applications to field conditions (Isachenko et al, 2003 b; Dattena et al, 2004).

Among all variables affecting embryo survival after cryopreservation, the stage of development and quality of embryos has a positive effect on the success of cryopreservation, only embryos grade 1 are elegible for cryo-preservation while embryos grade 2 and 3 will be transferred fresh. On the other hand, vitrification and warming procedures have less effect on the viability rate of ovine embryos in advances stages of development than those in earlier stages (Ali and Shelton, 1993; Szèll and Windsor, 1994). Dinnyés et al. (1995;1996) and Naitana (1997) have demonstrated that embryos vitrified at the expanded blastocyst stage had significantly higher viability than embryos vitrified at the early blastocyst stage (57% vs 88.4 %, p<0.01), this differences migth be related due to, comparing morphologically with early stage of embryos, blastocysts and expanded blastocysts have a fluid-filled cavity (i.e. the blastocoele) containing large amounts of water inside, while embryos at the morula stage (without a blastocoele) the larger proportion of the water is contained inside the cells (Van der Zwalment et al., 2002). Thus, it could be suggested that, the cellular membranes of embryos become more resistant to osmotic and toxic stress after formation of the blastocoele.

2.4 Embryo transfer

The first successful embryo transfer in farm animals was reported in sheep and goats by Warwick et al., (1934). Extraordinary progress and diversification in this embryo technology have been performed across decades, however its success still depends from several practical and technical factors.

2.4.1. Selection of recipients

The selection of recipients is a critical factor that can determinate the success of an embryo transfer program. For this reason the identification of a reliable criteria for the selection of the ideal recipient has been the objective of many studies during the last 40 years (Alabart et al., 1995; 2003).

The recipients should be identified and kept in separate groups for at least 2 months prior to the beginning of the embryo transfer program, to prevent unnecessary stress. Indeed, it has seen that sheep and goats are prone to stress by mixing (McKelvey and Bhattacharyya, 1992). Females with a history of good mothering are preferred, another considerable point is that they must be in good health conditions, animals with a history of mastitis, reproductive disorders or dystocia should be avoided. Potential recipients should be treated with parasiticides appropriate to the geographic location and season.

The reproductive status must be good and lack of any reproductive disorders. It has been described that ovarian alterations (e.g. follicular cysts and luteinised cysts) in preovulatory follicles, and inadequate luteal function lead to death of the embryo or fetus (Niswender et al. 1985; Reynolds et al., 1994).

The selection of recipients based on the characteristics of the corpus luteum can be an alternative to increase pregnancy rates. The assessment of the ovulatory response (size, number and colour of the corpora lutea) and uterine tone and morphology are usually done just before embryo transfer either during surgical or laparascopic techniques while, to avoid invasive handling the transrectal ultrasonography (non-invasive technique) first developed for ovarian scanning in ewes (Schrick et al., 1993) and thereafter in goats (Ginther and Kot 1994) can be used as alternative to determine if the recipient has ovulated and to evaluate the ratio between cavity diameter and total luteal tissue diameter as a means to distinguish the corpora lutea from luteinised follicles (Gonzalez-Bulnes et al., 1999).

2.4.2 Synchronization of recipients

In MOET programs when transfer is performed simultaneouslyto the number of donors is necessary to count whit the appropriated number of recipients in this way the effective synchronization of recipients becomes a primordial factor for success of an embyo transfer.

2.4.2.1 Pharmacological methods

The pharmacological methods are dived in depends of physiological principles in two types. The first type is based on administration of synthetic progestagens (or progesterone) to simulate the action of a natural CL. The second type is based on administration of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) or synthetic prostaglandin for lysis of CL.

Progestagen method

The progestagens used may be either progesterone itself or an analogue such as fluorogestone acetate (FGA), medroxy-progesterone acetate (MAP) or controlled internal drug releaser (CIDR) (Thibiert and Guérin., 2000) administered by different methods and routes.

For practical reasons the most widely used treatment involves the insertion of intravaginal sponges impregnated with 30 or 40 mg of fluorogestone acetate (Chrono-gest ®) or 60 mg of medroxyprogesterone acetate (MAP) generally for 12-14 d similar to the lifespan of a cyclic corpus luteum.

The treatment acts by suppressing the preovulatory pituitary release of gonadotrophins and, therefore, follicular, growth and ovulation. After the sponge removal, the increased amounts of gonadotrophin released lead to oestrus and ovulation.

The degree of syncronisation obtained and interval between the end of treatment and the onset of oestrus depends on the product used. Ewes will begin to come into oestrus from around 24 to 48 hours after sponge removal with a 80 – 100% of response to the treatment.

Prostaglandin method

Prostaglandin $F_{2\alpha}$ and its analogues have been widely used in small ruminants for synchronisation of oestrus, due the luteolytic effect of these compounds, they can be used to interrupt the luteal phase in cyclic ewes. When prostaglandin is injected the corpora luutea (CL) is removed and also the inhibitory effect of progesterone on the pituitary gland, thus the suppression of this inhibitory effect produce an increase on amounts of gonadotrophins released by the pituitary gland stimulating follicular growth and onsent of oestrus within 2-3 days with ovulation 24 hours later.

Traditional treatments consist of two $PGF_{2\alpha}$ doses separated by 9 to 14 days (Evans and Maxwell, 1990; Duràn Hontou, 1993) and is based on the concept of the refractoriness of the young CL. More later, in a study conducted by Rubianes et al. (2003) it was demostred that the refractoriness of CL might be restricted only to the first two days after ovulation because luteolysis, oestrus behaviour, ovulation and a newly formed CL can be observed in $PGF_{2\alpha}$ treated ewes just at day 3 after ovulation, this results have allowed the development of new protocols whit a shorter interval between the two $PGF_{2\alpha}$ doses to only seven days (Menchaca and Rubianes 2004).

2.4.2.2 Natural method (oestrus detection)

The natural method could represent an important alternative to select ewes in oestrus instead to the progestagens treatments, in simply terms this method involves the detection of spontaneous oestrus with the introduccion of vasectomized ram in the flock at least twice daily to detect ewes displaying oestrus behaviour and 6 or 7 days after this procedure they became used as recipients. Moreover, according to Evans and Maxwell (1987) during breeding season the 6-8% of females present in a flock show spontaneous natural oestrus daily. The simplicity of this method and the possibility to avoid the use of hormones make natural method an interesting alternative to be adopted in a low scale MOET programme to select recipients.

2.4.3 Factors influencing embryo survival rate

In small ruminants embryo survival and establishing of pregnancy after transfer is depended upon many factors.

2.4.3.1 Synchrony between embryo and recipient

Early investigators have described that pregnancy and embryo survival rates after transfer are highly related whit the degree of synchronization between donors and recipients.

Moore and Shelton (1964) reported maximum embryo survival rates in ewes when the recipient show oestrus between 12 hours before and 12 hours after their respective donors, although the best results were obtained when the onset of oestrus in the donor and recipient was exactly synchronised (Hancock and Hovell, 1961; Cumming, 1965) and more than seventy two hours of asynchrony appears to be incompatible or to result in very low pregnancy rates (Rowson and Moor, 1966).

Amstrong et al (1983) confirmed the importance of exact synchronization by comparing embryo survival as a function of degree of synchrony between donors and recipients. Their results revealed no significant difference in embryo survival as a degree of synchrony between donors and recipients on 1 day earlier, same day or 1 day later. However, Reichenbach et al., (1992) observed that synchronization between the embryo and recipient of −1 day resulted in a higher pregnancy rate than + 1 day. This reveal the importance that the transfer of older embryos into "younger" uteri is generally more successful than, transfer of younger embryos into "older" uteri.

The most likely reasons why the embryo dies is because the uterine environment may not be suitable for the "out-of-phase" embryo (Rowson and Moor, 1966). Another possibility could be that the "out-of-phase" embryo is incapable of exerting a sufficient luteotrophic action on the recipient's corpus luteum, resulting in luteolysis and consequently the embryo is lost (Wilmut et al., 1985). It is apparent that a successful pregnancy depends upon carefully orchestrated events which occur at the embryo-uterine interface allowing a "Maternal recognition of pregnancy" process where the conceptus signals its presence to the maternal system and prolongs lifespan of the corpus luteum (CL). It has been described that, ovine and bovine conceptuses secrete an array of proteins during the period of maternal recognition of pregnancy among all of them, ovine trophoblast protein-one (OTP-1) is the responsible for preventing luteal regression, by

attenuation of the uterine luteolysin prostaglandin $F_{2\alpha}$ (PGF$_{2\alpha}$) (Godkin et al., 1984; Roberts et al., 1999; Spencer and Bazer, 2004). In order to ensure a successful pregnancy, it is essential that these conceptus products are secreted in appropriate quantities at the appropriate time.

2.4.3.2. Site of transfer

It has been described that the success of transfer is highly related whit the development stage of embryos and site of transfer (tubal or uterine transfers).

Moore and Shelton (1964) demostred that when transferred of fertilized sheep eggs was performed, tubal transfer reaches more successful results than uterine transfer in number of ewes which lambed, by other hand Rowson and Moor (1966) found higher survival rates (70-75%) for embryos which were older than 3.5 days after estrus, if transferred to the uterus than oviducts.

In a study conducted by Amstrong and Evans (1983) not significant differences were found in terms of survival rate that can be attributed to the site of transfer, however, they concluded that embryos recovered below the eight-cell stage should be transferred to the oviducts of recipients and embryos wich had developed beyond eight cells should be transferred to the uterus this results were according with previous reports (Averill and Rowson 1958) were was described that the uterus does not provide an environment suitable for the survival and development of embryos of two and four cells.

2.4.3.3. Source of embryos

It has been described that, the source of embryos (fresh or frozen) is a factor that can affect the results of a embryo transfer program. Pregnancy rates are usually higher with fresh embryos (60-70%) than frozen embryos (50-60%) of a similar developmental stage, according to this Papadopoulos et al., (2002) described higher pregnancy rates after transfer of fresh in vivo derived embryos (90%) compared with those frozen (50%). On the contrary, Baril et al., (2001) found that when either fresh or vitrified 7 day embryos are transferred to synchronized recipients the preganancy rate (72% in both cases) and the number of lambs born (60% vs 50%) do not differ significantly.

The differences between source of embryos (fresh or frozen) can be explained, due the fact that freezing can results in the destruction of some cells of the embryos reducing their ability to develop normally (Dattena et al., 2000 a; 2004).

The embryo's quality has a significant effect on pregnancy rate. Wright (1981) demostred that the grade 1 embryos resulted in a 64% pregnancy rate as compared to 45% for grade 2 and 3 embryos. Kajihara et al., (1992) and Agca et al., (1998) showed that selection of embryos significatly improves the pregnancy rate (60%) compared whit non-selected embryos (27%).

Schneider et al., (1980) demonstrated that the stage of development and the grade has a significant effect on pregnancy rates. The best pregnancy rates were obtained when late morula, early blastocyst and hatched or collapsed blastocysts were transferred. Wright (1981) observed pregnancy rates from embryos transferred at the morula (44%) and advanced morula (53%) stage were reduced (p<0.05) when compared to those transferred at the early blastocyst (65%), blastocyst (66%) and advanced blastocyst (64%) stages.

2.4.3.4. Numbers of embryos transferred

The survival rate of transferred embryos varies according to the number of embryos present. Moore et al., (1960) found that the proportion of recipients becoming pregnant was the same when receiving five embryos or less, but the embryo survival rate following transfer of five embryos were lower, in agreement with this results, Larsen (1971) found that an increase in the number of ova transferred resulted in a lowering of the survival rate, whereas, when one or three ova were transferred although the difference was not statistical significant.

Amstrong et al., (1983) and Tervit et al., (1986) found that survival in goats improved when two embryos were transferred, over 80% of recipients of two embryos carried pregnancy to term, with approximately two-thirds of these giving births to twins. The improved embryo survival rates observed after twin transfer suggest that there is some type of synergism between embryos in influencing each other's survival upon transfer. Possible explanation for such co-operation is due an enhanced luteotrophic or antiluteolytic action resulting in improved luteal maintenance in recipients, or enhanced signals to the endometrium involved in the process of implantation (Ishwar and Memon, 1996). However, despite the advantages of twin transfers somes studies have revealed competitive factors between embryos when transfer is performed with embryos of different stages of development. When day 4 and day 8 embryos were transferred to day 6 recipients, the older more developed embryos had an improved chance of survival (Wilmut et al., 1988). This may reflect the fact that the more advanced conceptuses would secrete ovine

trophoblastic protein (OTP-1) earlier, stimulating changes in the uterine environment which may be detrimental to the younger embryo (Asworth, 1992).

2.4.4. Embryo transfer procedures

Sheep embryo transfer is performed usually at 6-7 days after onset of estrus and can be carried out by surgical (mid-ventral laparotomy and laparoscopic) procedures and by indirect or direct transfer method (Rowson and Moor, 1966; Walker et al., 1984; Cappai et al., 1988; Naitana et al., 1992).

2.4.4.1. Surgical techniques

Mid-ventral laparotomy

In embryo transfer the laparotomy technique is similar to the technique described for embryo recovery. Briefly, after surgical toilette recipients are sedated and anesthetized, a ventral midline incision is performed and the reproductive tract is exteriorised to allow visual observation of a corpus luteum, the embryos are transferred into the tip of ipsilateral uterine horn or the oviduct, dependending on the embryonic developmental stage which bear at least one functional corpus luteum.

Laparoscopy

Transfer of embryos to the uterus of a recipient female can be reliably achieved by laparascopy (McKelvey et al., 1985b; McMillan et al., 1994). If a normal corpus luteum is identified by laparoscopy, a small incision is made on the midline of the abdomen, cranial to the udder. The tip of the uterine horn corresponding to the ovary bearing the corpus luteum is pulled out and the embryos are transferred, the uterine horn is allowed to return to the abdomen and the small incisions are closed. This procedure reduces the time taken for each transfer and the conception rate is similar (70 to 75 %) to that obtained using mid-ventral laparotomy (Baril et al., 1993).

2.4.4.2 Embryo transfer methods

Indirect transfer

This method is considered the standard technique and has been widely used for both fresh and vitrified embryos. Briefly, after cryprotectant removal the frozen-thawed embryos are put into a petri and morphological evaluation is performed under microscopic. Soon after a laparatomy or laparascopy is performed and when the reproductive tract is exteriorised a fine TomCat catheter connected to a 1 ml syringe is used to transfer the embryos into the tip of the ipsilateral uterine horn bearing at least one corpus luteum.

According to Dattena et al. (2000a) increased pregnancy rates were observed following transfer of fresh embryos (93.7%) compared to those frozen (70.3%) (p<0.05) while lambing rates (81.2 % vs 75 %) do no show statistical differences between groups, on the other hand, Baril et al.(2001) observed similar results after transfer between fresh and vitrified embryo in terms of pregnancy rate (72% both cases) and the number of lamb born (60% vs 50%) respectively.

Direct transfer

In small ruminants, the use of embryo transfer technique is limited as compared with cattle (Thibier, 2000), probably due to the excessive costs of procedures when compared with the value of the animal, as an alternative the vitrification technique as cryopreservation method in combination with the direct embryo transfer technique offers a real possibility to reduce costs of procedures and increase the use of this techniques in ewes (Baril et al., 2001).

The direct transfer after thawing was first suggested by Heyman and co-workers (1987). Several authors reported that, direct transfer of cryopreserved embryos into recipients immediately after thawing reduces the need for stepwise freezing (time and equipment) without reducing pregnancy rates (Suzuki et al., 1990; Goto et al. 1992; Leibo and Loskutoff, 1993, Voelkel and Hu, 1992; Rall and Wood, 1994; Dinnyès et al., 1996).

In the last years, both techniques (indirect and direct) show to have similar results in terms of pregnancy rates (60-75%) and lambing rates (55-75%) (Baril et al., 2001; Isachenko et al., 2003 b; Dattena et al., 2004).

The advantages of this method are that warming is easily and rapidly achieved and embryo transfer is directly performed after thawing, without the need for optical equipment

(microscope-free) for embryo quality evaluation based in the aspect that only good quality embryos become vitrified. This method of transfer is considered ideal for use in field conditions (Isachenko et al., 2003 b).

2.5 Ultrasonography pregnancy diagnose

The ultrasonography pregnancy diagnose can be considered a very important skill to improve the management after a MOET programme, because with an accurate diagnosis of pregnancy it is possible to avoid the economical losses that means to maintenance non pregnant animals in the farm.

2.5.1 Transabdominal ultrasonography

Transabdominal ultrasonography offers an accurate, non invasive and a practical method of pregnancy diagnosis and determining fetal numbers in sheep. This technique is reliable to determinate pregnancy after day 28-30 of gestation (Blasco and Folch, 1989; Celorrio et al., 1994) while for determining fetal number, the optimal time is between 45-90 day of gestation, after that the fetuses become too large to be consistently differentiated from each other. (White and Russel, 1984; Haibel, 1990). The more commonly probes used in transabdominal ultrasonography are linear and sectorial tranducers with frequencies of 3,5 and 5 MHZ.

2.5.2 Transrectal ultrasonography

Transrectal ultrasonography is a reliable technique for early diagnosis of pregnancy. Is described that allows identification of the conceptus from 19-20 days after mating (Chevalier, 1988; Santiago Moreno et al., 1995), but for an accurate determination of pregnancy and fetal number is better if is used between 25-40 days of gestation. This technique is described to be efficient to observe pregnancy until day 90-91 because the foetus is not easily accessible later. (Gonzalez-Bulnes et al., 1998). The more commonly probes used in transrectal ultrasonography are linear tranducers with frequencies of 5 and 7,5 MHZ.

CHAPTER III

Material and Methods

3.1 First Experiment

" FSH-p superovulatory treatment without progestagen sponges"

3.1.1 Animals

The study took place during breeding season at the laboratory of biotechnologies of reproduccion in DIRPA-AGRIS Sardinia. From a flock of 70 animals. Twenty Sarda ewes in adult age (2-5 years) and healthy conditions were selected and divided into 2 groups: natural oestrus treatment (NT) (n=10) and single sponge (SS) (n=10) (control group). Throughout the experimental period, animals were kept at grazed on natural pastures and water ad libitum with access to indoor facilities.

3.1.2 Synchronisation of oestrus cycles

The group NT was selected throughout oestrus detection (Day 0) by the introduction of a vasectomized ram in the flock twice daily (8:00 am – 16:00 pm). The ewes were considered to be on oestrus when they show oestrus behaviour and were mated by the vasectomized ram.

The group SS was synchronized by insertion of intravaginal sponges containing 40 mg fluorogestone acetate (FGA) (Chronogest ®, Intervet, Boxmeer, Holland) for 12 days. After sponge removal all ewes were kept in pens of 5 animals each and tested to determine the onset of oestrus behaviour by visual observations, twice daily (8:00 am – 16:00 pm) with the introduction of a vasectomized ram.

3.1.3 Visualisation of oestrus behaviour

The visualisation of oestrus behaviour take at least one hour and was performed according to Ortman R. (2000) with some modifications. After the introduction of the vasectomized rams in the flock the presence of following parameters were considered: 1) the ewe-ram seeking activity, 2) active immobilization followed by mating, 3) ewe's tail fanning and 4) movement of the head toward the male. The intensity of oestrus was divided in three categories (Table 1): **high** (when all 4 parameters were present at the observation), **medium** (when at least two of the parameters where observed and one was the parameter n°2) and **low** (when at least parameter n°2 was present).

Table. 1. Intensity of oestrus observed during visualisation of oestrus behaviour

Intensity of oestrus	Parameters observed
High	1, 2 , 3 and 4
Medium	1, 2
Low	2

3.1.4 Superovulation treatment

Superovulatory treatment consisted in 350 I.U. of porcine FSH (Folltropin®, Bioniche Animal Health, Ireland) administered in eight (i.m.) decreasing doses at every 12 h (2 ml x 2, 1.5 ml x 2, 1.0 ml x 2 and 0.5 ml x 2) starting on day 4 after oestrus detection (Day 0) in the NT group and 48 h before sponge removal in the SS group. A single dose of 125 μg (i.m) cloprostenol (Estrotek®, Azienda Terapeutica Italiana, Italy) was injected on day 6 after oestrus detection in the NT group to induce luteolysis. The superovulatory treatment is summarized on figures 10 and 11 for NT and SS groups respectively.
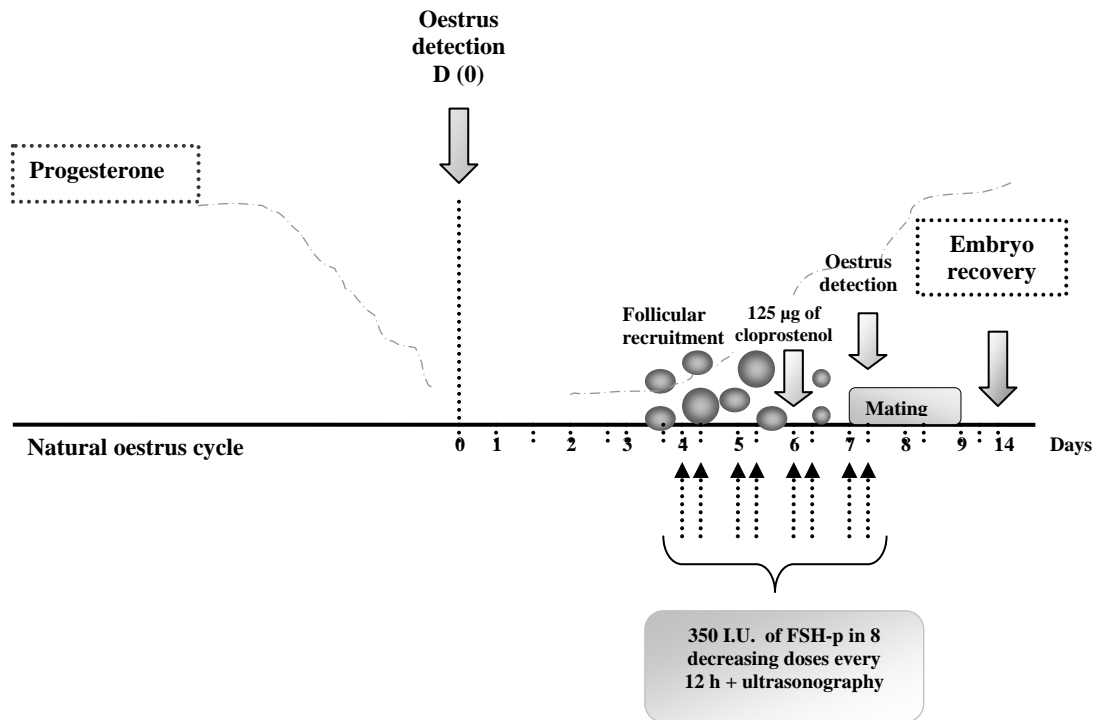
Fig. 10. Schematic representation of superovulatory treatment in ewes during natural oestrus (NT group). The eight FSH-p injection were started at day 4 after oestrus detection (Day 0: day of oestrus detection) and were finished at day 7. At Day 6 a cloprostenol injection was administered to induce luteolysis.
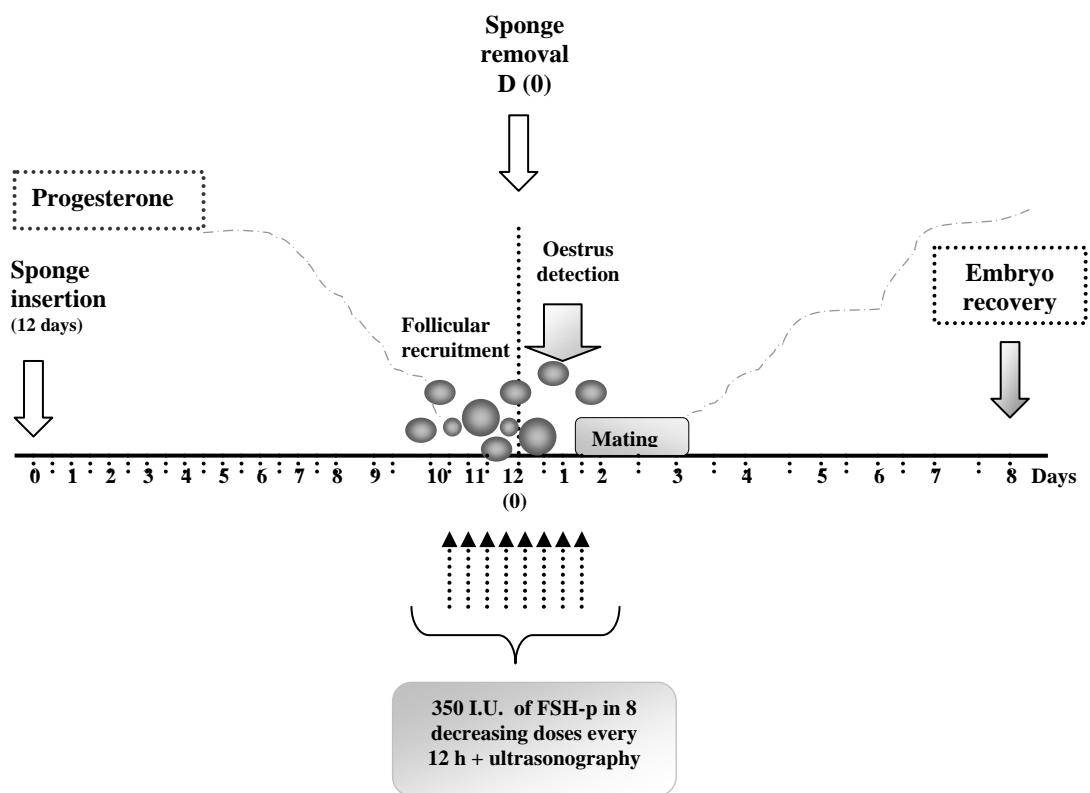
Fig. 11. Schematic representation of superovulatory treatment in progestagen treated ewes (SS group). The FSH-p administration started 48 hours before sponge removal.

3.1.5 Ovary ultrasonography

The follicular dynamics was monitored by transrectal utrasonography using an ALOKA ECHO CAMERA SSD-500 (Japan), with 7.5 MHz linear-array probe designed for examination of the human prostate to follow the reponse of the ovaries during the superovulatory treatments. The observations were carried out twice daily starting at the first FSH dose and continued for all period of FSH treatment in both groups. Briefly, a tilting squeeze chute was used to restrain the ewes in dorsal recumbency, feces were removed digitally before ewes were restrained gel was applied to the transducer to act as a coupling medium between the rectal wall and transducer. Images of the urinary bladder and the uterine horns were used as references points to locate ovaries. In each ultrasonography the largest diameter of each follicle ≥ 2 mm was measured and its position

was recorded for each animal on a diagram of the ovaries (Fasc.1: see apendix) to evaluate its development during successive observations. The last day of ultrasonography the number of expected CL was recorded to predict the ovulation response after treatment avoiding surgery in animal with none or very low (< 3 CL) ovulation rate (Table A: see apendix).

3.1.6 Oestrus detection and mating

Oestrus detection was performed twice daily (8:00 am – 16:00 pm) with the help of vasectomized rams for visualizations of oestrus behaviour from cloprostenol injection or from sponge removal in NT and SS groups respectively. When oestrus was detected donors were mated with fertile rams whichs were replaced by a another one at 12 h intervals until the animals refused mating.

3.1.7 Recovery embryos

Surgical embryo recovery was performed at Day 8 after cloprostenol injection or sponge removal in NT and SS groups respectively. Twenty four hours before surgery donors were deprived of food and water.

At surgery ewes were sedated with acepromazine maleate (Prequillan ®, Fatro, Italy) at total dose of 3 mg/ewe (i.v.) and general anaesthesia was induced with penthotal sodium (Penthotal sodium®, Intervet, Italy) with a dose of 10 mg/kg body weight (i.v.). The abdominal area anterior to the udder was shaved and sprayed with iodine solution and

70% alcohol. An inguinal mini-laparotomy was performed with an incision of 5 – 6 cm and the reproductive tract was exteriorised to assess the number of corpora lutea (CL) (Fig. 12 and 13).

Embryos were recovered by flushing each uterine horn according to the technique of Tervit and Havik (1976) with some modifications (Fig. 14). Briefly, each uterine horn was flushed by the insertion of a needle (19g) attached to a sterile syringe with flushing media (HTCM 199 + BSA 0,4% + HEPARIN) near the utero-tubal junction. A foley catheter (N°10) was inserted in the base of the uterine horns for recovery of the embryos. Each uterine horn was flushed with 20-40 ml flushing media aprox, collected in Petri dishes and examined for the presence of embryos under a stereo microscope. The surgery was performed with minimal manipulations and the time never overcome the 10-15 minutes.

Fig. 12. Inguinal mini-laparotomy.
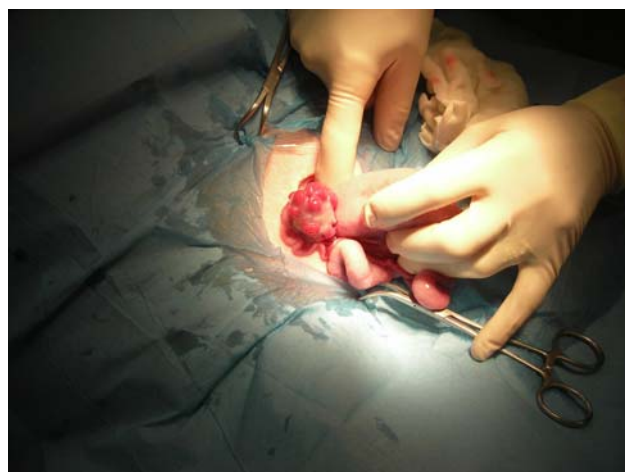
Fig. 13. Exteriorization reproductive tract.

Fig. 14. Flushing of uterine horns.

3.1.8 Embryo classification

The recovered embryos were evaluated morphologically using a stereomicroscope (magnification 80x) (Fig.15) and classified according to the stage of development. Their quality was scored on a scale of 1 to 3 according to the guidelines of the International Embryo Transfer Society. Embryos with a score of 1 were those in the appropriate developmental stage with perfect morphology, and considered the highest quality (Q_1). Only Q_1 embryos were selected for vitrification. Embryos of quality Q_{1-2} were considered for fresh transfer purposes. Embryos Q_3 those with poor quality and major morphological deviations were not considered in the experiment.

Fig. 15. Embryo evaluation.

3.1.9 Storage of embryos

After flushing, embryos were kept less than 30 min at room temperature in HTCM 199 + BSA 0.4% before to be transferred or vitrified.

Vitrification of Blastocysts

The vitrification procedure was based on the method originally designed by Yang et al. (1992) and Vajta et al. (1998) for cow embryos and modified for ovine embryos by Dattena et al. (2004). Briefly, all vitrification solutions were prepared using Dulbecco's PBS supplemented with 0.3 mM sodium pyruvato, 3.3 mM glucose and 20 % FBS. Expanded blastocysts were exposed at room temperature to equilibration solution (V1) 10 % ethylene glycol (EG) and 10% dimethylsulfoxide (DMSO) for 4-5 min, then to the vitrification solution (V2) 20% EG; 20% DMSO and 0.5 M sucrose for $\leq$ 45 sec., then were loaded into open pulled straws (OPS) and inmediately plunged into LN_2 (2 blastocysts per straw) (Fig. 16 and 17).

Fig. 16. Vitrification of embryos with the OPS.

Fig. 17. OPS plunged into LN$_2$

3.1.10 Analysis of data

Incidence and distribution of oestrus

The incidence and distribution of oestrus between NT and SS groups was analized by a Chi Square analysis.

Ovulatory response and Embryo yields

Data on number of corpora lutea (CL), embryos recovered (ER), embryos fertilized (EF), and high quality embryos (EQ$_1$) per ewe were analysed by ANOVA (GLM SAS procedure), whereas, data on recovery (RR), fertility (FR) and embryo quality (Q$_1$R) rates per treatment were analysed by a Chi Square analysis.

The ovulation response wich was divided in two classes: medium ($3 - 10$ CL/Ewe) and high (≥ 11 CL/Ewe) categories and embryo quality rates (Q1, Q2 and Q3) per treatment were analyzed by Fisher's Exact Test.

Follicular data

Data on follicular development at each ultrasonogaphy were recorded and classified as small (2 to 3mm), medium (4mm) and large (≥5mm) follicles.

In order to test the impact of large follicle on the variables of interest a linear model was applied to CL, ER, EF and EQ1 per ewe. The fixed effect included in the analysis were treatment (NT and SS groups) and large follicles (2 classes, presence or absecence) and their interaction. The effect of the presence or abscence of a large follicle on RR, FR and Q1R rates by treatments and between treatments was analysed by Chi Square anlysis.

To assess the effect of the presence or absence of a large follicle at starting FSH treatment on the distribution of low (0-5 CL) and high (6-10 CL) ovulatory response classes a Chi Square analysis was applied in NT and SS groups. Data were considered such as single ovary (1 ovary = 1 data).

When the number of observations (< 5 records) in the experiment did not allow to use the Chi Square analysis the Fisher's Exact test was condidered

The relation between the number of the follicular population at start the FSH treatment and the superovulatory responses obtained was analyzed by the Pearson correlation and linear regression procedures.

3.2 Second Experiment

"Using the open-pulled straw as catheter (OPS-C) to symplify the embryo transfer technique with vitrified embryos"

3.2.1 Animals

The study took place during breeding season at the laboratory of biotechnologies of reproduccion in DIRPA-AGRIS Sardinia. From a flock of 70 animals, 21 Sarda ewes in adult age (2-5 years) and healthy conditions were selected as recipients and divided into 2 groups: direct embryo transfer with OPS-catheter (OPS-C) (n=11) and indirect embryo transfer with TomCat-catheter (TomCat) (n=10) (control group).

3.2.2 Recipient management

Throughout the experimental period the animals were identified with plastic ear tags and kept at grazed on natural pastures intended to maintain constant body weight and drinking water was supplied ad libitum. During the experiment the animals were tested for onset of oestrus twice daily (8:00 am–16:00 pm) with the introducion of vasectomized rams. A ewe was considered to be on oestrus and selected as a recipient when she allows be mated by the vasectomised ram..

3.2.3 Source of embryos

The number of experimental animals was reduced thus only embryos of NT group (Exp. 1) were considered for embryo transfer. Among all recovered embryos (N°72) after flushing of NT group, 52 were vitrified but only 22 were utilised for transfer in the OPS-C group, while, 20 fresh embryos were utilized for transfer in the TomCat group. In both OPS-C and TomCat groups embryos were transferred in pairs into D7 synchronised recipient ewes (D7: day 7 after natural onset of estrus).

3.2.4 Embryo transfer

3.2.4.1 OPS-C with vitrified embryos

Warming of embryos

The OPS-C containing the vitrified embryos were warmed by holding for 6 sec. in air and then dipped into a falcon tube contain HTCM199+ 20% serum+ 0.5 M sucrose in a water bath at 37°C for aprox 15 sec. (Fig. 18).

Fig. 18. Warming of embryos at 37°C in water bath.

In the OPS-C group surgical embryo transfer was performed at day 7 after the onset of natural oestrus was detected. All recipients were deprived of food an water 24 hours before surgery.

At surgery ewes were sedated with acepromazine maleate (Prequillan ®, Fatro, Italy) at total dose of 3 mg/ewe (i.v.) and general anaesthesia was induced with penthotal sodium (Penthotal sodium®, Intervet, Italy) with a dose of 10 mg/kg body weight (i.v.). The abdominal area anterior to the udder was shaved and sprayed with iodine solution and 70% alcohol. An inguinal mini-laparotomy was performed with an incision of 5 – 6 cm and the reproductive tract was exteriorised with minimal manipulations to asses the number and quality of corpora lutea (CL) after this procedure 2 vitrified - thawed embryos per recipient were transferred directly without any morphological evaluation under stereomicroscope into the top of the uterine horn ipsilateral to the ovary showing at least

one functional corpus luteum (CL) using the OPS as the catheter for transplantation and a TomCat[TM] catheter (Kendall Co., Mansfield, MA, USA) was used to connect the straw to a 1 ml syringe (Fig. 19 and 20).
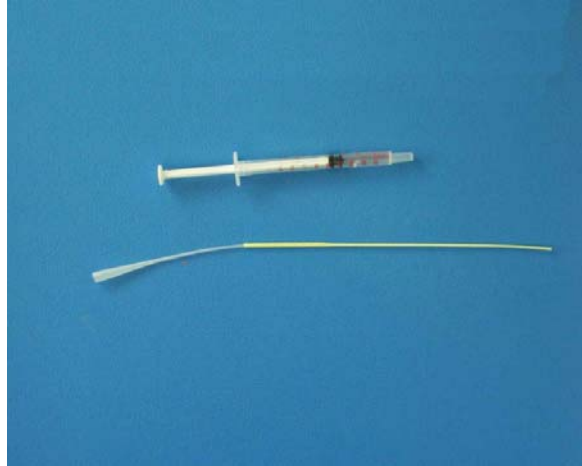
Fig. 19. OPS connected to TomCat[TM] for embryo transfer.

Fig. 20. Direct embryo transfer with OPS- C into the uterine horn.

3.2.4.2 TomCat with fresh embryos.

In the TomCat group surgical embryo transfer was performed at day 7 after the onset of natural oestrus. All recipients were deprived of food an water 24 hours before surgery.

.   At surgery ewes were sedated with acepromazine maleate (Prequillan ®, Fatro, Italy) at total dose of 3 mg/ewe (i.v.) and general anaesthesia was induced with penthotal sodium (Penthotal sodium®, Intervet, Italy) with a dose of 10 mg/kg body weight (i.v.). The abdominal area anterior to the udder was shaved and sprayed with iodine solution and 70% alcohol. As in Experiment 1 an inguinal mini-laparotomy was performed with an incision of 5 − 6 cm the reproductive tract was exteriorised with minimal manipulations to asses the number and quality of corpora lutea (CL) after this procedure 2 fresh embryos (blastocysts) per recipient were transferred into the top of the uterine horn ipsilateral to the ovary showing at least one functional corpus luteum (CL) using a TomCat[TM] catheter (Kendall Co., Mansfield, MA, USA) connected to a 1ml syringe (Fig.21 and 22).

Fig. 21 TomCat[TM] connected to a 1ml syringe for embryo transfer..
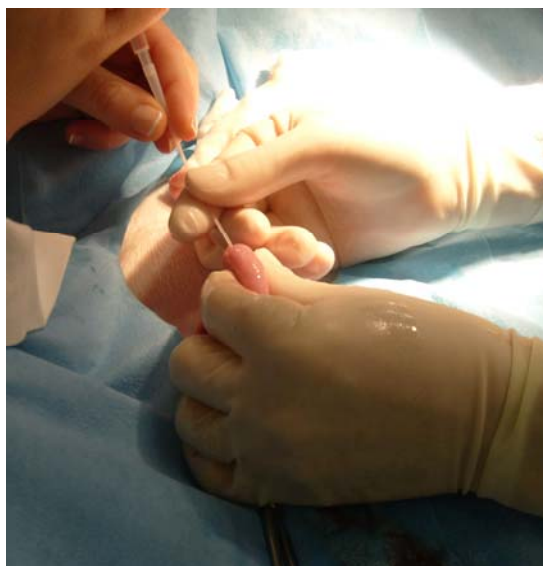
Fig. 22. Indirect embryo transfer with TomCat^{TM} into the uterine horn.

3.2.5 Pregnancy diagnose

Pregnancy diagnose was performed 45 days after transfer by abdominal ultrasonography using a 50S TRINGA ESAOTE (U.S.A.) ultrasound scanner with a 5 MHz transducer .

3.2.6 Lambing

At 146 day of pregnancy ewes were allocated indoor (3 ewes/pen) for monitoring the onset of lambing. The lambs borned were weight and identified within 6 h after birth.

3.2.7 Analysis of data

Comparisions in pregnancy rate, embryo survival rate (Lambs born/embryos transferred), twining rate and birth weight among groups were performed using the Chi Square analysis.

CHAPTER IV

Results

4.1 First Experiment

4.1.1 Incidence and distribution of oestrus.

After NT and SS treatment 100% of the animals were observed on heat starting 24 hours after cloprostenol injection or sponge removal. The 90% of NT group and the 70% of SS group were on heat within 36 h the other respective 10% and 30% coming on heat 12 h later.

Analysis of data did not show any significant effect of treatment on the incidence and distribution of ewes showing oestrus following cloprostenol injection or sponge removal. Fig. 23 shows the incidence and distribution of oestus in NT and SS groups.
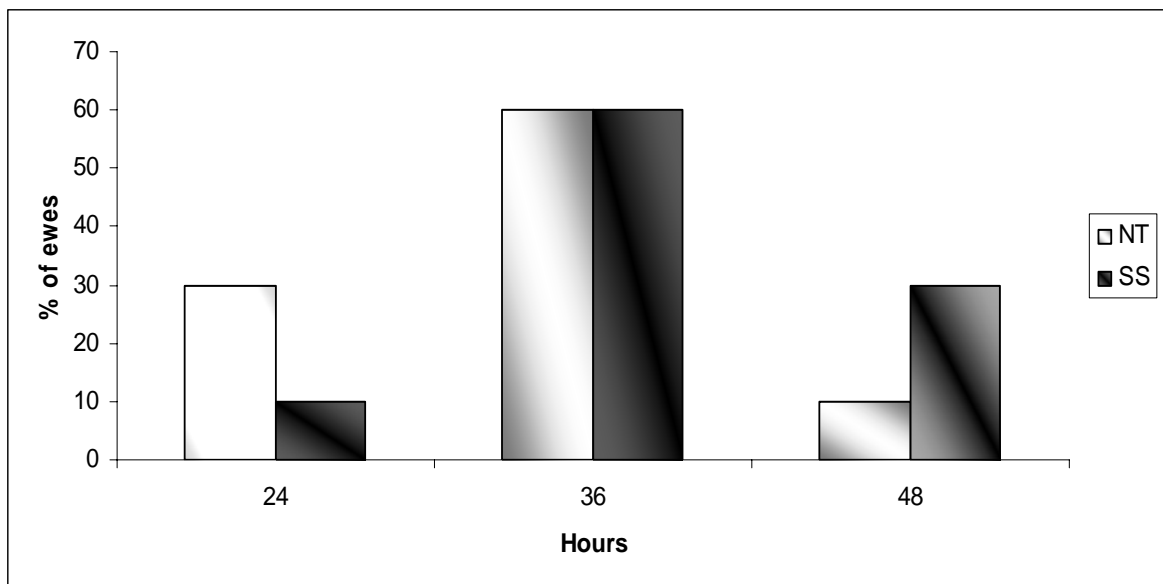
Fig. 23. Effect of the treatment on the incidence and distribution of oestrus in natural oestrus (NT) and single sponge (SS) groups after cloprostenol injection or sponge removal respectively.

4.1.2 Ovulatory response and embryo yields after FSH-p treatment

4.1.2.1 Ovulatory response

All ewes exhibited multiple ovulation ($\geq$ 3 corpora lutea) after FSH-p treatment. Statistical differences in mean number of CL/Ewe (10.7±3.4 vs 7 ±3.2) ($p < 0.05$) were found between NT and SS groups respectively (Table 2).

The percentage of ewes distribuited according ovulatory response classes (medium vs high) are shown in (Fig. 24). Analysis of data did not show any significant effect of treatment.

Table 2. Mean number (± sd) of CL/ewe after FSH-p treatment in natural oestrus (NT) and single sponge (SS) groups.

Group	Days of treatment	N°ewes	% ewes responding ($\geq$ 3 CL/ewe)	Overall N° Cl	N° CL/ewe
NT	14	10	100	107[a]	10.7± 3.4[a]
SS	20	10	100	70[b]	7 ±3.2[b]

Different superscripts indicates treatments with significant differences ($p < 0.05$).
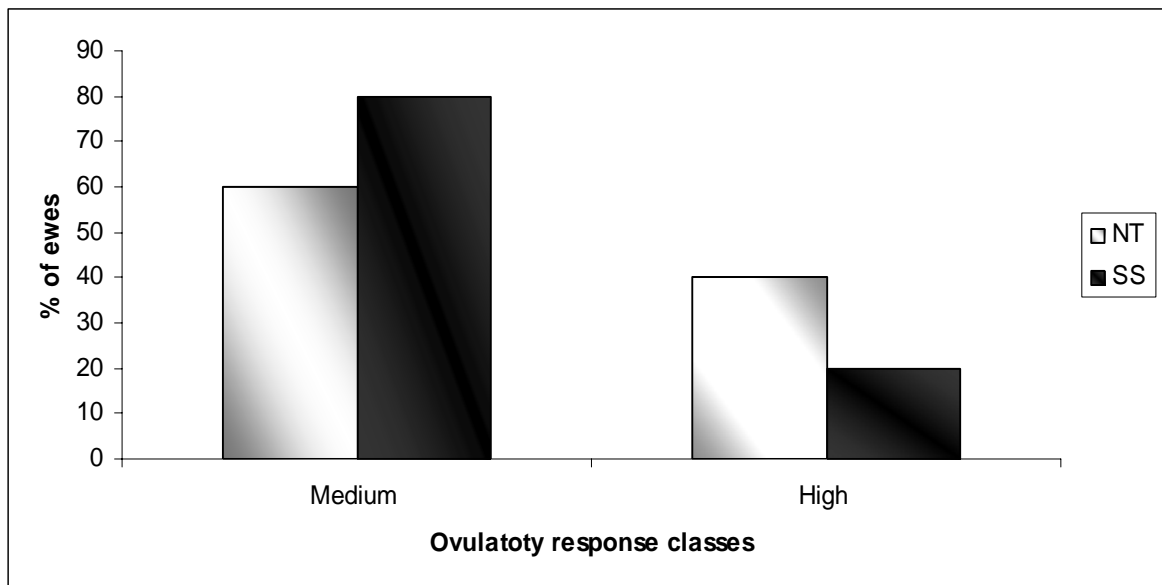
Fig. 24. Effect of the treatment on the number of ewes showing medium (3-10 Cl/Ewe) or high (≥11 Cl/Ewe) ovulatory response classes in natural oestrus (NT) and single sponge (SS) groups.

4.1.2.2 Recovery rate

No statistical differences in either mean number of ER/Ewe (7.2 ± 3.9 vs 5.6 ± 3.2) or RR% (67 vs 80) were found between NT and SS groups respectively (Table 3).

Table 3. Mean number (± sd) of embryos recovered per ewe (ER/ewe) and recovery rate (RR%) after FSH-p treatment in natural oestrus (NT) and single sponge (SS) groups.

Group	Animals	CL total	Total embryos recovered	ER/Ewe	RR%
NT	10	107	72	7.2 ±3.9	67
			NS	NS	NS
SS	10	70	56	5.6± 3.2	80

NS: Not statistical significance

4.1.2.3 Fertility rate

Results did not show statistical differences in mean number of EF/Ewe (7.2 ± 3.9 vs 4.5 ± 3.5) between groups, whereas, statistical differences in FR% (100 vs 80) were found between NT and SS groups respectively (p<0.05) (Table 4).

Table 4. Mean number (± sd) of embryos fertilized per ewe (EF/ewe) and fertility rate (RR%) after FSH-p treatment in natural oestrus (NT) and single sponge (SS) groups.

Group	Animals	Total embryos Recovered	Total embryos fertilized	EF/Ewe	FR%
NT	10	72	72	7.2 ±3.9	100[a]
				NS	
SS	10	56	45	4.5 ± 3.5	80[b]

NS: Not statistical significance.
Different superscripts indicates treatments with significant differences (p<0.05).

4.1.2.4 Quality embryo rate

No statistical differences in either mean number of EQ_1/Ewe (6.2 ±3.8 vs 4 ± 3.0) (Table 5) or in the distribution of Q_1 (86 vs 88), Q_2 (8.3 vs 6.6) and Q_3 (5.5 vs 4.4) embryo quality rates (Fig. 25) were found between NT and SS groups.

Table 5. Mean number (± sd) of high quality embryos per ewe (EQ_1 /Ewe) after FSH-p treatment in natural oestrus (NT) and single sponge (SS) groups.

Group	Animals	Total embryos recovered	Total embryos fertilized	Total embryos Q_1	Mean of EQ_1 /Ewe
NT	10	72	72	62	6.2 ±3.8
				NS	NS
SS	10	56	45	40	4 ± 3.0

NS: Not statistical significance
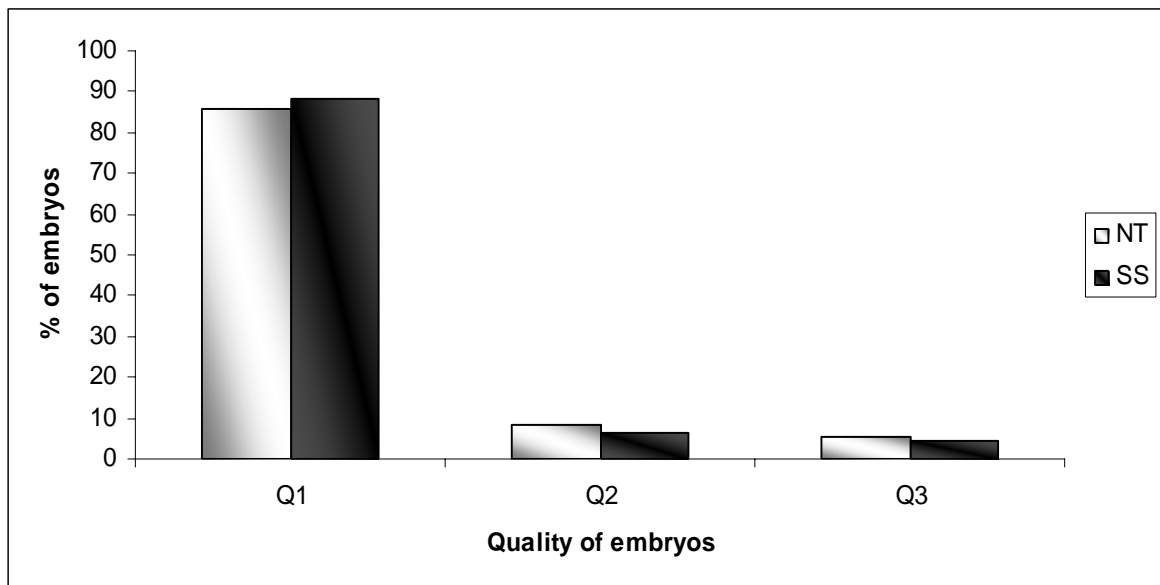
Fig. 25. Effect of the treatment on the distribution of embryo quality rates (Q_1, Q_2 and Q_3) in natural oestrus (NT) and single sponge (SS) groups.

4.1.3 Effect of the presence of a large follicle on ovulatory response and embryo yields.

A total of 6 sheep (60%) in NT group and 5 sheep (50%) in SS group had a large follicle ($\geq$5mm) in diameter at first FSH dose.

Results for CL/Ewe, EF/ewe, EQ_1/Ewe, $Q_1R\%$ and FR% did not show statistical differences between ewes bearing or not a large follicle when data were analized within or between NT and SS groups respectively (Table 6).

Table 6. Ovulatory responses and embryo yields in relation to presence or absence of a large follicle (≥ 5 mm) in diameter when starting FSH-p treatment in natural oestrus (NT) and single sponge (SS) groups.

	Group NT		Group SS		Significance
	Presence large follicle	**Absence large follicle**	**Presence large follicle**	**Absence large follicle**	
Animals	6	4	5	5	NS
CL/Ewe	10.6 ± 4.4	10.7 ± 1.7	7.8 ± 3.8	6.2 ± 2.6	NS
EF/Ewe	7.5 ± 4.8	6.7 ± 2.8	4.2 ± 4.3	4.8 ± 2.9	NS
EQ$_1$/Ewe	6.1 ± 4.3	6.2 ± 3.7	3.6 ± 3.4	4.1 ± 3.1	NS
Q$_1$R (%)	82.2	92.5	85.7	91.6	NS
FR (%)	100	100	77.2	82.7	NS

NS: Not statistical significance.

4.1. 4 Effect of large follicle on distribution of ovulation classes.

A total of 6 ovaries in NT group and 5 ovaries in SS group bear a large follicle at first FSH dose. Results did not show any statistical differences in terms of distribution of ovulatory responses classes (low vs high) in ovaries bearing or not a large follicle in the NT (Fig. 26) and SS (Fig. 27) groups respectively.
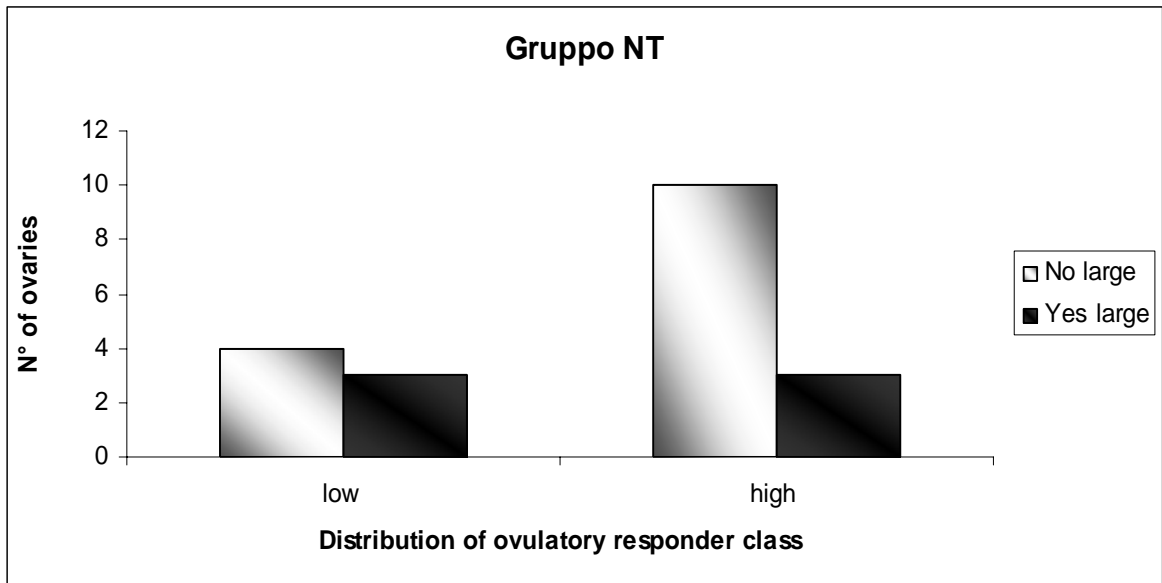
Fig. 26. Effect of the treatment on the distribution of ovulatory response class low (0-5 Cl) vs high (6-10 Cl) in ovaries bearing (yes) or not bearing (no) a large follicle in natural oestrus (NT) group.
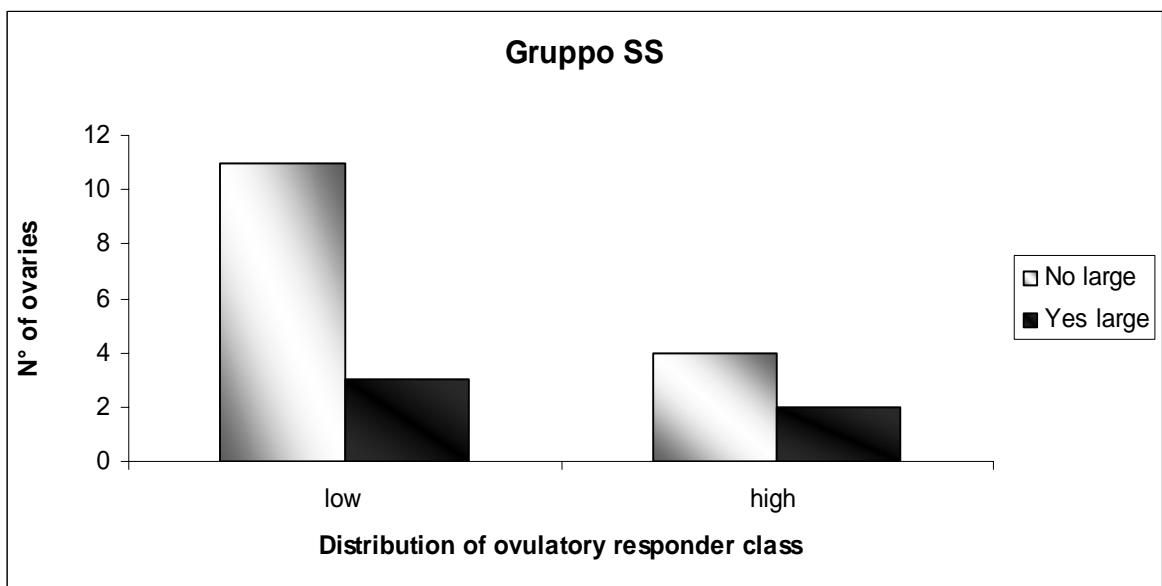
Fig. 27. Effect of the treatment on the distribution of ovulatory response class low (0-5 Cl) vs high (6-10 Cl) in ovaries bearing (yes) or not bearing (no) a large follicle in single sponge (SS) group.

4.1. 5 Effect of number of follicle popolation at the start of FSH treatment on ovulatory responses and embryo yields.

Analysis of possible relationships between the superovulatory responses obtained and the number of follicular population (small, medium and large) at start of FSH treatment did not show any statistical differences.

4.2 Second Experiment

4.2.1 Pregnancy rate

 No statistical differences in pregnancy rates on day 45 (82% vs 70%) were found between OPS-C vitrified and TomCat fresh embryos respectively (Table 7).

4.2.2 Embryo survival rate

No statistical differences in embryo survival rates at lambing (59% vs 60%) were found between OPS-C vitrified and TomCat fresh embryos respectively (Table 7).

4.2.3 Twining rate

No statistical differences in embryo twining rate (44,4% vs 71,4%) were found between OPS-C vitrified and TomCat fresh embryos respectively (Table 7).

4.2.4 Birth weight

No statistical differences in the birth weight (2.8 ± 0.8 vs 2.7 ± 0.4) were found between OPS-C vitrified and TomCat fresh embryos respectively (Table 8).

Table 7. Pregnancy, embryo survival and twining rates after transfer in OPS-C and TomCat groups respectively.

Group	Source of embryos	Recipients	Embryos transferred (n)	Pregnancy rate (day 45)	Embryo survival rate (lambs born/embryos transferred)	Twining rate
OPS-C	vitrified	11	22	82% (9/11)	59% (13/22)	44,4% (4/9)
TomCat	fresh	10	20	70% (7/10)	60% (12/20)	71,4% (5/7)

Table 8. Mean (± sd) of birth weight in OPS-C vitrified and TomCat fresh embryos respectively.

Group	Embryos transferred	Lambs born	Birth weight (kg)
OPS	22	13	2.8 ± 0.8
TomCat	20	12	2.7 ± 0.4

CHAPTER V

Discussion and Conclusion

5.1 First experiment

5.1.1 Incidence and distribution of oestrus

The study reveal that NT and SS treatments resulted in 100% of ewes showing oestrus.

To our knowledge, there are not data available related to superovulation treatment without sponges. Thus, oestrus responses in NT group after cloprostenol injection can only be compared with some studies were a dose of cloprostenol was given in early luteal phase to induce luteolysis during a superovulation protocol (Rubianes et al., 1995; Rubianes et al., 1997), whereas, in the case of SS group results are in agreement with traditionals studies were a high oestrus response was observed in progestagen FSH-p treated animals (Chagas e Silva et al., 2003, D'Alessandro et al., 2005; Bettencourt et al., 2008). Thus, it can be assumed that the use of NT treatment was satisfactory as much as SS treatment to induce oestrus after supeorvulatory treatment.

NT treatment had an earlier occurrence of oestrus, possibly due to the early administration of $PGF_{2\alpha}$ (~ 4 days after ovulation). This findings are in agreement with other studies were in ewes (Rubianes et al., 1995;1997) as in goats (Menchaca and Rubianes, 2002; Menchaca et al., 2007) an early onset of oestrus was observed using $PGF_{2\alpha}$ during superovulatory treatment in early luteal phase (3 days after ovulation). According to Houghton et al. (1995) when PGF2α is given in early luteal phase the interval between oestrus and ovulation is shorter than when is given later in the cycle possibly due to that a longer time is required to reduce progesterone concentrations to basal levels as the luteal phase progress and the CL acquires its full endocrine function.

Moreover, an earlier occurrence of oestrus has been associated with higher ovulation rate (OR). Torres and Cognie (1984) and Torres et al., (1987) indicated that a higher OR corresponded with an oestrus time interval from sponge removal of about only 24 h . This, suggest that animals with high follicular activity result in high ovulation rates reaching the levels of oestrogen that will bring them into oestrus earlier than in animals with lower ovulation rate.

5.1.2 Ovulatory response and embryo yields after FSH-p treatment

Ovulation Rate

MOET efficiency decrease by the presence of females not bearing any or with very low ovulation response after exogenous hormone supply. In the present study results showed that after gonadotrophin treatment the 100% of ewes responded with a multiple ovulation (≥3 CL/ewe). This findings are superior to previous reports, (Bartleski et al., 2008) (67%) and (Chagas et al., 2003) (95%). The FSH dose level given in the present study resulted higher than the others reports mentioned above, possibly leading to a large follicular recruitement whitin the ovary. In fact Tervit (1989) found a significant linear increase in ovulation rate as level of FSH-p or folltropin was increased.

In the NT protocol the total number of corpora lutea (CL) increased statistically ($P<0.05$) compared with the SS protocol. This demonstrate that a good superovulatory response can be elicited in sheep in early luteal phase (Day 4) of the oestrus cycle avoiding the use of intravaginal progesterone devices.

Thus, the increased P4 concentrations have a positive effect on follicular turnover increasing the number of young large follicles with the potential to ovulate. According to Rubianes et al. (1996) and Menchaca and Rubianes (2002), a high progestereone levels during follicular recruitment, affect the dominance of the largest follicle of wave 1, inducing early regression and accelerating the emergence of next follicular wave, which results in the ovulation of heatly young follicles in ewes.

The lowest ovulatory response in the SS protocol may be related to the progesterone serum profile induced by intravaginal devices opposite to that observed during the normal oestrus cycle, when progesterone concentrations are low and then increased until luteolysis. (Menchaca and Rubianes, 2004). Moreover, it has been described that after 6 days (Greyling et al., 1994; Rubianes et al., 1998) of progestagen treatments serum progesterone concentrations decline to subluteal levels and remain low until the device is withdrawn. To overcome this, some authors suggest the use of a second progestagen sponge from day 7 to 14 to maintain constant concentrations of progesterone during treatment (Dingwall t al., 1994; Gonzalez-Bulnes et al., 2002).

In ewes, subluteal progesterone levels promote excessive growth and persistence of the largest follicle (Viñoles et al., 1999) increasing the age of the ovulatory follicles (Johnson et al., 1996), in these conditions it has been reported by Rubianes et al. (1997) that follicles in early atresia can be rescued and stimulated to growth by exogenous

gonadotrophins and their development has been associated with lower ability to ovulate and lower developmental competence of their oocytes (Gonzalez-Bulnes et al., 2005; Berlinguer et al., 2007).

Taking into consideration the effect of treatments on the distribution of ovulatory response classess results did not show any statistical significance between groups. Interesting, NT treatment show better results in terms of high ovulatory response class than SS treatment (40% vs 20%) respectively, possible due to the better conditions for a higher follicular recruitement during the FSH-p treatment in the NT group.

Recovery Rate

In this study, although the recovery rate in the NT group (67%) was somewhat low when compared with SS group (80%), NT treatment increase the number of embryo recovered per ewe (ER/ewe) after FSH-p treatment due to a better fertilization rate than SS treatment.

The success of a embryo recovery in a MOET programme depend on many factors. One of the variables that strongly affect is the expertise revealed by the operator during the flushing of embryos either surgical or by laparoscopy techniques. On the other hand, it has been described in ewes a relation between and increased ovulation rate and lower embryo recovery (Betterige and Moore 1977). This observations are in contrast with thoses reported in ewes by Wright et al., (1981) and in goats by Armstrong and Evans (1983) were it was found that the number of embryos recovered increased progressively with increased ovulation rate after p-FSH treatment.

Moore recently reports by Chagas e Silva et al. (2003) and Veiga-Lopez et al. (2006) described that the lower efficiency of recovery can be associated with a significantly higher number of follicles at time of flushing, this is in agreement with the observations of Jabbour and Evans (1991) who reported that the presence of large follicles with high periovulatory estradiol peak could interfere with ova capture by the fimbria or with transport of ova through the oviduct, moreover these prolonged high estradiol levels have been also associated with a decreased fertility related to abnormal oocyte development (Wherman et al., 1993) with significant decreases in embryo recovery, suggesting alterations in the embryo transport through the reproductive tract (Misra et al., 1998) and with abnormal embryonic development (Breuel et al., 1993).

Fertility rate

It has been reported that, fertilization after superovulation is one of the most important factors which greatly limits the success of embryo transfer technology in sheep (Mutiga and Baker, 1982; Amstrong and Evans, 1983; Hawk et al., 1987).

Analysis of the results show a significant difference in the fertility rate between SS and NT groups respectively. In general, despite the differences observed between treatments these results are similar to other MOET programme reported on the literature (Veiga-Lopez et al., 2005; Bettencourt et al., 2008).

Possible causes related to a decrease in the fertility rate could include the failure of sperm to fertilize ovulated oocytes and/or failure of oocytes to be fertilized. It is reported that, superovulatory treatment results in an impairment of sperm transport to the uterus and oviduct through the cervix (Mutiga and Baker 1982; Evans and Amstrong 1984). Other workers described that the failure of fertilization in superovulated ewes is related to the ovulatory response (Baril et al., 1993;Cognie Y, 1999) according to this Amstrong and Evans (1983) have shown that fertility was markedly reduced in naturally mated superovulated ewes showing higher ovulatory response when compared with those having fewer ovulations, contrary to this findings Torres and Cognie (1984) described that the presence of unfertilized eggs cannot be attribuited to either sperm failure or excessive superovulation since these eggs were found more frequently in ewes with few corpora lutea.

It is well known that, synchronisation with progestagens leads to a lower fertility rates than in untreated animals, possibly due the alterations in the sperm transport and survival in the female tract (Lopez-Sebastian et al., 1997) this alterations caused by progestagens are even increased in superovulatory protocols, when combined with high FSH doses (Gonzalez-Bulnes et al., 2004). On the other hand, it has been described that the use of intravaginal progesterone devices in ewes induce subluteal progesterone levels at the end of treatment promoting an excessive growth and persistence of the largest follicle (Viñoles et al., 1999) increasing the age of the ovulatory follicles (Johnson et al., 1996). In cattle, the ovulation of an aged follicles is followed by low fertility (Savio et al., 1993; Stock and Fortune 1993; Mihm et al., 1994; Austin et al., 1999) apparently because an early resumption of meiosis occurs in the oocyte (Revah and Butler 1996), a similar detrimental effect on the conception rate has been observed in the ewe (Ungerfeld and Rubianes, 1999; Viñoles et al., 2001; Evans A., 2003).

Hawk and Conley (1975) described that, the permanence of anovulatory estrogenic follicles could be associated with fertilization failures and this permanence and estrogenicity of anovulatory follicles might affect fertilization and viability rates of oocytes embryos from follicles that ovulated in response to FSH treatment.

We can concluded that, in this experiment the good response with NT protocol may be related to the fact that the detrimental effects of progestagens treatments on oocytes quality and sperm transport into the genital tract are avoided.

Quality of embryos

In this experiment, NT quality embryo were similar to those in SS group. This findings are very interesting because reveal that the new NT protocol was effective as much as traditional sponge treatment.

Due to the lack of data available related to superovulation treatments without sponges, quality of embryos in NT group can be only compared with results reported by Rubianes et al. (1995;1997) although superovulation treatment was quite different.

The good quality of embryo, can be related to the good response of animals to the use of FSH-p in combination with the endogenous progesterone. It has been suggested by Gonzalez-Bulnes et al. (2002) that, the starting of follicular recruitment on Day 4 during early luteal phase could improve the quality of embryos, due to the fact that , the presence of an early corpora lutea might have a benefical effect on embryo quality since the embryo degeneration rate was increased in sheep without a CL.

On the contrary, progestagen synchronisation treatments affect the embryo quality because, insufficient concentrations of progestagen at the end of the treatment induce a high variability in the onset of oestrus and LH peak between animals in the same group (Gaston-Parry et al., 1988), causing mis-timing between superovulatory treatment and ovulation (Scudamore et al., 1993), ovulatory failures (Kafi and McGowan 1997) and abnormalities in the developmental processes of fertilization and early embryo development (Greve et al., 1995).

Despite the alterations, related to progestagen synchronisation treatments, some factors such as the source of the gonadotrophin preparation, its purity and the protocol of the administration influence the final response. Donalson and Ward, (1985) have shown that LH imbalance may results in disturbance of normal oocyte and follicle maturation and consequently in poor ovum quality and reduced fertilisation rate. Thus, different ratio of

FSH/LH in the gonadotropin preparation could affect the oocyte development and formation of the embryo. High LH content was shown to decrease ovulatory response (Torres et al., 1987;Chupin et al., 1987). However excessively low amounts of LH at the end of treatment decrease the ovulatory response (Chupin et al., 1987), thus a better control of the level of LH might therefore yield a higher incidence of good quality embryos.

The use of FSH-p wich had a constant by weight (5%) quantity of LH (Tervit, 1989) could contribute to a good quality embryo in the two groups of our experiment.

5.1.3 Effect of the presence of a large follicle on superovulatory responses and embryo yields.

The present study reveal that, among all animals treated more than 50% had a large follicle at starting of FSH-p treatment. Analysis of data failed to find any possible effect of the presence of a large follicle on superovulatory responses and embryo yields. Results are in agreement with Driancourt et al. (1991) who reported no differences between animals bearing o not a large follicle on superovulatory response to eCG. In contrast some authors described that the presence of a large growing follicle at the onset of gonadotropin treatment increase variability response to superovulatory trials in cattle (Guilbaut et al., 1991) and sheep (Rubianes et al., 1995; Lopez-Sebastian et al., 1999; Gonzalez-Bulnes et al., 2000;2004).

In the NT protocol superovulatory reponses were similar between animals bearing or not a large follicle at start of FSH treatment suggesting that the administration of FSH at Day 4 of the oestrus cycle contemporary to the presence of a young corpora lutea promotes good results despite the presence of a large follicle. This observations are in agreement with Campbel et al. (1995; 1998) and Adams (1999) which suggested that, there is a lack of follicular dominance when the FSH treatment is applied in presence of a young corpora lutea because LH concentration decrease and large follicles become critically dependent on FSH without establishing dominance. This effect, induced by LH reduction, explains the suppressive effects of endogenous progesterone from the corpus luteum on dominant follicles in sheep (Adams 1999) and goats (Menchaca and Rubianes et al., 2002).

On the other hand, Hunter and Southee (1987) hypothised that progesterone exposure during FSH treatment reduce the capacity of the follicle to secrete estradiol by directly inhibition of the aromatase enzyme complex, as was demonstrated in anoestrus ewes.

The quality of embryos in ewes not bearing a large follicle in the NT group was increased when compared to ewes bearing the large follicle, this effect can be related to an increase of the rate of embryo degeneration in sheep with large follicle, since fertilization rate was not affected.

In summary, the good results of the NT group may be related to the hormonal condition of the early luteal phase capable   to sustain full growth and maturation of follicles an oocytes.

In the SS group ovulation rates were similar between animals bearing or not a large follicle. Similar results are reported in the paper of Gonzalez-Bulnes et al. (2000). In fact Gonzalez-Bulnes et al. (2004) suggested that,   in sheep the preovulatory follicle inhibits the growth of other follicles present in the ovaries at the time of emergence while the appearance of new growing follicles is decreased, but not inhibited.

Although ovulation rate is not affected in the SS group, both number of high quality embryos per ewe and quality rate are decreased by the presence of a dominant follicle. An interpretation of this observation is that, when a exogenous FSH treatment is administered the selection of large presumptive follicle process is not yet complete and subordinate follicle were still able to be rescued. However, a post selection treatment appears to be associated with rescue of static or regressing subordinate follicles. Apparent regrowth of these follicles have been associated with lower ability to ovulate, subnormal luteal gland formation, and lower developmental competence of the oocytes (Rubianes et al., 1997).

Staigmiller et al. (1995) suggest that, size alone is not a sufficient criteria for establishing a large follicle as dominant. Moreover, Veiga-Lopez et al. (2006) found increased ovulation rates in ewes bearing a large follicle in static or decreasing phases in comparision with thoses bearing a large follicle in growing phase. These observations suggest that, FSH treatment in presence of large follicles in decreasing phase (ie: in an interwave period with absence of dominant effects) would provide better conditions for recruitment and development of follicles (Rubianes te al., 1997) and later ovulation (Bo et al., 1995; Rubianes et al., 1997, Rubianes and Menchaca 2003).

Gonzalez-Bulnes et al. (2005) and   Berlinguer et al. (2007) reported that, superovulatory yields would be increased by starting the treatment during the presence of a corpus luteum either in natural oestrus or during the progestagen treatments when LH concentration is low.

On the other hand, Rubianes et al., (1997) and Menchaca et al. (2002;2007) describe that, when FSH treatment is initiated soon after ovulation (day D0) there is a better response in terms of increased follicle recruitment, ovulation rate, embryo quality and the number of embryos recovered compared to treatment initiated on Day 3, possibly due to the presence of a homogenous cohort of growing small follicles (in the absence of a large dominant follicle) promoting better follicular recruitment. Thus, in our NT protocol it could be interesting to considered the possibility of advance the FSH treatment from Day 4 to Day 2 after oestrus detection (Day 0) to avoid the presence of a large (presumptive dominant) follicle.

5.1.4 Effect of the presence of a large follicle on distribution of ovulation classes.

It has been widely described that the presence of a large follicle in the ovary surface at the time of gonadotrophin treatment can affect the ovulatory response. Cahill and Fry (1986) suggest that factors in the follicular fluid can have a direct effect on the ovary and therefore modulate the gonadotrophin effects, leading to the possibility that local factors may play an important role in determining the ovulation rate.

In any of the groups of this study the response to FSH-p supply on the ovary ipsilateral or controlateral to the large follicle did not reveal any statistical differences in terms of distribution of ovulatory response class (low vs high). This findings are in agreement with Driancourt et al. (1991) who reported that, no effect of a large follicle on response to eCG was observed when in vivo studies were performed, suggesting that the dominance is essentially passive in ewes and can easily be overcome by raising gonadotrophin concentration.

On the other hand, Rubianes et al. (1995) observed that, although better superovulatory responses were observed in ewes without large follicles on the ovarian surface at the time of eCG treatment, results did not show differences in the individual ovulatory response when comparing ovaries ipsilateral or contralateral to the large follicle in a same animal suggesting that, if there is a inhibition of superovulatory response the effect is fundamentally systemic, exerted through a systemic pathway by means of follicular factors such as inhibin and/ or estrogens (Adams et al., 1992). These observations are in agreement with results reported by Castonguay et al. (1990) were no active inhibitory paracrine role of the dominant follicle was found in sheep.

5.1.5 Effect of number of follicle population at the start of FSH-p treatment on ovulatory responses and embryo yields.

Some authors described that, the ovarian follicular status at the start of a superovulatory treatment in sheep influences the ovarian response to FSH in terms of ovulation rate and embryo out put showing a positive correlation with the number of small follicles (2-3mm) at 1st FSH dose (Brebion and Cognie 1989, Gonzalez-Bulnes et al., 2002;2004; Veiga-Lopez et al., 2005).

In this experiment, in any of the groups, analysis of possible relationships between the responses to the superovulation treatment and the follicular population at start of FSH regime did not show any statistical correlation and are in agreement with those reported by Bartlewski et al. (2008).

It would appear that, the lack of correlation between number of small follicles and ovulatory responses is related to the fact that only a proportion of small antral follicles are able to acquire gonadotropin receptors and thus utilize exogenous FSH for further growth culminating in ovulation (Scaramuzzi et al., 1993).

The reasons for discrepance between results in this experiment and with the previous reports are difficult to explain, but among all, genetic factors may be related with the variability in the superovulatory response to the gonadotrophin treatments. Indeed , some authors suggested the existence of a breed-related variability and provides evidences supporting that breed differences in superovulatory response would be mainly explained by breed differences in follicular dynamics in response to exogenous FSH rather than to any differences in the dynamics of FSH absorption and clereance. Such differences might be related to a higher expression, or a higher sensitivity to FSH receptors in the ovary (Driancourt et al., 1986; Dufour et al., 2000).

Ammoun et al. (2006) described that, the number of small follicles were correlated with the corpora lutea at 1st FSH dose, while, with the number of embryos recovered at the 2nd FSH. In addition, it has been observed that, although any correlation between ovulatory responses and small follicles at start FSH treatment was founded, the number of luteal structures and viable embryos were correlated with the number of sized antral follicles 12h after the 1st FSH dose (Bartlewski et al., 2008).

Taking into consideration the information mentioned above and the fact that in the present study the analysis between superovulatory responses and follicular population was performed only at the 1st FSH dose, it is important to note that, possible further studies are necessary to find any correlation between follicular population and ovulatory responses,

perhaps involving analysis of follicular population growing in response during all FSH treatment.

5.2 Second experiment

5.2.1 Pregnancy rate

In the present study, pregnancy rates after transfer of vitrified (82%) and fresh (70%) embryos did not show statistical differences. This findings are in agreement with those reported by Baril et al. (2001) and Papadopoulos et al. (2002) although the transfer technique performed was quite different.

It has been described that, among all factors affecting pregnancy rates, the quality of embryos transferred plays a fundamental role. (Wright, 1981; Kajihara et al., 1992; Agca et al., 1998). Thus, in this study the good pregnancy rate with vitrified embryos could be related to the embryos high quality (Q_1) selected before vitrification. In the fresh embryos group the lower pregnancy rate might be related to the slight lower embryo quality (Q_{1-2}) often choose for the transfer.

This is in agreement with the consideration of Baril et al. (2001) who reported that, the direct transfer of vitrified embryos could represent a potential gain of 7 to 8% in terms of lambs born due to, about a 15 to 20% of the embryos are discharded at morphological examination, leading to the elimination of viable embryos.

It has been suggested that, among the limits of OPS technology the direct contact between the medium containing the embryos and the non sterile liquid nitrogen could be a problem. Indeed, there has been much discussion about the need to avoid contact between embryos and liquid nitrogen thus, the sterile application of the OPS has been proposed (Vajta el al., 1998b). However, Dattena et al. (2004) reported that in their experience direct transfer of embryos vitrified in OPS into non-sterile liquid nitrogen has not been a problem.

The results in this experiment demonstred that, vitrification of embryos and the direct transfer with the OPS- catheter is possible avoiding the use of microscope and expertise technicians. Thus, with this technique we decrease the cost of procedures and increase the use of embryo transfer in field conditions.

5.2.2 Embryo survival rate

In this experiment results were similar in terms of embryo survival rates following transfer of vitrified and fresh embryos in the OPS-C and TomCat groups respectively. This findings are in agreement with results reported by other authors in similar experimental conditions (Mermillod et al., 1999; Baril et al., 2001; Papadopoulos et al., 2002; Isachenko et al., 2003; Dattena et al., 2004).

Despite the similar embryo survival rates, recipients bearing vitrified embryos tended to keep pregnancy less than recipients bearing those fresh. This observation is in agreement with Mermillond et al., 1997 and Baril et al. (2001) and explained by fact that, freezing can results in the destruction of some cells of the embryos reducing their ability to develop normally (Dattena et al., 2000a; 2004) .

The good results obtained in this study, confirm the suitability of OPS-C together with vitrification technique to be applied in embryo transfer programmes without affect embryo survival in comparison to more complex traditional procedures. This findings are very interesting, because a quick and simple technique would be of particular value to widespread the use of embryo transfer in sheep especially in difficult farm conditions were geographical and economical aspects can limit ready access to technical expertises and facilities.

5.2.3 Twining rate

The twining rate of recipients bearing vitrified embryos (44.4%) was lower compared with recipients bearing those fresh (71.4 %). However, it was not statistically different, possibly due to, the limited number of observations. Data are similar to those reported by Papadopoulos et al. (2002) although experimental conditions were quite different.

Moore and Shelton (1964) described that, embryo survival after transfer is highly related with the degree of synchronization between donors and recipients. According to Amostrong et al. (1983) and Tervit et al. (1986) improved embryo survival rates were observed after twin transfer suggesting that, there is some type of synergism between embryos in influencing each other's survival upon transfer. Possible explanation for such co-operation is due an enhanced luteotrophic or antiluteolytic action resulting in improved luteal maintenance in recipients, or enhanced signals to the endometrium involved in the process of implantation (Ishwar and Memon, 1996).

However, despite the advantages of twin transfers somes studies have revealed competitive factors between embryos in different stages of development the older embryo had an improved chance of survival (Wilmut et al., 1988). This may reflect the fact that the more advanced conceptuses would secrete ovine trophoblastic protein (OTP-1) earlier, stimulating changes in the uterine environment which may be detrimental to the younger embryo (Asworth, 1992).

It has been described that, when the vitrified/thawed embryos are transferred they needs time to fully restore the biological metabolism and repair the structural damage due to cryopreservation procedures (Dobrinsky, 1996; Gardner et al., 1996; Kaidi et al., 2001). In this contex, Naitana et al. (1995) reported that after thawing, vitrified embryos needed 16- 24 h to re-expand the blastocoelic cavity, while, 29-35 hr to reacquire its full capacity of protein secretion (Leoni et al., 2003). Thus, this delay in restoring cavity can affect the embryo survival by altering the degree of synchronisation between embryo and recipient promoting a detrimental uterine environment . Therfore, it could be suggested that, differences on twinnig rates in the current experiment between fresh and vitrified embryos are related to the interval time needed by the cryopreserved embryos to acquire morphology and physiological functions like fresh blastocysts .

5.2.4 Birth weight

It has been describe that, maternal administration of progesterone in very early stages of pregnancy (Kleeman et al., 1994), nuclear cloning of embryos (Willadsen et al., 1991), in vitro culture (Walker et al., 1992; Holm et al., 1994) and vitrification procedures (Naitana et al., 1995; Leoni et al., 2003) in domestic animal may result in fetuses that are heavier at birth than natural breeding. For this reason, it was of our interesting to assess a possible effect of vitrification on the birth weight at lambing.

Analysis of the data failed to find any possible effect of vitrification on the birth weights. Similar considerations have been reported by other workers after transfer of in vivo derived (Papadopoulos et al., 2002) and in vitro produced embryos (Hollinshead et al., 2004; Morton et al., 2004; Dattena et al., 2007).

Conclusions

The study reveal that the use of the spontaneous oestrus cycles is an efficient natural tool to simplify some steps in a MOET programme.

The use of OPS-catheter in combination with vitrification technique for embryo transfer demonstred to be efficient as much as more traditional techniques. The transfer can be performed without the use of microscope and costly specific expertise are avoided.

The inguinal mini-laparotomy surgery proved to be effective as well as other more conventional techniques, making possible to use the same animal more than one time because surgery can be performed by both inguinal sides. It is likely that this technique will play a substantial role in adoption of MOET programme by the sheep industry.

The main passages involved in the simplification of this MOET programme are related to decrease the use of hormones, length of treatments and cost of procedures. All together these factors open a new alternative to widespread the use of these technologies.

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Fascimil 1. Diagram of ovaries during a FSH-p treatment

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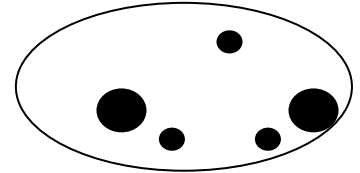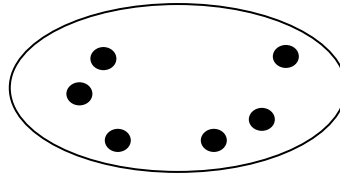
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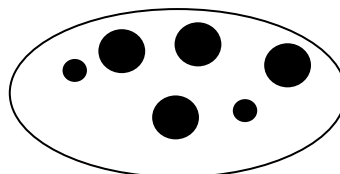N°FSH: 2
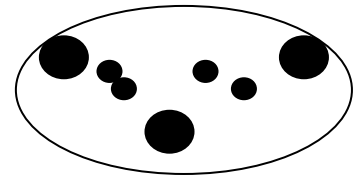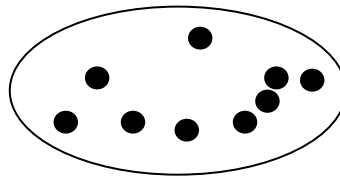
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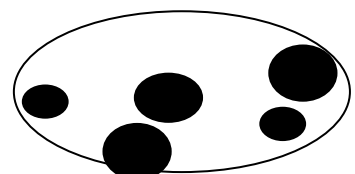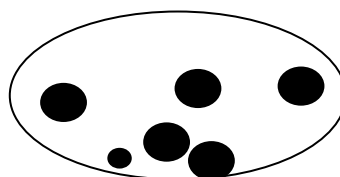N°FSH: 3

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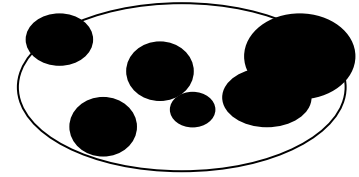DATA: 13/09/07
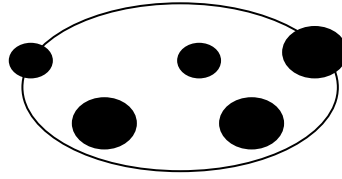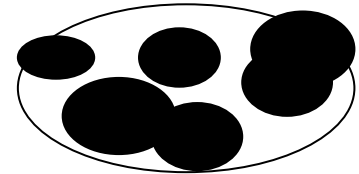TIME: 20:00 pm
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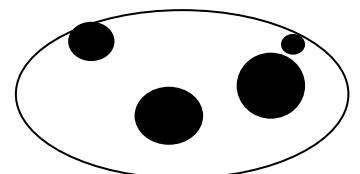
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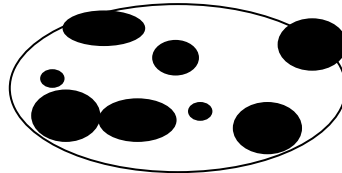DATA: 14/09/07
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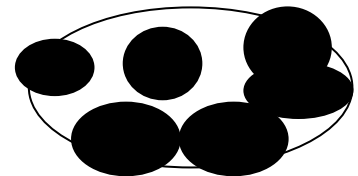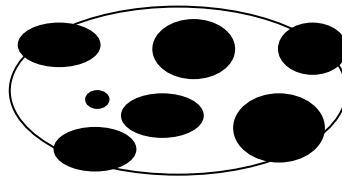DATA: 15/09/07
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Table A. Number of expected and effective corpora lutea recorded at last ultrasonography (CL*) and at surgery (CL**) respectively.

Treatment	Animals	Cl *	Cl **
NN	10	122	107
SS	10	77	70

Characteristics of Sarda sheep

According to Owen, (1976) and Mason, (1988) the Sarda sheep like the majority mediterranean breeds, is probably originated from the large asian stock and its first illustration goes back to the Proto-Sardic art of 2000 B.C. (Bonelli, 1961).

Originally was a small-sized animal with rams and ewes both horned and with a low milk yield in comparison with the current levels, this breed was divided into three varieties: The small mountain variety, the medium sized hill variety and the large lowland variety (Passino, 1936; Bonelli, 1950; Molina et al., 1991). The small variety probably corresponded to the original type and has been identified with the typical migratory sheep of the eastern highlands of Sardinia.

Today, the whole breed can be considered of medium size with a range of highness about 60-80 cm and live-weight ranging from 60-80 kg for males and from 40- 55kg for females. It has a light, polled head (sometimes vestigial horns are present in males) with rectilinear profile and medium-sized horizontal ears, an extended trunk, deep thorax, straight back and broad abdomen.

Its udder is well-developed with a large cistern and nipples of medium size (16mm in diameter and 27 mm in length) implanted at quite a high level (60°-70°), globular with great sinuses (Casu et al., 1983;1989, Carta et al., 1999). Their milk yield reach levels of 130 lt in 100 days for the primiparus and 180 lt in 180 days for the mature ewes. It is a typical coarse–wooled breed showing a white (black spots appear sometimes) open fleece of mixed wool and hair with pointed staples stretching half way down the foreleg and a little further up the hock (Molina et al., 1991).

Among all breeds of the national sheep stock Sarda sheep represents the 41% with about 4.3 millon heads, more than 3.1 millon in Sardinia and the rest distribuited on the mailand, especially in central Italy (Latium, Tuscany, Umbria and Emilia-Romagna). In the past, some large stocks were exported to North Africa an small stocks of some hundreds heads were exported to southern Europe and Israel (Mason, 1967).

The natural mating is the usual way of reproduction in the commercial flocks, while, artificial insemination system is more limited. The reproductive cycle is characterized by an out-of season mating period: between may and july for mature ewes and early autumn (September-October) for the yearlings of 8-10 months of age (28-32 kg of live- weight). Thus, lambing occur from October-December for the mature ewes and at

the end of winter- early spring (February-April) for the primiparous at 14-15 months of age (35 kg live-weight)

The annual fertility rate with natural mating is between 87-93% for mature ewes and 75% for primiparus with large differences between flocks and years. The average prolificacy rate is of 1.5 in mature ewes and 1.2 in primiparous (ARAS, 1997).