# CHAPTER 2

# 2.0 LITERATURE REVIEW

## 2.1 BACKGROUND

The introduction of multiple ovulation and embryo transfer (MOET) techniques together with embryo cryopreservation methods in the early 1980s provided a constructive tool for the livestock management to expedite the propagation of breeding stock with high genetic merit for desirable traits. In addition, the programme also increased the potential in the marketing of the frozen embryos internationally for the purposes of livestock upgrading and conservation of endangered species. MOET is now being in practice with millions of embryos transported all over the world (Thibier and Guerin, 2000). The advantages of MOET in sheep and goats are to generate progenies from genetically superior females and males and to facilitate their proliferation by shortening of the generation interval (Wuliji *et al.,* 1995; Morand-Fehr and Boyazoglu, 1999).

Goat MOET programme involves synchronisation of oestrus in both the donors and recipients by using progestagen treatment, followed by the administration of exogenous gonadotrophins for stimulation of the follicles growth (superovulation) at the end of the progestagen treatment of the donors (Ishwar and Menon, 1996). The embryos are then recovered surgically, generally from the uterine horns of the reproductive track on days 6 to 8 (Pendleton *et al*., 1992,1998; Corderio *et al*., 2003) or from ovi-duct on days 2 to 5 (Amstrong and Evans, 1983; Freitas, Baril and Saumande, 2003) following progestagen withdrawal. Although this is rare, non-surgical embryo collection has also being performed in goats, (Bondurant *et al*., 1984; Pereira *et al*., 1998; Suyadi *et al*., 2000). The recovered embryos can either be cryopreserved (Suyadi *et al.,* 2000), transferred as fresh (Cognie, 1999) or manipulated or frozen before transferred to

recipient does (Nowshari *et al.,* 1993; Sźell, MacLeod, Windsor and Kelly, 1994; Oppenheim *et al*., 2000)

The variation in superovulation response as well as the fact that the superovulated donors fail to produce viable transferable embryos for consistent production remained a major restraining factor to the routine application of MOET in small ruminants (Bari *et al*., 2003). Intrinsic and extrinsic factors are been identified as the contributing to the variation in embryo production. Manipulation and origin of embryos are also among the factors that affect the viability and survivability of the embryos after transferred (Heyman, 1985; Wells *et al*., 1990; Cognie *et al*., 2003). This review covered the goat follicular and reproductive hormonal dynamics during natural oestrous cycle and protocols for induction of superovulation in small ruminants, with special focus on the extrinsic and intrinsic factors that affect the effectiveness of a MOET programme as well as to describe approaches that reduce the variability in superovulatory responses in sheep and goats.

## 2.2 GOATS FOLLICULAR AND REPRODUCTIVE HORMONAL DYNAMICS DURING NATURAL OESTROUS CYCLE

### 2.2.1 Ovarian Follicular Dynamics Patterns

The whole process of the ovarian follicular development or folliculogenesis from primordial to preovulatory follicle in goats started from early age of the females. The ovarian folliculogenesis does not differ from the spermatogenesis. Once gametogonia enter the gonadal ridge as somatic cells, development proceeds and the gametogonia turn into oogonia, which become fully surrounded by a layer of cells (pre-granulosa cells). Oogonia multiply by dividing mitotically; this proliferation ends when the oogonia enter meiosis that are now called primary oocytes and stop dividing. At this stage, the cells enter a prolonged ‘resting phase’. Total number of gametes was

established at this time. Only one functional oocyte is produced for each primary oocyte that undergoes meiosis. The other two or three cells form structure called polar bodies that have no function, and eventually deteriorate. The primary oocyte turns into a secondary oocyte in mature ovarian follicles. The egg is arrested in the secondary stage of meiosis until fertilisation. Upon fertilisation by sperm, the secondary oocyte continues the second part of meiosis and becomes a zygote.

In most goats, recent ultrasonographic studies shown that ovarian follicular development and growth emerge in four follicular waves in the oestrous cycle and suggested that follicular dominance occurred in first and last waves (Ginther and Kot, 1994; de Castro *et al*., 1998, 1999; Gonzalez-Bulnes *et al*., 1999; Padilla, Sohnrey and Holtz, 2005). A follicular wave is characterised by the synchronised emergence of a group of small antral follicles from which one or two follicles will be selected and grow to become a dominant follicle of >5 mm in diameter and eventually ovulate, while the other follicles will regress. When a dominant follicle arises, the other ovarian follicles regress and during this dominance period no new follicular recruitment occurs. The number of waves within a goats oestrous cycle ranges from 2 to 6. Most goats with a normal oestrous cycle length ranging between 19 and 22 days, exhibit 4 follicular waves (Ginther and Kot, 1994; de Castro *et al*., 1998, 1999; Schwarz and Wierzchos, 2000; Menchaca and Rubianes, 2002; Evans, 2003; Cruz *et al*., 2005; Simões *et al*., 2005). The emergence of follicular waves seems to differ among females depending on the length of the oestrous cycle. However, it could be divided as waves 1, 2, 3, and 4 (ovulatory wave), occurring approximately on days 0-1, 4-7, 8-11, and 13-17 post-ovulation, respectively (Ginther and Kot, 1994; de Casto *et al*., 1999; Rubianes and Menchaca, 2003; Simões *et al*., 2005).

There are several significant characteristics of the goat follicular waves, e.g. the differences in diameter of the large follicle between the waves. The diameter of the dominant follicle in wave 1 and the ovulatory wave in goats is usually larger than the diameter of follicles in wave 2 and 3. The maximum diameter of the dominant follicle in wave 1 was reported to be 8.7±0.3 mm, while a mean of 9.7±0.3 mm in the ovulatory wave 4. The mean diameter of a dominant follicle in waves 1 and 3 at averaged 7.2 ± 0.2 mm and 7.3 ± 0.2 mm, respectively. The diameter of a dominant follicle in the ovulatory wave is usually the largest among other large follicle in the unovulatory follicular waves. This could suggest that follicular dominance in goats could also be more during wave 1 and the ovulatory wave, compared to the middle follicular waves (Ginther and Kot, 1994; de Castro *et al*., 1999; Menchaca *et al*., 2002). Frequently, two or more follicles attain 5 mm or more in diameter per wave (Ginther and Kot, 1994; Schwarz and Wierzchos, 2000). The diameter growth rate of dominant follicles between the day of emergence and the day of maximum diameter is around 1mm per day (Ginther and Kot, 1994; Gonzalez-Bulnes *et al*., 1999a,b; Schwarz *et al*., 2000). Follicular emergence increases as the luteal phase progresses, and the inter-wave intervals are shorter than during the early luteal phase (Ginther and Kot *et al*., 1994; de Castro *et al*., 1999). The follicles that do not grow beyond 4 mm during the mid-late luteal phase often are not part of the present follicle-wave, but they represent part of a dynamic primary follicles pool (Ginther and Kot, 1994; de Castro *et al*., 1999;). The majority of the ovulatory follicles are the largest follicles on the day of luteolysis, (Ginther and Kot, 1994; de Castro *et al*., 1999). In double ovulation, the ovulatory follicles emerged as part of the same follicular wave but also possible as a part of different waves and the double ovulation occurs on the same day in most cycles (Ginther and Kot, 1994). Within pairs of ovaries, the onset of last wave occurred later and was less variable in ovulatory ovaries than in anovulatory ovaries. The length of the

growing phase was longer in the last waves of ovulatory ovaries than in the last waves of anovulatory ovaries (Simões *et al*., 2005). The CL were first appeared around day 3 after the day of ovulation (day 0), reached their maximum size (12.5±1.6 mm) on day 11 and regressed two days before the following ovulation (Simões *et al*., 2007).

### 2.2.2 Endocrine Functionality During Follicular Wave

In goats, blood reproductive hormone concentrations observed to be fluctuating during the oestrous cycle. These fluctuations correlated with the emergence of follicular waves. The emergence of a follicular wave in the doe is usually preceeded by a rise in the circulating FSH concentration. This FSH concentration declines as the large follicle increases in diameter (Adams *et al*., 1992; Ginther and Kot, 1995; Leyva-ocariz *et al*., 1995; Souza *et al*., 1998; Bartlewski *et al*., 1999; Schwarz *et al*., 2000; Diskin *et al*., 2002; Evans *et al*., 2003; Rubianes and Menchaca, 2003). One or two FSH peaks have been observed in goats during an oestrus cycle. Generally, FSH peak occurred during the time of estrous in goat that a showed one FSH peaks, and this increases in FSH level is normally associated with a pre-ovulatory LH surge (Leyva-ocariz *et al*., 1995; Schwarz and Wierzchos, 2000; Medan *et al*., 2003). The LH and FSH surges during pre-ovulatory period induce the final stages of oocyte maturation to metaphase II. The pre-ovulatory surges of LH and FSH, last for 6 to 12 hours, are responsible to ovulation. In the case of two FSH fluctuations were observed, one peak was still associated with the pre-ovulatory surge while, the other peak was reported to occur 48 hours following the pre-ovulatory LH surge due to lack of a negative feedback from gonadal-steroid. (Hafez and Hafez, 2000).

In goats, 2 peaks of oestrogen have been reported during the oestrous cycle. One peak occurs 2 days before ovulation particularly during the follicular phase and the additional peak during the early luteal phase. The concentration decreases rapidly from

day 5 post-ovulation for the entire luteal phase. These 2 peaks correlate well with the 2 follicular waves. The first peak occurring during oestrous period, coinciding with the ovulatory wave, with the second peak occurring during the early luteal phase and coinciding with wave 1 (Leyva-Ocariz *et al*., 1995; de Castro *et al*., 1999; Rubianes and Menchaca, 2003; Gaafar *et al*., 2005). The dominant follicle present is responsible for the production of ovarian oestrogens, while the subordinate follicles are responsible for the production of about 10% of the ovarian oestrogens (Badinga *et al*., 1992; Mann *et al*., 1992). The highest concentrations of oestrogen coincide with follicular wave 1 and the ovulatory wave in the oestrous cycle, because the largest dominant follicle occurs during these two waves (Ginther and Kot, 1994; de Castro *et al*., 1999; Menchaca *et al*., 2002). The dominant ovarian follicles growing during the midluteal phase do not produce high oestrogen levels and this be attributed to the inhibitory factor from the increasing progesterone concentration that suppresses the pulsatile LH secretion (Bauernfeind and Holtz, 1991; Rubianes and Menchaca, 2003; Gaafar, Gabr and Teleb, 2005).

The frequency of the LH pulses in goats is reported to be high during the first half of the luteal phase. This increase in LH concentration and pulses has also been shown to coincide with the growing phase of the dominant follicle, as well as the time when the concentration of progesterone is low (Adams, 1992; Kawate *et al*., 2000; Menchaca *et al*., 2002). This thus justifies the suggestion that a dominant follicle escapes its regression by changing its reliance from FSH to LH. It also implies that if the concentration of LH can remain low, large follicles will continue to depend on FSH and it will not experience the detrimental effect of a dominant follicle. This has been observed in sheep and goats, where out of season and during the luteal phase (when the concentration of LH is low), there is a lack of dominance (Baird, 1983; Campbell *et al*., 1995, 1998; Adams, 1999; Menchaca *et al*., 2002).

During the mid-luteal phase and the rest of the luteal phase, both the concentration and pulses of LH decline, due to an increase in the progesterone concentration. Thereafter, the LH concentration and frequency of pulses increase following luteolysis (decline in progesterone concentration), coinciding with the onset of estrous and the ovulatory wave (Baird, 1983; Campbell *et al*., 1995, 1998; de Castro *et al*., 1999; Gaafar *et al*., 2005). This indicates that progesterone levels control follicular development and growth, possibly mediated through the control of the LH pulses. In goats, small follicles have been observed in waves developing under high levels of progesterone. It was observed that high circulating progesterone concentrations during the early mid-luteal phase in goats leads to 4 follicular waves, while low progesterone levels during the mid-luteal phase lead to 2 or 3 waves. Moreover, circulating oestrogen levels decline earlier in goats with 4 waves, probably due to the early increase in progesterone concentration, which suppresses the LH pulses, hence leading to the early or quick emergence of follicular wave 2. In contrast, goats with 3 waves have a delayed wave 2 occurrence, which happens with the decline in oestrogen concentration occurring later. When inducing a high progesterone concentration with the aid of intravaginal progestagen pessaries early during the luteal phase, the lifespan of a dominant follicle is reduced, hence advancing the emergence of follicular wave 1 (Ginther and Kot, 1994; de Castro *et al*., 1999; Rubianes and Mechaca, 2003).

### 2.2.3 Control of the Follicular Development

The synchronisation of follicular waves is compulsory for initiating a superovulation treatment. In cattle, many superovulation protocols have been designed to synchronised follicular wave emergence by removal of a dominant follicle through aspiration. The absence of a dominant follicle leads to the brief secretion of FSH concentration and trigger the emergence of a new follicular wave within 1 to 2 days (Bergfelt *et al*., 1994;

Diskin *et al*., 2002). These techniques have been reported to improve the superovulatory response. In goats and other small ruminant, however, this approach may seem infeasible because of the small body size and; therefore, the employment of exogenous hormones would be more appropriate. For this reason, progesterone and oestrogen have been used to synchronise follicular emergence as the two hormones can suppress FSH and LH secretion and terminate the existing follicular wave, hence stimulating the beginning of a new follicular wave. Another approach involves the administration of GnRH to induce luteinisation or ovulation of the dominant follicle, leading to the emergence of a new follicular wave (Bo *et al*., 1995; Twagiramungu *et al*., 1995; Diskin *et al*., 2002). In sheep and goats, methods for inducing the synchronisation of follicular wave in superovulatory protocols are currently being considered. This involves starting superovulation soon after ovulation, which coincides with emergence of a follicular wave, known as a day 0 protocol or the pretreatment with a GnRH agonists or antagonists to avoid the development of a dominant follicle (Cognie, 1999; Rubianes and Menchaca, 2003).

In small ruminant and cattle MOET programme, oestrus of donors and recipients were usually synchronised by insertion of Y-shaped silicone-coated devices ("controlled internal drug release", CIDR), impregnated with progesterone for period of 10-15 days. Beside CIDR, other progestagen implants that frequently employed with equal effectivess are vaginal pessaries. These are polyurethane sponges impregnated with fluorogestone acetate (FGA) (Freitas *et al.,* 1997; Espinosa-Marquez *et al.,* 2004) or medroxyprogesterone acetate (MAP) (Greyling and Van Der Nest, 2000; Mellado, Olivas and Ruiz, 2000). As an alternative to vaginal pessaries, an implant impregnated with the highly potent synthetic progestagen norgestomet may be inserted under the skin on the upper side of the ear (Bretzlaff and Madrid, 1985; Holtz and Sohnrey, 1992; Yuswiati and Holtz, 1996; Freitas *et al.,* 1997b; Graff *et al.,* 1999; Mellado, Olivas and

Ruiz, 2000; Oliveira *et al.*, 2001; Medan *et al.*, 2003) or on the underside of the tail (East and Rowe, 1989).

## 2.3. EXTRINSIC FACTORS AFFECTING OVARIAN RESPONSE IN SUPEROVULATED DONOR DOES

### 2.3.1 Gonadotrophins in Superovulation Protocol.

Superovulation plays an essential role in the MOET programmes in goats. MOET procedure depends on the provision of sufficient numbers of viable embryos for the routine embryo transfer operations. In goats, the superovulation treatment, generally, is a method of inducing the development of large number of ova released from the female ovaries by administration of exogenous gonadotrophins at the end of a progestagen treatment, which is employed to synchronise oestrus. The aim of superovulation treatment is to generate high yield of good quality embryos. Many practitioners consider superovulation to be the most challenging aspect of all MOET procedure. The significant variable response of the donor to superovulatory treatment and the numbers of embryos available for transfer from each donor is the main drawback aspect, since the results vary from complete failure to total success. The main factors contributing to the unpredictability of this technique are the variability of the superovulatory response, the poor fertilisation associated with high ovulatory responses, and early regression of corpora lutea (Cognié, 1999; Cognié *et al.*, 2003). These unpredictable results, combined with high costs and the use of surgical procedures for collecting and transferring embryos, have prevented large-scale use of MOET in goats' improvement programmes.

Variation and unpredictability in the ovulatory response obtained by superovulation can be attributed to the type and preparation of exogenous gonadotrophins used (Nowshari *et al*., 1995a; Pintado *et al*., 1998; Holtz, 2005). The

gonadotrophins are complex glycoproteins and carbohydrates belong to the glycoprotein hormone family (Chemineau *et al*., 1991a). Sialic acid, which is a part of the carbohydrate content, is essential for full expression of the biological activity of the gonadotrophins. All gonadotrophins are composed of two dissimilar subunits, alpha and beta, whose non-covalent association is required for their biological activities. Most of the superovulatory regimes involve treatment with gonadotrophins because gonadotrophins play an important role in the control of gametogenetic and endocrine activities of gonads (Herve *et al*., 2004). The two most widely used gonadotrophins preparations for superovulation treatment of goats presently are eCG and FSH.

#### 2.3.1.1 Pregnant Mare's Serum Gonadotrophins (eCG)

Pregnant Mare's Serum Gonadotrophins (PMSG) or eCG was the only product available for superovulation in previous day. It is obtained from the serum of pregnant mare during pregnancy and was found to be concentrated in cup-shaped depression in the endometrium (Papkof, 1981; Perry, 2001). eCG was used extensively in small ruminant research for oestrus synchronisation and superovulation and embryo transfer programmes (Larson *et al.,* 1970; Armstrong and Evans, 1983; Maxwell and Wilson, 1986; Tsunoda *et al*., 1987; Rosnina *et al*., 1989; Pendleton *et al.,* 1992). This hormone is administered as a single injection subcutaneous or intramuscular given one to two days before or at the end of progestagen treatment. The use of eCG with or without a follow-up with eCG antibodies (Pintado *et al*., 1998), in many cases did not deliver the expected response (Saharrea *et al*., 1998; Cognič, 1999). This might be associated with its long half-life being 10–15 hours (Holtz, 2005), that reported to lead to the production of a large number of follicles that fail to ovulate (Amoah *et al*., 1990; Cognič, 1999). The occurrence of anovulatory follicles is constantly associated with lower numbers and quality of embryos recovered following ovulation (Boland *et al.,* 1983; Saumande *et al.,*

1984). These anovulatory follicles often continually stimulated after ovulation become multiple cystic follicles and keep producing high blood oestrogen concentrations for an prolonged period of time (Armstrong and Evans, 1983a; Amoah *et al*., 1990; Mahmood *et al.,* 1991; Saharrea *et al.,* 1998). The elevated oestrogenic response from these unovulated follicles is believed to create an unsupportive environment for the sperm, oocyte and embryo survival in the female reproductive tract, resulting in reduced fertilisation and embryo recovery rates (Evans *et al*., 1984).

Other disadvantage reported in eCG superovulated goats is high incidence of premature luteal regression (Battye *et al*., 1988; Cameron *al et*., 1988; Saharrea *et al.,* 1998; Espinosa-Ma´rquez *et al.,* 2004). High levels of plasma oestradiol from the unovulatory follicles due to eCG stimulation result an early increase in the secretion of endogenous prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$), from either follicular or endometrium might be the causative factors of premature luteal regression (Armstrong and Evans, 1983a; Battye *et al.,* 1988, Ishwar and Menon, 1996). The high blood oestrogen concentration in ruminants known to increase endometrial oxytocin receptor synthesis and activate the enzymes associated with $PGF_{2\alpha}$ secretion (Flint and Shedrick, 1983). In sheep, the infusion of oestrogen ($E_2$-$17_\beta$) resulted in the formation of oxytocin receptors, 6 hours following treatment, with oxytocin stimulating the secretion of $PGF_{2\alpha}$ (McCracken *et al.,* 1984).

The administration of exogenous oxytocin might bind to the endometrium, causes greater stimulation of the endometrium to release $PGF_{2\alpha}$, and induces early luteal regression (Garverick, Zollers and Smith, 1992), while immunisation against oxytocin has been shown to delay luteolysis in sheep and goats (Burgess, Ralph, Jenkin and Thorburn, 1990; Garverick *et al.,* 1992; Seals, Lemaster, Hopkins, dan Schric, 1998; Lemaster, Seals, Hopkins and Schrick, 1999). The end result of premature luteal regression being a decline in progesterone concentration 1 to 6 days following the onset

of oestrus (Espinosa-Mârquez *et al.,* 2004; Lu *et al.,* 2008). This inhibitory effect of progesterone on oestrogen receptor synthesis then declines, leading to an increase in endometrial oestrogen and oxytocin receptors. This situation will lead to a loss of embryos before the scheduled collection on day 6 or 7 after mating or insemination (Stubbings, Bosu, Barker and King, 1986; Burgess, Ralph, Jenkin and Thorburn, 1990; Saharrea *et al.,* 1998). The premature regressing corpora lutea do not only leads to a decline in the progesterone concentration, but also showed an embryo toxicity effect (Buford *et al.,* 1996; Hernandez-Fonseca, Sayre, Butcher and Inskeep, 2000; Costine, Sayre and Inskeep, 2001). Treatment with an exogenous progestagen (FGA) increased embryonic survival in eCG-superovulated goats recovered on day 6, although it did not prevent premature CL regression (Espinosa-Mârquez *et al.,* 2004), while the pregnancy rate was not maintained or improved (Butcher *et al.,* 1992; Breuel *et al.,* 1993).

The undesirable side effects observed following the utilisation of eCG in superovulation protocols can be minimised by the administration of anti-eCG, LH, HCG or GnRH at the onset of oestrus in sheep and cattle (Monniaux, Chupin and Saumande, 1983; Saumande, Procureur and Chupin, 1984; Moyaert *et al.,* 1985; Martemucci *et al.,* 1995; Cognie, 1999). However, the treatment with HCG or GnRH unsuccessful to improve the ovulation rate, or to reduce the number of large follicles that failed to ovulate in goats superovulated with eCG (Armstrong *et al.,* 1982; Saharrea *et al.,* 1998). The administration of anti-eCG in goats superovulated with eCG has lead to variable ovarian responses and failed to reduce the incidence of premature luteal regression in superovulated dairy and Murciana goats (Stubbings, Bosu, Barker, and King, 1986). On the other hand, an increase in the number of viable goat embryos was reported when goats treated with anti-eCG (Pintado, Gutierrez-Adan and Perez Llano, 1998)

### 2.3.1.2 Follicle Stimulating Hormone (FSH)

Currently the most common used gonadotrophin preparations in caprine MOET programmes are ovine (oFSH) and porcine (pFSH) derived FSH (Baril and Vallet, 1990; Dattena *et al*., 2004, Cognie, 1999). Because the short half-life of FSH (110 minutes; Fry *et al*., 1987) optimal responses have been achieved by providing the total dosage as multiple injections over a 2–3-day period (Holtz, 2005). Satisfactory ovulation rates ranging between 8.4±0.9 and 28.7±2.3 embryos have been recorded in FSH superovulated goats, although individual variability is huge (Senn and Richardson, 1992; Rosnina, Jainudeen and Nihayah, 1992; Ishwar and Menon, 1996; Greyling, Van Der Nest, Schwalbach and Muller, 2002).

Primary factors limiting embryo production in FSH caprine MOET programmes are the variability of the ovarian response following induction of superovulation with commercially available FSH preparations and the competence of the oocytes to ovulated. FSH preparations proved to be more effective than eCG (Armstrong and Evan, 1983a; Mahmood, Koul and Biswas, 1991), provided it contains a proper FSH/LH ratio in the preparation (Nowshari, Beckers, and Holtz, 1992; Kelly, Duffy, Roche and Boland, 1997; Crosby and Gordon, 1997; D'Alessandro, Martemucci and Taibi, 1997). FSH preparations with high LH content results in poor embryo yields (Murphy, Mapleton, Manns and Humphrey, 1984; Donaldson, Ward and Glenn, 1986; Chupin and Saumande, 1979), while highly purified FSH preparations significantly lower ovulation rates (Chupin and Saumande, 1979; Donaldson, Ward and Glenn, 1986). LH content in the range of 40% does not only provide the best superovulatory response but also superior embryo viability (Nowshari, Beckers and Holtz, 1995a). In other ruminants, FSH preparations with high LH content have been found to result in lower ovulation and fertilisation rates and poor quality embryos (Murphy, Mapleton, Manns and Humphrey, 1984; Donaldson, Ward and Glenn, 1986). The high blood LH

levels cause premature ovulation of the large follicles, which are present at the onset of the superovulation regime. This ovulation leads to the production of progesterone during the pre-ovulatory period of the newly induced follicles. The progesterone and oestrogen ratio of the prematurely stimulated follicles are then altered, leading to disturbances in the process of maturation of the follicles, hence the production of poor quality embryos (Callesen, Greve and Hyttel, 1986; Callesen, Greve and Hyttel, 1987).

When LH was removed from the pFSH, the effectiveness of the FSH increased as the lower doses of LH in pFSH gave the highest ovulatory rate and number of transferable embryos (Donaldson, Ward and Glenn, 1986). In goats, as the ratio of FSH/LH was reduced in a superovulation treatment, more corpora lutea and transferable embryos were obtained, compared to when a constant FSH/LH ratio was utilised (Baril, Casamitjana, Perrin and Vallet, 1989). This observation and the adverse effects demonstrated by the FSH preparations with LH, lead to the production and utilisation of a more purified commercial preparation of FSH with a low LH content (oFSH: OVAGEN and pFSH: FOLLTROPIN and STIMAFOL) (Kelly, Duffy, Roche, and Boland, 1997). When the efficiency of oFSH and pFSH were evaluated in goats, the ovulation rate and number of transferable embryos induced were similar (McNatty *et al.*, 1989). However, superovulation with a highly purified FSH preparation has led to a lower superovulatory response than the LH supplemented FSH preparations (Herrler, Elsaesser, Parvizi and Niemann, 1991; Cognie, 1999). Moreover, there has been an increase in the frequency of ovulation abnormalities observed following superovulation with these commercially purified FSH batches, for example premature ovulation and unovulatory follicles (Cognie, Baril, Poulin and Mermillod, 2003). This occurrence indicates a minimum amount of LH being required following progestagen treatment termination. In goats, the supplementation of FSH with 40% purified LH has been suggested to be the optimal dose, and has been proven to produce satisfactory ovulation

rates and a high number of transferable embryos (Pulskleingeld, Nowshari and Holtz, 1992; Nowshari, Beckers and Holtz, 1995a).

The efficiency of FSH and eCG as superovulation agents has been compared in goats and other species. Superovulation with FSH in most protocols produced higher ovulation and embryo recovery rates, when compared to eCG treated goats (Goel and Agrawal, 1990; Selgrath, Memon, Smith and Ebert, 1990; Mahmood, Koul and Biswas, 1991). The higher number of abnormal corpora lutea, which is an indication of premature luteal regression was observed in eCG treated does, compared to FSH treated does (Pendleton *et al.*, 1992). Furthermore, the incidence of a large number of unovulatory follicles was higher in eCG stimulated does, compared to following FSH treatment (Armstrong and Evans, 1983a, Armstrong, Pfitzner, Warnes, Ralph and Seamark 1983b). The difference in ovarian response between these two gonadotrophins can largely be attributed to the differences in biological half-life activity (approximately 5 hours for FSH, as opposed to 20 hours for eCG). This means that FSH can be cleared from the circulation more quickly, compared to eCG (Ishwar and Memon, 1996; Holtz, 2005).

Even though the utilisation of FSH in the goats superovulation programme has demonstrated better results, this regime is more labour intensive and imposes more stress to the animals, due to excessive handling. Several attempts to simplify the administration procedure of FSH treatment without compromising the superovulatory response had been made over the time (Batt, Killeen and Cameron, 1993; Gordon, 1997; Phua, 2006; Amir, 2007; Rahman, 2008 and Chan, 2008). In ewes, observed a three-fold increase in ovulation rate in cyclic ewes with a single injection of FSH dissolved in propylene glycol (PGL) (Lopez-Sebastian, Cognie, Cocero, De La Fuente and Poulin, 1993). A single injection of FSH dissolved in polyvinylpyrrolidone (PVP) resulted in similar or enhanced ovulation rates and numbers of recoverable embryos,

compared to the conventional multiple injections in rabbits (Kanayama, Endo and Sakuma, 1993), ewes (Dattena *et al*., 1994; Simonetti *et al*., 2008) and heifers (Takedomi, Kaneko, Aoyagi, Nakanishi and Taya, 1995). Additionally, elevated concentrations of FSH were maintained significantly longer in heifers treated with FSH in PVP than in heifers treated with FSH in saline (Takedomi *et al.*, 1995). Doubling the dose of FSH while administrated at 24 hours instead of 12 hours intervals resulted in an average ovulation rate of 8.9 (Suyadi, Sohnrey and Holtz, 2000). Compatible ovulation rates reported by substituting the last three of six FSH-injections by a single dose of 200 IU eCG (Pintado *et al*., 1998). Almost equalled the embryo yield obtained with the conventional multiple injection regimen by applying a "one shot"- treatment regime consisting of a single dose of FSH combined with a moderate dose of eCG (e.g. 80 µg FSH and 300 IU eCG) (Batt, Killeen and Cameron, 1993 and Baldassarre *et al*., 2002, 2003). On the other hand, Baldassarre *et al*. (2002) recorded no difference in superovulation response, based on the number of follicles aspirated and oocytes collected between a multiple FSH regime and a combination of FSH and eCG. The replacement of certain FSH injections with eCG also gave similar results with respect to the ovulation rate, the number of follicles and the total number of embryos recovered, compared to a FSH treatment alone (Pintado, Gutierrez-Adan and Perez Llano, 1998).

Although eCG has the advantage of being administered as a single injection, which is simpler and more practical than 6 to 8 injections given when using FSH, its use in goats is currently not preferred. The main reason being the high incidence of premature luteal regression, low embryo recovery rates and poor quality embryos collected in the eCG superovulated goats, compared to FSH treated goats. The improvement of embryo recovery and embryo quality following the supplementation with LH, hCG or GnRH in the FSH protocols which failed in the eCG regime, also support the use of FSH for superovulation in goats as opposed to eCG.

### 2.3.2 Hormones Used in a Oestrus Synchronisation Protocol

#### 2.3.2.1 Progestagens

Oestrous cycle control in goats serves the purpose of synchronising oestrus in groups of animals to be bred or inseminated at a particular time. The control of oestrus and ovulation in farm animals remains the basis and a prerequisite for the success of MOET programme. Oestrous synchronisation in goats, using progestagen combined with PMSG administration 48 h before or at progestagen withdrawal, has been extensively applied achieving an acceptable oestrous response (Greyling *et al*., 2005; Baril *et al*., 1993; Baril and Saumande, 2000). In a MOET programme, oestrus synchronisation allowed the donor and recipient animals to be at the same stage of reproductive cycle at the time of embryo transfer, which is vital for maximum survival of embryos (Moor, Kruip and Green, 1984), since the degree of synchrony is one of the factors influencing the success of embryo transfer (Torres, Cognie, and Colas, 1987).

Oestrus is a period when animals exhibit sexual activity or heat and be able to undergo insemination. A doe exhibits sexual receptivity with restless behaviour, allowing her to be mounted, mounting other animals, bellowing, and having her vulva sniffed by other bucks (Gordon, 1997). The oestrous cycle is a complex relationship between a range of hormones in the animal including progesterone and oestradiol. Oestrus occurs at day zero in the cycle, with ovulation occurring at day one. There is a decline in the blood plasma progesterone concentration before oestrus occurs. Pro-oestrus occurs during this stage, with an increase in oestradiol levels, which cause the behavioral changes during heat. During this phase, the increasing concentration of oestradiol increases the amount of luteinising hormone, which results in further increases of the oestradiol level. The surge in luteinising hormone results in ovulation.

**Table 2.1.** Timeline of selected finding in the number of ovulation and viable embryos of FSH superovulation protocols.

| Year | Author | Goat Breed | Gonadotrophin type | Total dosage | Pattern administration | Days recovery | Ovulation (CL) | Viable embryos |
|---|---|---|---|---|---|---|---|---|
| 1989 | Rosnina, | Kambing Katjang | pFSH | 10 mg<br>15 mg<br>20 mg | Decreasing<br>Decreasing<br>Decreasing | Day 5 and 6<br>“<br>“ | 7<br>7<br>0 | 3<br>3<br>3 |
| 1997 | Gootwine *et al*. | Saanen Nubian | oFSH | 180 iu | - | 36 and 48 hours | 14 | |
| 1997 | Lee e*t al*., | Korean | oFSH | 5.6 mg | - | 68 hours | 12 | - |
| 1998 | Pintado *et al*. | Murciano-Granadina | pFSH | 200 mg | Decreasing | Day 6 | 10 | 6 |
| 2002 | Greyling *et al*. | Indigenous Boer | pFSH | 20 mg | Decreasing | Day 6 | 15<br>18 | |
| 2002 | Menchaca and Rubianes | Alpine | pFSH +GnRH | 200 mg | Equal | Day 6 | 10-14 | - |
| 2003a | Senthil Kumar *et al*. | Tellicherry | oFSH<br>pFSH | 180 mg<br>180 mg | Equal<br>Decreasing | Day 3<br>Day 3 | 22<br>10 | 13<br>7 |
| 2003b | Gonzalez-Bulnes *et al*. | Murciano-Granadina | oFSH | 8 x 1.25 ml | Equal | Day 8 | 16 | 6 |
| 2006 | Lehloenya *et al*. | Boer | pFSH<br>pFSH + GnRH | 200 mg<br>200 mg | -<br>- | Day 6<br>Day 6 | 21<br>16 | 18<br>13 |
| 2007 | Menchaca *et al*. | Alpine | pFSH<br>day 0 protocol<br>traditional | - | Decreasing | Day 6 | <br>10<br>6 | <br>5<br>2 |
| 2008 | Melican and Gavin. | Dairy goat | pFSH<br>oFSH | 25.6 mg<br>10.56 mg | Equal<br>Equal | Day 5-7 | | 2-4 |
| 2010 | Lehloenya *et al*. | Boer | pFSH<br>day 0 protocol<br>traditional | 200 mg | Equal | Day 6 | <br>4<br>15 | <br>1<br>12 |

Exogenous progesterone or one of its synthetic analogues treatment is preferred for control the oestrus cycle in most situations, as its suppresses the secretion of luteinising hormone and therefore prevents oestrus and ovulation (Gordon, 1997). Once the progesterone or its analogues treatment removed the decline in progesterone restart the natural conditions prior to the onset of oestrus. In goats, the MOET programme usually follows by the stimulation of the ovaries by administration of gonadotrophins during the last few days of progesterone treatment. Typically the progestagen treatment extends over 16 to 21 days (Gordon, 1997), a period that is long enough for corpora lutea to undergo appropriate regression in all animals no matter at what stage of the cycle the animals were at the onset. Currently, these are progestagen containing vaginal pessaries such as polyurethane sponges impregnated with 45mg fluorogestone acetate (FGA) or 60mg medroxyprogesterone acetate (MAP) or Y-shaped silicone-coated devices, (CIDR, "controlled internal drug release" impregnated with 30mg progesterone) (Gordon, 1997).

CIDR devices were developed in early 1980s and serve as an alternative method of administering exogenous progesterone for oestrous synchronisation (Wheaton *et al*., 1993; Godfrey *et al*., 1999). Unlike the intravaginal sponges, the CIDR devices do not absorb nor impede drainage of vaginal secretions, with the result that it has less foul-smelling discharge on removal. The devices also induce earlier and more compact synchronisation and have a better retention rate during treatment (Greyling and Brink, 1987; Carlson *et al*., 1989). For these reasons CIDR devices may be more preferable for oestrous synchronisation programmes in does. As an substitute to vaginal pessaries, implants impregnated with the highly potent synthetic progestagen norgestomet may be inserted under the skin on the upper side of the ear or tail (Bretzlaff *et al*., 1985, East and Rowe, 1989; Holtz and Sohnrey, 1992; Yuswiati and Holtz, 1996; Freitas *et al*., 1997; Graff *et al*., 1999; Mellado *et al*., 2000; Oliveira *et al*., 2001; Medan *et al*., 2002).

The administration of these progestagens suppresses the LH secretion and in turn assures the spontaneous occurrence of oestrus and ovulation in a controlled manner following progestagen removal. However, the administration of progestagens has been reported to modify the normal pattern of LH secretion as well as the pattern of follicular growth and dominance (Noel, Bister and Pierquin, 1994; Gonzalez-Bulnes *et al*., 2004a). In goats and other ruminants, a high level of progesterone following the administration of exogenous progesterone suppressed both the LH pulse and oestradiol and induced LH surge. High blood progesterone concentrations will coincide with the onset of the progestagen treatment, and towards the end of the treatment, the progesterone levels are sometimes lower than the basal levels, which cannot imitate the normal luteal phase (Leyva, Buckell and Walton, 1998). In goats, low progesterone levels completely suppress the LH surge, without any effect on the LH pulse until secretion (de Castro, Rubianes, Menchaca and Rivero, 1999; Kim, Tanaka and Kamomae, 2003).

In sheep and cattle, the induction of a low progesterone environment has been indicated to extend the life span and increase the size of the dominant follicle (Savio *et al*., 1993a, b; Vinoles, Meikle, Forberg and Rubianes, 1999). These persistent large follicles lead to an increased secretion of oestrogen towards the end of the progesterone treatment and the subsequent follicular phase. The high level of oestrogen induced by a low progesterone concentration also disturbs the sperm transport and could reduce the fertilisation rate (Johnson, Dailey, Inskeep and Lewis, 1996). Moreover, a high concentration of oestrogen had also been reported to alter the developmental competence of the oocytes. It has been suggested that oocytes from persistent ovarian follicles are normally at a more advance stage of nuclear maturation before the onset of the pre-ovulatory LH surge. This advancement of meiosis leads to the production of abnormal oocytes, which would be less fertile and result in increased embryonic

mortality rates. Ovulation of these abnormal follicles may also disturb the function of the subsequent corpus luteum. The other subsequent effect of a high oestrogen environment has been reported as an alteration in the oviduct or uterine environment (Kojima *et al*., 1992; Revah and Butler, 1996; Wehrman *et al*., 1997; Binelli, Hampton, Buhi and Thatcher, 1999).

Superovulation protocol at the end of synchronisation treatment further deteriorate all effects from low blood progesterone concentrations and result in the variability regarding the interval between oestrus onset and LH peak. Hence, the miss-timing of ovulation, which ends up in reduced fertility, as indicated by the recovery of more unfertilised ova, especially following fixed-time fertilisation by AI or natural mating. Fertility of goats, which came into oestrus later than 30 hours after sponge removal, was significantly lower than those, which were first observed in oestrus 24 or 30 hours after sponge removal (Baril *et al.,* 1993**).** This delay in the onset of estrous behaviour is associated with a delay in the LH preovulatory surge (Maure, Hunt and Foote, 1992) and in a delay in the time of ovulation (Leboeuf *et al.*, 1996; Leboeuf, 1998). Time of ovulation cannot be predicted from either the time of sponge removal or the occurrence of oestrus following progestagen treatment and superovulation (Baril and Vallet, 1990).

Prolonged progestagen treatment is associated with suboptimal conception rates, presumably because of impaired sperm transport in the female genital tract (Quinlivan and Robinson, 1969; Hawk, 1988; Corteel, Leboeuf and Baril, 1988). The disturbance on oocyte maturation and alteration of the uterine environment then also leads to the increased incidence of abnormal embryos, reducing the number of transferable embryos (Scudamore *et al*., 1992, 1993a, b). The low levels of circulating progesterone recorded towards the end of synchronisation treatment suggests an inadequacy of the progestagen used to block the LH pulse secretion. This may be

attributed to the dose or type of the progestagen used. In sheep when using different progestagen treatments following superovulation (FSH, eCG or HAP), the sponge pessaries led to a higher ovulation rate, and thus a higher number of ova recovered, compared to synchronisation with CIDR's (Boland, Lonergan and O' Callaghan, 1983; Thompson *et al*., 1995).

In a study comparing the effect of the dose of progestagen on the response to superovulation, ovulation and embryo recovery rates, the responses were not affected by the different dosages of progesterone priming used. However, the number of transferable embryos recorded was lower in the lesser dosages of FGA, compared to the higher dosages. This Indicates that the low progesterone at the beginning of superovulation leads to a reduction in embryo quality (Scudamore *et al*., 1992; Wallace, 1992). When two CIDR's were successively used in the superovulation programme (first CIDR replaced on day 9 by a new CIDR then removed on day 12), in attempt to maintain higher progesterone concentrations, the superovulation response was higher, compared to when a single CIDR was used throughout the synchronisation treatment (Thompson *et al*., 1995). On the other hand, when two individual intravaginal sponges were used consecutively to synchronise oestrus in a superovulation programme (compared to a single sponge utilisation), the ovulation rate and the number of recovered embryos did not differ. Although, the utilisation of two intravaginal sponges improved the number of embryos recovered. To shorten the treatment period to between 5 and 12 days, a luteolytic dose of $PGF_{2\alpha}$ is injected either at the onset (Rubianes and Menchaca, 2003) or, more commonly, at the end (24–48 hours) before the end of the progestagen treatment.

### 2.3.3 Repeated Superovulation and Embryo Recovery

The potential commercial exploitation of MOET to accelerate the genetic progress in goats can be accomplished through repeatedly superovulate selected high-genetic merit donor, with predictably high mean ovarian responses and minimal variation in ovulation rate and high numbers of transferable grade embryos. These certainly could accelerate rates of genetic improvement compared to a single collection of embryos from superior donors above that achieved in a natural breeding management system. Although the idea is possible, repeated superovulation in small ruminants in the past has always associated to unfavourable result. Repeated superovulation with pFSH in goats has been reported to reduce the number of ovulations and embryos recovered as well the number of transferable embryos (Nuti *et al.*, 1987; Baril *et al*., 1989; Beckers, *et al*., 1990). Similar observations have been reported in other species (Chupin and Saumade, 1979; Al-Kamali, Boland, Crosby and Gordon, 1985; Bavister, Dees and Schultz, 1986). In addition, work, involving superovulation repeated up to 5 times, showed a progressive reduction in the proportion of ewes superovulating, and a significant drop in mean ovulation rate, especially between first and second treatments (Al-Kamali *et al*., 1985; Fuki, Kano, Kobayashi, Tetsura and Ono, 1985; McKelvey, Robinson and Aitken, 1985; Tervit, Thompson, Mcmillan and Amyes, 1991)

This reduction in superovulation response in the past was suggested to be attributed to the refractoriness of the ovaries if superovulation was repeated within an interval of 2 to 6 months (Willett and Bucker, 1953; Al-Kamali *et al.*, 1985; Nuti *et al*., 1987; Brebion, Baril, Cognie and Vallet, 1992., Gootwine *et al*., 1997). However, other study found that 2 - 6 months interval between superovulation procedures should not lead to a reduction in ovarian superovulation in sheep (Bolan *et al*., 1982). This was based on the fact that 20 to 30 gonadotrophin-responsive follicles have been developed on the ovary of an ewe during each normal oestrous cycle, and also that the primordial

follicles take about 40 days to achieve the ovulatory stage (Web, Dombo and Roets, 2004). No significant change in either mean ovulation rate or embryos recovered per donor was observed ewes were repeated superovulation treatments up to 4 times at yearly intervals (Bari, Khalid, Haresign and Merrell, 2001).

Another explanation regarding the reduction in ovulation rate and the number of females responding to repeated superovulation was that production of gonadotrophin antibodies following successive superovulation (Holtz, 2005). The anti-eCG antibodies produced in goats indicated to have a negative effect on reproduction, especially when fixed-time AI is performed. This was confirmed by Roy *et al*. (1999), where the high concentration of anti-eCG antibodies was correlated with a decrease in fertility. The reduction in fertility is believed to be due to the alteration in the timing of the induced oestrus in the does repeatedly exposed to eCG treatment (Baril *et al*., 1993). The alteration in the preovulatory LH surge and time of ovulation observed following repeated eCG treatment has also been associated with the formation of anti-eCG antibodies (Herve, Roy, Bertin, Guillou and Maurel, 2004). More anti-gonadotrophins have been detected in poor and non-responder goats, following repeated superovulation with pFSH (Beckers *et al*., 1990). Antibodies was also detected to p-FSH in plasma samples as early as after the first injection in goats that were treated at 6-week intervals for a period of 7 months, and concentration increased following booster injections. (Remy *et al*., 1991). It has also been advisable to use caprine or ovine FSH preparations for superovulation in goats that need repeatedly treated if embryos are to be collected from a same donor several times (Chemineau *et al*., 1999).

Melican and Gavin, (2008) in their study on in transgenic dairy goats concluded that repeated oestrus synchronisation, superovulation and the non-surgical embryo retrieval, coupled with surgical embryo transfer at 4-5 week intervals expedited the production of progeny from transgenic founder does. Beside high ovulation rate

achieved, embryo recovery rate over successive collections also influences the success of repeated MOET procedures. The procedure employed for embryo collection is important in repeated embryo recovery in goats. Goat embryos in a MOET programme are usually collected and transferred surgically (Armstrong, *et al.,* 1983b; Bessoudo, Davis, Coonrod and Kraemer, 1988; Nowshari *et al*., 1995a; Baril *et al*., 1989). Surgical recovery procedure are known to result in a significant decline in embryo recovery rates and a decline in the proportion of donor being flushed due to post-operative scar tissue formation beside surgical trauma during exteriorisation of the reproductive tract through laparatomy (Agrawal, Mongha and Bhattacharyya, 1982; Al-Kamali *et al.,* 1985; Fuki *et al*., 1985, Kano, Kobayashi, Tetsura and Ono, 1985; Torres *et al*., 1987; Tervit *et al*., 1991). This limits the number of times surgical flushings may be performed on the same donor (McKelvey *et al.,* 1985; Ishwar and Memon, 1996; Pereira, Sohnrey and Holtz, 1998; Suyadi *et al*., 2000. The post-operative adhesions have been identified to be the main factor leading to a reduction in ovulation rate and embryo yield following repeated superovulation treatment (Al-Kamali *et al*., 1985; Cognie, 1999).

Non-surgical methods of embryo collection such as the use of laparoscopy (McKelvey *et al*., 1985) and the transcervical passage of a catheter by mechanical dilation of the cervix or ripening of the cervix with $PGF_{2\alpha}$, or/with oxytocin have been performed with success in goats (Bondurant *et al*., 1984; Nagashima, Matsui, Sawasaki and Kano, 1987; Bessoudo *et al*., 1988; Flores-Foxworth, McBride, Kraemer and Nuti, 1992; Pereira *et al*., 1998; Suyadi *et al*., 2000). Laparoscopic embryo collection has the advantage of leading to fewer adhesions and putting less strain on the animal, when compared to surgical collection, but the approach requires special instruments and skilled personnel. Embryos recovered by laparoscopy was reported lower than surgical procedure but the recovery under laparoscope could be repeated successfully on the same goats compared to surgery procedure due to the problem of adhesions (Baril, *et*

*al*., 1989; Gordon., 1997). The embryo recovery rates recorded range from 60 to 79% following laparoscopic embryo flushing (Baril, *et al*., 1989, Flores-Foxworth *et al*., 1992). This is comparable to recovery rates reported in goats ranging from 60 to 90% following surgical embryo recovery (Nowshari *et al*., 1995a; Suyadi *et al*., 2000; Holtz, 2005). As laparoscopic embryo collection also leads to the formation of adhesions and limits the number of times a donor can be collected.

Attempts are being made regarding transcervical embryo collection in goats (Armstrong *et al*., 1983b; Nowshari, Nayudu and Hodges, 1994). Transcervical embryo collection in sheep and goats has been limited in the past because of the difficulty of passing the catheter through the cervix. Hence this technique was performed under anaesthesia, either in ventral or dorsal recumbency (Nagashima *et al*., 1987; Ishwar and Memon, 1996; Suyadi *et al*., 2000). In goats, early research on transcervical embryo collection has demonstrated a lower embryo recovery rate (36.9%) (Flores-Foxworth *et al*., 1992). However, the discovery of the induction of luteolysis and contractility of the uterus during flushing by injecting the animals with $PGF_{2\alpha}$ before collection of the embryos has contributed to an increased success of transcervical embryo collection in goats. Using this technique, transcervical embryo collection can be performed without sedating the donor animal (Ishwar and Memon, 1996; Pereira *et al*., 1998; Holtz, 2005). Treating of goats with $PGF_{2\alpha}$ 8 or 16 hours before embryo flushing has led to higher number of embryos or ova being recovered, as well as a higher recovery rate (Pereira *et al*., 1998; Suyadi *et al*., 2000). With this approach embryo recovery rates ranging from 60 to 80% have been obtained in goats, comparable with the surgical embryo recovery procedure rates ranging from 60 to 90% (Nowshari *et al*., 1995a; Suyadi *et al*., 2000; Holtz, 2005). As previously stated, transcervical embryo collection has several advantages over surgical and laparoscopic embryo collection procedures e.g. less trauma to the animal, no need for sedating the animal and no limitation to the number of

times a donor can be flushed. This procedure hence holds the potential for more popular utilisation in goats, if further investigation could improve the efficiency this procedure. Holtz *et al*. (2000) reported goats to be flushed 10 times within one year when transcervical embryo collections were performed.

Although the treatment of donors with $PGF_{2\alpha}$ before the transcervical flushing of embryos has been a breakthrough in goat MOET programmes, this procedure still has a major constraint in the time required to recover the embryos from an individual animal. Pereira *et al.* (1998) reported 24 embryo flushes to be required, of which 12 flushes took about 45 minutes plus a 2 hours pause between the first and the second 12 embryo flushes. The whole procedure was recorded to take about 4 hours. In an attempt to reduce the time spent flushing embryos, Suyadi *et al.* (2000) evaluated the effect of injecting the donor animals with $PGF_{2\alpha}$, 24 hours prior to flushing, plus an additional oxytocin injection. These researchers observed a reduction in the duration of the flushing period (63 minutes), but no effect of both treatments on embryo recovery rate. Holtz *et al*. (2000) also reported a duration period of 30 to 40 minutes flushing per doe, when the flushings were reduced to 20. However, the time of the flushing procedure when the goats are treated with PGF2α and oxytocin 24 hours before flushing for commercial embryo recovery purposes is still the major constraint facing transcervical embryo collection in goats. Thus, at the end of the day, surgical embryo collection still remains the most commonly utilised method in goat MOET programmes.

### 2.3.4 Fertilisation Failure in Goats

#### 2.3.4.1 Poor Synchronisation of Oestrus and Ovulation

The yield of fertilised ova/embryos following superovulation is mainly related to the poor synchronisation of oestrus and ovulation, especially following fixed-time AI (Baril *et al*., 1989; Baril *et al*.,1990; Kafi and McGowan,1997; Cognie *et al*., 2003). In goats,

asynchrony of oestrus has been commonly observed following progestagen withdrawal in FSH treated females. The onset of oestrus in Angora, Alpine and Saanen goats has been reported to range from 24 to 54 hours following intravaginal progestagen withdrawal. The does with the shortest Interval from intravaginal sponge withdrawal to the occurrence of oestrus recorded a higher ovulation rate, compared to does taking a longer time to exhibit behavioural signs of oestrus. This large variation in the time interval from progestagen withdrawal to the onset of the induced oestrus period indicates that the time of ovulation cannot be predicted based only on the onset of oestrus. Poor fertilisation rates result, especially in fixed-time AI, as a result of poor synchronisation of oestrus and ovulation (Baril *et al*., 1989; Baril *et al.,* 1990). Poorer synchronisation efficiency of ovulation is usually observed following superovulation in goats compared to sheep. It has been reported that in goats only 8.8% ovulations occurred 50 hours following intravaginal sponge withdrawal, while 88% of the ovulations occurred from between 50 to 80 hours, a clear indication that most goats in a fixed-time AI programme (e.g. 36 hours and 48 hours) will be inseminated too early with respect to the time of ovulation.

In goats, it has also been reported that the distribution of ovulation within females can be attributed to the time interval from the onset of oestrus to the onset of the pre-ovulatory LH surge, as well as the interval from the first to the last ovulation (Cognie *et al*., 2003; Gonzalez-Bulnes *et al*., 2004a). In sheep, efficient synchronisation of the LH surge has been reported following progestagen removal (mean of 56 ± 6 hours) and the average time from the first to the last ovulation reported as 6 hours, which is relatively short. However, in goats the distribution in the time of the LH surge has been reported to range from 24 to 64 hours following progestagen termination (mean of 63 ± 9 hours) and the average time from the first to the last ovulation to be 12 hours - which is long, compared to the 6 hours in sheep (Cognie *et al*., 2003). In a

MOET programme involving fixed-time AI, the timing of ovulation is critical for overall fertility results and therefore the induction of the LH peak with exogenous GnRH and LH may give better results. In sheep superovulated with either FSH or eCG, the number of fertilised ova recovered was increased by treating the ewes with GnRH (Walker *et al*., 1989; Naqvi and Gulyani, 1998). In goats, not only was the ovulation rate increased by GnRH treatment following superovulation with eCG, but also the ovulation time was synchronised - leading to 91% of the ovulations occurring between 36 and 48 hours following sponge removal. In goats treated with only eCG, ovulations continued to occur until up to 77 hours following intravaginal sponge withdrawal. This observation indicates that the fertilisation rate could be improved in goats superovulated with FSH by treating the animals with GnRH, as the occurrence of ovulation could be synchronised (Cameron, Battye and Trounson, 1988).

#### 2.3.4.2 Failure of Sperm to Capacitate and Impeded Sperm Transport

Low embryo recovery rates due to fertilisation failure following superovulation has been reported in most species as a result to the failure of sperm to capacitate and obstacle of sperm transport through the uterus to the Fallopian tube where fertilisation takes places (Armstrong and Evans, 1983; Evans and Amstrong, 1984; Hawk, 1988; Kafi and McGowan, 1997; Cognie, 2003). Impeded sperm transport has been largely associated with the method of mating, where natural mating and cervical insemination led to high incidences of fertilisation failure and intrauterine inseminations resulted in improved fertility in ewes following superovulation (Boland *et al*., 1983; Rexroad and Powell, 1990; Ishwar and Memon, 1996). There is, however, ample research indicating that superovulation also leads to the impairment of sperm transport (Moore and Eppleston, 1979; Baril *et al*., 1989; Brebion *et al*., 1992). In most species, fertilisation failure is normally indicated by a lack of sperm recovery and sperm numbers in the

uterus and oviducts (site of fertilisation) following superovulation, with either FSH or eCG (Evans and Amstrong, 1984; Hawk, 1988). This impairment of sperm transport following superovulation has been suggested to result from prior ovulatory elevated oestrogen levels (Armstrong *et al.,* 1983b; Greve *et al.,* 1995). In goats impeded sperm transport following superovulation has also been a result of premature ovulation, as early ovulations leads to an increase in circulating progesterone concentrations during the preovulatory period (Cameron *et al.,* 1988). Lastly, this phenomenon of sperm impairment reduces the fertilisation rate, as those ova released early will never be fertilised by the time the sperm reach the oviduct (Moor *et al.,* 1984).

#### 2.3.4.3 Abnormal Maturation of the Oocytes

Fertilisation failure in goats could also be attributed to the abnormal maturation of the oocytes following superovulation (Kumar *et al*., 1990, 1991). Abnormalities in oocyte maturation are generally reflected by the premature condensation of chromatin in the goat oocytes, which is a sign of premature activation of the initial stages of meiotic maturation (Cameron *et al*., 1988; Kumar *et al*., 1990). Premature activation of oocyte maturation has also been also reported in other species following superovulation. The occurrence of this phenomenon has been reported to be higher in animals superovulated with eCG than in FSH treated animals (Moor *et al*., 1984; Moore, 1985; Callesen *et al*., 1987; Kumar *et al*., 1990; Hyttel *et al*., 1991). This phenomenon is the result of increased blood LH concentrations, which is predominant following eCG treatment with LH-like bioactivity inherent to this gonadotrophin (Moore and Eppleston, 1985, Cameron *et al*., 1988, McNatty *et al*., 1989; Kumar *et al*., 1991). This condition contributes to major embryonic loss following superovulation, as some of these activated oocytes will be left as luteinized follicles, while others will be ovulated as old eggs and will end up being abnormal embryos (Moor *et al*., 1984).

### 2.3.4 .4 Nutritional Effect on Reproduction

Several reproductive events including hormone production, gametogenesis, fertilisation and early embryonic development in farm animals has been known be control by the level of nutrition intake (Cox, 1997; Butler, 2000; Boland, Lonergan and O'Callaghan, 2001; Lucy, 2003; Peura *et al*., 2003; Paula *et al*., 2005; Fitz-Rodriquez *et al*., 2009). In goats, a low level of nutrition for example, can lead to a loss in body weight, body condition and a reduction in ovulation rate (Mani, Watson and Mckelvey *et al*., 1992). Nutrition may play a role in oocyte development, the competence and morphology of the oocytes and ovulation rate (Smith, 1991; Downing, Joss, Connell and Scaramuzzi, 1995). While trying to determine the interaction of nutrition on ovulation rate, it has also been shown that the nutritional effects are mediated at the hypothalamo-pituitary level. Based on this aspect, several nutritional effects have been recognised when animals are in a negative energy balance, e.g. the inhibition of GnRH secretion by the hypothalamus, absence of LH pulses, low FSH concentrations, inhibition of folliculogenesis and a high negative feedback sensitivity (Downing and Scaramuzzi, 1991; Gong, 2002; Wade and Jones, 2005; Scaramuzzi *et al*., 2006). Moreover, the feeding of the animals with diets above maintenance has been associated with an increase in blood glucose, insulin and leptin, leading to increased folliculogenesis and ovulation rates (Abecia, Sosa, Forcada and Meikle, 2006; Scaramuzzi *et al*., 2006).

Dietary energy has been indicated to alter the follicular dynamics in superovulated ruminants (Gong, 2002). However, the response to the nutritional influence following superovulation and embryo transfer has been variable and inconsistent. There is a lack of information regarding the effects of dietary energy on the response to superovulation in goats. Some studies indicated that sheep nutrition has failed to affect the response to superovulation, with no differences being recorded in the number of follicles aspirated and oocytes recovered between high and low energy diet

groups (McEvoy *et al.*, 1995; Peura *et al*., 2003; Kakar *et al*., 2005; Borowczyk *et al*., 2006). On the other hand, superovulated ewes on a lower energy diet have been shown to produce fewer follicles (O'Callaghan *et al*., 2000). In trials designed to determine the effect of energy restriction or nutritional intake before and during superovulation in cattle, an increase in the number of follicles and improvement in the quality of embryos has been reported in animals fed a low energy diet, compared to those on a high-energy diet (Nolan *et al*., 1998). However, the quality of embryos matured in vitro was influenced by the restriction of feed. In this case oocytes derived from underfed animals yielded lower cleavage and blastocyst rates (Borowczyk *et al*., 2006).

The effect of nutrition and metabolic hormones on the response to superovulation has been widely documented in sheep and cattle and less in goats. While expecting an increase in the response to superovulation following supplementation with higher energy diets, the contrary was true. In sheep and cattle lower superovulatory response, in terms of animals ovulating, ovulation rate per animal ovulating and the quality of embryos recovered have been observed in animals fed diets of high energy concentrates (Blanchard *et al*., 1990; Yaakub, O'Callaghan, O'Dohertyt and Hyttels, 1997; Lozano *et al*., 2003). Poor superovulatory responses have been associated with an increase in insulin or IGF-I (insulin-like growth factor I), observed in animals on high energy intakes. In supporting this observation, it has been found that under-nutrition can reduce the concentration of IGF-I in sheep and cattle (Gong, 2002; Lozano, Lonergan, Boland, and O´Callaghan, 2003). High concentrations of insulin and IGF-I have been reported to reduce the amount of FSH needed to support the gonadotrophin-dependent follicles (Adashi *et al*., 1985; Downing and Scaramuzzi, 1991; Yaakub *et al*., 1997), this explains the low superovulatory response induced following high energy intake diets. In other trials, diets with a high urea concentration increased the embryo mortality and reduced the pregnancy rates following embryo transfer in sheep (McEvoy *et al*., 1997).

Other studies indicated that, diets supplemented with crude protein have been shown to have no effect on the ovulation rate and the number of embryos recovered following superovulation in cattle (Garcia-Bojalil *et al*., 1994; Gath, Lonergan, Boland and O'Callaghan *et al*., 1999; Mikkola *et al*., 2005). Although these results are inconsistent, it can be suggested from these observations that the type of nutrients has different effects on the response to superovulation. However, in cattle different types of concentrates fed to an animal failed to have any effect on the response to superovulation, although the type of concentrates influenced the quality of embryos recovered (Yaakub *et al*., 1997). Blanchard *et al*. (1990) supported this finding by reporting an inferior embryo quality following the feeding of excess crude protein. The effect of supplementary feeding with energy or protein concentrates on superovulated animals is also highly dependent on the body condition of these treated animals. This has been demonstrated by an observation in goats where does in a poor body condition recorded a higher superovulatory response as indicated by the increase in ovulation rate and high number of oocytes collected. Nutritional priming however reduced the number of oocytes produced in over-conditioned does (Buzzel *et al*., 2003).

Overfeeding and underfeeding can delay embryonic development and increase the embryo mortality rate following fertilisation (Parr, Davis, Fairclough and Miles, 1987; Mani *et al*., 1992; Rhind, 1992; Abecia *et al*., 1996, Abecia, Lozano, Forcada and Zarazaga, 1997). Following superovulation, *ad libitum* diets may also Reduce the quality and quantity of embryos recovered. The high energy diet intake does not only have a detrimental effect on the morphological classification and cleavage rate of the embryos produced *in vivo*, but also exerts negative effects on the development of the *in vitro* embryo collected as early as day 2 of pregnancy (Creed *et al*., 1994; Lozano *et al*., 2003). In support of these finding it has also been found that dietary restriction can increase the number of follicles and ovulation rate in superovulated heifers (Nolan *et*

*al*., 1998). These phenomena have led to the conclusion that a high-energy intake can negatively affect the embryonic development, partially before fertilisation and during the attainment of oocyte developmental competence. The effect of dietary intake on embryonic development has always been related to the circulating progesterone concentration, as this hormone is responsible for the maintenance of pregnancy (Lozano *et al*., 2003).

There are many findings consistently reporting an inverse relationship between the dietary intake and blood progesterone concentration in ruminants (Parr *et al*., 1987; Creed *et al*., 1994; McEvoy *et al*., 1995; Abecia *et al*., 2006). Animals on a high energy diet have been reported to have lower progesterone concentrations compared to those on lower dietary energy intakes or on restricted diets (Nolan *et al*., 1998; Yaakub *et al*., 1997; O'callaghan *et al*., 2000; Lozano *et al*., 2003). The decrease in blood progesterone concentration of animals on a high plane of nutrition is the result of the increased metabolism of progesterone by the liver (Symonds and Prime, 1989; Parr, 1992; Parr, Davis, Miles, and Squires, 1993; Abecia *et al*., 2006). The findings of Parr *et al*. (1993) confirmed that the mean blood flow in the portal veins of ewes fed high-energy diets was higher, when compared to ewes on low energy diets and 90% of the progesterone passing through the gut was metabolised. The reduced circulating progesterone concentration due to a high-energy intake in superovulated ewes has been reported to reduce the viability of the embryos produced *in vivo* and cultured *in vitro*. This has been emphases by the reduction in number of embryos reaching the blastocyst stage in ewes fed high-energy diets. This confirms that a lower concentration of Progesterone, as a result of feeding higher energy diets during pre-ovulatory period (superovulation), can affect embryo survival rate by altering the oocyte development and oviduct environment (McEvoy *et al*., 1995).

High concentrations of insulin and IGF-I have been reported to reduce the amount of FSH needed to support the gonadotrophin-dependent follicles (Adashi *et al*., 1985; Downing and Scaramuzzi, 1991; Yaakub *et al*., 1997). This explains the low superovulatory response induced following high-energy intake diets. In other trials, diets with a high urea concentration increased the embryo mortality and reduced the pregnancy rates following embryo transfer in sheep (McEvoy *et al*., 1997).

## 2.4. INTRINSIC FACTORS AFFECTING MOET IN GOATS

### 2.4.1 Breed

Breed or genotype has been indicated in several studies to be a factor to be taken into consideration in MOET programmes (Holness, Hale and McCabe, 1980; Donaldson, 1984; Bindon *et al*., 1986; Nuti *et al*., 1987; Baril *et al*., 1989; Dufour *et al*., 2000). It should be noted that in the first step (oestrus synchronisation) of a MOET programme, different breeds respond differently. This requires different timings of AI with respect to ovulation. When Alpine and Nubian does were compared, 87% of the Nubian goats came into oestrus 36 hours following sponge removal, while only 50% of the Alpine goats showed signs of oestrus (Nuti *et al*., 1987). The onset of oestrus has a big influence on the ovulation rate following superovulation. When this parameter was evaluated, a higher ovulation rate was recorded in alpine goats that exhibited signs of oestrus within 24 hours after progestagen withdrawal, compared to does that showed overt oestrus 24 hours following progestagen removal. To the contrary, no differences were observed with respect to the ovulation rate between Saanen does which exhibited signs of oestrus before or 24 hours following progestagen treatment termination. This observation indicates that the response to oestrus synchronisation and superovulation to be breed specific (Baril *et al*., 1986).

Besides the different times to the onset of oestrus recorded in different ruminant breeds, breed has been indicated as a major factor contributing to the variation recorded in the ovarian response to superovulation (Donaldson, 1984; Torres *et al*., 1987; Vivanco, Greany, and Varela, 1994; Ammoun *et al*., 2006). In cattle, sheep and goats the number of corpora lutea recorded and number of transferable embryos recovered following superovulation differ between breeds (Donaldson, 1984; Baril *et al*., 1989; Goel and Agrawal, 2005; Ammoun *et al*., 2006). In goats for example, a higher number of embryos (average of 10.1) were recovered in Alpine does, compared to Angora does (average of 7.5) (Baril *et al*., 1989). In addition, a higher Refractoriness was recorded in Alpine goats, compared to Nubian goats following superovulation (Nuti *et al*., 1987). However, the breed effect has been associated with the different prolificacy trends of the breeds, where a high prolific breed has been reported to respond better to exogenous gonadotrophins (Bindon *et al*., 1986). It has also been found that sheep selected for prolificacy tend to be more sensitive to gonadotrophin treatment (Kelly *et al*., 1983; Bindon *et al*., 1986). In contrast, Picazo *et al*. (1996) failed to establish a clear breed difference in ovarian response in three sheep breeds superovulated with FSH.

Breed has been recognised to have a major effect on ovarian follicular development. Even though gonadotrophin treatment increases the follicular development in all breeds, the numbers of ovarian follicles, which are recruited to ovulate, differ in the different breeds (Dufour *et al*., 2000; Ammoun *et al*., 2006). In sheep, for example, it has been observed that ewes carrying the *fec* gene have a higher ovarian follicle selection rate. Therefore, a lower ovulation rate following superovulation has been recorded in this breed, due to the high atretic rate recorded among recruited follicles. On the other hand, ewes without the *fec* gene have shown less follicular atresia, making more follicles available to be recruited and proceeding to the

ovulation stage, hence leading to a high ovulation rate following superovulation (Dufour *et al*., 2000).

### 2.4.2 Seasonal

Small ruminants are seasonal breeders that breed spontaneously during autumn to winter and in spring (short daylight length). Thus MOET is more frequently conducted during the breeding season. However, it would be a great advantage to increase the number of offspring born per donor per year by being able to conduct this programme throughout the year. However, conflicting results have been reported in studies designed to determine the effect of season on MOET. There is general agreement that season has an effect on the ovarian response to superovulation treatment. Differences have been observed in ovarian response concerning corpus luteum formation and function, LH peak and progesterone concentration as well as the number and quality of embryos recovered during the different seasons. A higher ovulation rate and the number of embryos recovered have also been recorded early in the breeding season compared to late in the breeding season in goats (Pendleton *et al*., 1986; Walker *et al*., 1989; Sebastian-Lopez *et al*., 1990; Mitchell *et al*., 2002). The number of embryos recovered and viable embryos has been shown to be highest during the breeding season. However, the differences recorded have not been that great between the breeding season and the other months of the year. This observation has produced more doubt regarding the effect of season on the ovarian response to superovulation in goats.

It has been observed that in does, the number of large unovulatory follicles following superovulation to be more prominent during the seasonal anoestrous period (Baril *et al*., 1996; Senn *et al.*, 1992; Gonzalez-Bulnes *et al.*, 2003a). In sheep however, the number of unovulatory follicles at embryo recovery following superovulation with eCG was recorded to be higher in autumn than in spring. This led to a lower number of

embryos being recovered in autumn, than in spring (Chagas de Silva *et al*., 2003). In cattle, the number of transferable embryos recovered was shown to be greatly influenced by season (Tegegne, Lahlou-Kassi and Mukasa-Mugerwa, 1997). Contrary, season in both sheep and goats have been reported to have no effect on the number of corpora lutea, large unovulatory follicles, fertilisation rate, ova and embryo recovered, quality of transferable embryos, as well as the embryonic survival rates observed following transfer. These contradictory results with respect to the effect of season on ovarian response to superovulation, may indicate that MOET programmes in small stock can be performed throughout the year, without a reduction in ovarian response to superovulation and the quality of embryos recovered (Lopez-Sebastian *et al*., 1990; Greaney *et al*., 1991; Samartzi, Boscos, Vainas and Tsakalof, 1995; Mitchell *et al*., 2002; Gonzalez-Bulnes *et al*., 2003b).To highlight the use of MOET throughout the year, Greaney *et al*. (1991) recorded a higher ovulation rate outside the breeding season in sheep compared to during the breeding season. The low ovulation rate observed during the breeding season could largely be attributed to the presence of a large follicle at the onset of the superovulation treatment. In sheep the occurrence of a large ovarian follicle at the time the first superovulation treatment was more prominent during the breeding season. This, although the presence of large follicles at the onset of superovulation did not have any effect on the ovulation rate, the number and quality of embryos recovered was reduced during the breeding season. This therefore, emphasises that follicular dominance is more profound during the breeding season than during the anoestrous period (Gonzalez-Bulnes *et al*., 2003b).

### 2.4.3 Donor Age

For MOET to be efficient in accelerating genetic improvement, one method is by shortening the generation interval. This could be achieved by utilising young animals in

the programme. Several studies have been conducted to evaluate the effect of age on the ovarian response to superovulation. There is general agreement that juveniles can be superovulated and the follicles are sensitive to gonadotrophin stimulation (Donaldson, 1984; Driancourt, Fry, Campbell and McNeilly, 1990; Rangel-Santos, McDonald and Wickham, 1991; Hasler, 1992; Kuhholzer and Brem, 1999). However, contradictory results have been reported regarding the effect of age following superovulation in young animals. A poorer response to superovulation in terms of lower ovulation and fertilisation rates, as well as lower embryo recovery and survival rates have been recorded in young females, compared to adult females (Quirke and Hanrahan, 1977; Rangel-Santos *et al*., 1991; Driancourt and Avdi, 1993). More trials evaluating the effect of age on the ovarian response to superovulation have been conducted in sheep and cattle than in goats. Ova and embryos recovered from young ewes have been reported to have a lower potential for development when compared to ova produced by adult ewes (Quirke and Hanrahan, 1977; Wright *et al*., 1981; Mcmillan and McDonald, 1985). Baril *et al*. (2000) reported adult ewes to have higher ovulation rates. In cattle, the effect of age on the superovulatory response is not always taken as a deciding factor, as there is either a too small difference in superovulatory response due to age or no effect of age on the total number of embryos recovered (Donaldson, 1984; Hasler,1992).

### 2.4.4 Reproductive Status

#### 2.4.4.1 Ovarian Status and Response Following Superovulation in Goats

Irrespective of the advances in animal management, gonadotrophin preparations and administration protocols achieve, the ovulation rate and the yield of transferable embryos following superovulation continues to vary between treatments and individual animals in the same treatment. Currently, with the aid of endoscopy and ultrasonography more studies on the variation in ovarian response to superovulation

ware to focus on an individual animal compared to in a group of animals. Via regular monitoring of the ovary of an animal in a MOET programme from the beginning of the superovulatory treatment, it was discovered that the ovulation rate and quality of embryos obtained are greatly influenced by the state of the ovarian follicles at the onset of the superovulatory treatment. These technologies are also able to indicate that the donor animals are at different ovarian status at the onset of the superovulatory treatment. This explained the occurrence of large variation in ovulation rate and embryo yield following superovulation between individual animals (Cognie *et al*., 2003; Gonzalez-Bulnes *et al*., 2003b, 2004a; Holtz, 2005).

It is well documented that the treatment of sheep and goats with high doses of exogenous FSH will stimulate the follicular growth of the so-called gonadotrophin-responsive ovarian follicles following the first FSH injection, to reach a pre-ovulatory phase. These gonadotrophin-responsive ovarian follicles are small follicles with diameter of approximately 2 to 6 mm in goats. However, the number of viable and transferable embryo is positively correlated to a narrower group of ovarian follicles (4-6 mm). This observation indicates that in goats, although all small follicles (2-6 mm) can be stimulated to grow with the aid of exogenous gonadotrophins, very small follicles (2-3 mm) are seldom reach maturity. This is supported by the findings that the ability of developmental competence in goat oocytes is acquired by follicles with diameter 3 mm or more. On the other hand, full developmental competence of oocytes is also indicated by the secretion of inhibin, the follicles capable of secreting inhibin in goats are reported to be follicles of a diameter 4 mm or more (Brebion and Cognie, 1989; de Smedt, Crozet and Gall, 1994; Gonzalez-Bulnes *et al*., 2000, 2003a, 2004b, d).

Although the administration of exogenous gonadotrophins stimulates the growth of small ovarian follicles until the pre-ovulatory and ovulation stages, it has been observed that the number of ovulations and embryos recovered is negatively influenced

by the presence or absence of a dominant follicle at the onset of the superovulatory treatment. In small ruminants, it has been observed that there are a higher number of gonadotrophin-responsive follicles when no dominant follicle is present. The presence of a dominant follicle at the onset of superovulation reduces follicular recruitment, ovulation rate, yield and the number of transferable embryos. Moreover, the presence of a dominant follicle at the start of a superovulation treatment negatively affects the ovarian follicles maturing and eventually the quality of embryos recovered (Rubianes *et al*., 1995, 1997; Lopez-Sebastian *et al*., 1999; Menchaca and Rubianes, 2002; Cognie *et al*., 2003; Gonzalez-Bulnes *et al*., 2004d; Holtz, 2005). In goats it has been found that the presence of a dominant follicle at the beginning of superovulation treatment leads to delayed of follicular recruitment and decrease number of large follicles during the oestrous period. This also suggests that the presence of a dominant follicle in small ruminants does not completely inhibit the growth of new follicles. However, it could reduce the number of new emerging follicles and suppress their subsequent growth. It was also observed that the ovulation rate in goats was reduced if superovulation treatment began when a high number of large follicles with diameter of 7 mm or more (dominant follicles) were present (Menchaca and Rubianes, 2002; Gonzalez-Bulnes *et al*., 2003a, 2004b).

The number of quality embryos obtained following superovulation is not only influenced by the presence or absence of a dominant follicle at the onset of superovulation treatment but, also by the presence or absence of a corpus luteum. It has been observed that when superovulation treatment was initiated in the absence of a corpus luteum, it led to high incidences of degenerated embryos being produced therefore, reducing the number of viable embryos at the time of recovery. These results were also supported by an increase in embryo viability and decreased incidences of degenerated embryos observed in ewes with a corpus luteum present. This emphasises

the importance of the presence of a corpus luteum for growth of ovarian follicles (Gonzalez-Bulnes *et al*., 2002b; Gonzalez-Bulnes *et al.*, 2004a, 2005; Veiga-Lopez *et al*., 2005).

In cattle, it has been found that a low concentration of progesterone at the beginning of superovulation treatment leads to a reduced number of quality embryos being produced (Callesen, Greve and Hyttel, 1988). It has also been reported that the number of viable embryos in sheep could be improved by starting superovulation treatment during the early luteal phase (Gonzalez-Bulnes *et al.*, 2005). It has therefore been hypothesised that if superovulation treatment began in the absence of a large follicle and the presence of a high number of gonadotrophin-responsive follicles in the early luteal phase when there is a presence of a corpus luteum, the ovarian response in term of ovulation rate and yield of transferable embryos to superovulation could be increased.

This situation could be created artificially, including the ablation of the dominant follicle, induction of high enough progesterone levels to suppress follicular development before superovulation, the pretreatment with a GnRH agonist or antagonist before superovulation to prevent emergence of a dominant follicle or the initiation of superovulation during the first follicular emergence after ovulation. In conclusion, it is important to understand the pattern of ovarian follicular development and hormonal fluctuations before creating an ideal time for starting superovulation treatment (Bungarts and Niemman, 1994; Cognie, 1999; Menchaca and Rubianes, 2002; Rubianes *et al.*, 2003; Holtz, 2005).

#### 2.4.4.2 Day 0 Protocol

The Day 0 protocol involves the introduction of the superovulation treatment on the day of ovulation (day 0) or soon after ovulation to target the pre-wave FSH increase or emergence of a follicular wave before the selection of a dominant follicle (Rubianes *et al*., 1997; Rubianes and Menchaca, 2003; Gonzalez-Bulnes *et al*., 2004a; Holtz, 2005). The superovulation treatment of day 0 protocol at the absence of in the doe could be initiated under these circumstances the variation in the ovarian response could be minimised. For example, the number of large follicles recruited and the number of ovulations recorded following superovulation have been increased in goats using a day 0 protocol at the onset of oestrus when compared to a superovulation treatment initiated on day 3 after ovulation. The number and quality of the embryos were, however, not evaluated (Menchaca and Rubianes, 2002; Rubianes and Menchaca, 2003). In sheep, the number of ovulations and transferable embryos were higher when implementing a day 0 protocol compared to initiating a superovulation treatment on day 3 after ovulation. From the literature it is clear that more research is needed in goats with respect to the utilisation of this day 0 protocol - as the high ovulation rate achieved does not necessarily lead to high fertilisation rate and embryo viability (Rubianes *et al*., 1997).

## 2.5 FACTORS INFLUENCING THE SURVIVABILITY OF TRANSFERRED GOAT EMBRYOS

### 2.5.1 Synchronisation Between Stages of Embryo Development and Recipient Reproductive Tract Environment

Final stage in the MOET programme is embryo transfer. Usually embryos are transferred to recipients, which have shown signs of oestrus, concurrently with their respective donors (Moore, 1974; Bessoudo *et al*., 1988; Wallace, 1992). It is clear that

the synchronisation of the donor and the recipient's oestrous period is extremely important to achieve a favourable environment for the development of the transferred embryos. Optimal embryo survival rates have been obtained when the onset of oestrus of the donor and the recipients is within 24 hours of each other. However, acceptable embryo survival rates have also been obtained even when the recipients show oestrus signs between 12 and 48 hours before or after their respective donors (Moore, 1974; Moore and Eppleston, 1979; Ishwar and Memon, 1996; Oppenheim *et al*., 2000; Holtz, 2005). Besides the synchrony of oestrus between the donor and recipients, there are other fundamental parameters of the goat recipients influencing the survival rate of the embryos transferred e.g. health, the reproductive status, nutrition status, breed, as well as the age and parity of the recipient. For example, a low embryo survival rate has been reported in goats undernourished before and after embryo transfer compared to recipients on a maintenance diet. In addition, poor pregnancy rates have been reported in recipient goats in which embryos were transferred outside the natural breeding season (Mani *et al*., 1994; Gonzalez-Bulnes *et al.,* 2004b; Holtz, 2005). In sheep, the survival rate of embryos has been reported to be 52% in young recipients compared to 73% adult ewes. In contrast, other findings have reported the embryo survival rate to be higher in yearling recipients compared to multiparous dams (2 to 6 years of age), as well as similar survival rates in all age groups (Wallace, 1992; Mani *et al.,* 1994; Gordon, 1997; Bari *et al*., 1999, 2003). There are several other factors influencing the survivability of embryos following transfer. These include the stage of development of the embryo transferred, the number and quality of embryos transferred, the embryo origin, the presence and the number of corpora luteum present (Armstrong, Pfitzner, Warnes and Seamark, 1983c; Ishwar and Memon, 1996; Bari, Haresign and Merrell, 2003).

### 2.5.2 Embryonic Factors

#### 2.5.2.1 The Origin of the Embryos Transfered

The production of *in vitro* embryos can be utilised to increase the number of the embryos for transfer in a MOET programme. Advanced research on the production of *in vitro* goat embryos provides an alternative to unpredictability response of the superovulation as a source of *in vivo* embryos for transfer in a MOET programme. The first kids born after complete *in vitro* maturation, fertilisation and culture were reported by Keskintepe *et al*., (1994) and Pereira *et al*., (1995). During the following years, there were only haphazard reports on *in vitro*-derived offspring (Poulin *et al*., 1996; Traldi *et al*., 1999; Cogniè *et al*., 2001). Baldassarre *et al.,* (2003) reported the birth of an appreciable number of 150 *in vitro*-derived kids.

For the production of *in vitro* embryos, mature oocytes may be recovered from the oviduct of the donor within 5 hours following ovulation. However, this procedure is infeasible due to difficulty of detecting the exact time of ovulation. In goats, immature oocytes recovered from the ovaries obtained from an abattoir or from life animals through ovarictomy surgery, ultrasound guided either transvaginal aspiration or laparoscopy aspiration (Baldassarre *et al*., 1994; Pawshe, Totey and Jain, 1994; Graff *et al*., 1999; Crozet, Dahireland and Gall, 2000; Han *et al*., 2001; Reggio *et al*., 2001; Baldassarre *et al*., 2003; Baldassarre and Karatzas, 2004). In brief, recovered oocytes undergo *in vitro* maturation (IVM) before being exposed to in vitro-capacitated sperma to be *in vitro*-fertilised (IVF fertilised embryos are cultured *in vitro* for 5–7 days (IVC) and, upon reaching the morula or blastocyst stage, transferred to recipients or cryopreserved for future use (Crozet *et al*., 1995; Izquierdo, Villamediana and Paramio, 1999; Ongeri *et al*., 2001; Cogniè *et al*., 2003; Koeman *et al*., 2003; Baldassarre *et al*., 2003a).

A special version of *in vitro* fertilisation is the injection of single spermatozoa into the cytoplasm of *in vivo* or *in vitro* matured oocytes. The technique, called "intracytoplasmic sperm injection" (ICSI), requires a micromanipulation unit and a skilled operator. Amongst other advantages the attraction of the technique lies in the avoidance of potential polyspermia (Palomo, Izquierdo, Mogas and Parami, 1999; Bhatia, Wang, Baldassarie and Keefer, 2002) and the intriguing perspective of being able to predetermine the sex of the offspring by using spermatozoa sex sorted by flow cytometry (Parrilla, Vazques, Roca and Martinez, 2004). When it will be possible to utilise sperm as carriers for altered chromosomal material, ICSI could also become a useful way of generating transgenic animals (Perry *et al*., 2001). The first live kid born to 1 of 4 recipients receiving 6 ICSI-derived two cell embryos each was reported by Wang *et al*. (2003). Part of the success of Wang *et al*. (2003) might be due to their cutting of the sperm tail distal to the mid piece, which seems to be sufficient to accomplish the necessary destabilisation of the sperm membrane without an exorbitant medium influx.

Low survival rate after transferred were reported of *in vitro* produced embryos, when compared to *in vivo* produced embryos (Greve, Avery and Callesen, 1993; Cognie, 1999). In goats, *in vitro* produced embryo survival rates of 47% have been recorded compared to 71% for *in vivo* produced embryos. Several factors attributed to the structural and biochemical differences components have been indicated to contribute to the low survivability of *in vitro* produced embryos, when compared to the *in vivo* derived embryos (Cognie *et al*., 2003). The main structural components factors differences are in the gene expression observed between the *in vivo* and *in vitro* produced embryos. The cell-cell coupling has been prominent in the *in vivo* produced blastocysts compared to blastocysts produced *in vitro*. The cell number of *in vitro* produce embryos has also been observed to be slightly lower then *in vivo* derived

embryos. In addition, the inner cell mass (ICM) from *in vitro* produced embryos are also lower and less compact due to a higher degree of vacuolisation and have fewer and shorter junction complexes between the cells. These differences between *in vitro* and *in vivo* derived embryos in goats are similar to those observed in other ruminant animals. The *in vitro* produced embryos have been reported to have a darkened appearance. This contributed by biochemical differences components and in relatively more intracellular lipids in relation to protein and small ICM and a more fragile zona pellucida compared to *in vivo* derived embryos (Iwasaki *et al.*, 1990; Greve *et al*., 1993; Pollard and Leibo, 1994; Thompson, 1997; Lonergan *et al*., 2001; Papadopoulos *et al*., 2002).

The size of the ovarian follicles used to produce an *in vitro* embryo may also reduce the survival rate following embryo transfer. This is indicated by the low blastocyst development rate observed in oocytes from small follicles compared to large follicles. Blastocysts from small and medium sized goat oocytes possessed a smaller number of cells as well as ICM, which demonstrates a poor subsequent developmental capacity (Koeman *et al*., 2003). It is, therefore, essential to selected oocytes from large follicles for producing embryos *in vitro*. In goats, the survival rate to blastocyst of embryos developed *in vitro* from follicles that larger >5 mm was higher than the survival rate of embryos from oocytes from smaller follicles (2-3 mm) (Crozet *et al*., 1995; Cognie, 1999).

#### 2.5.2.2 Method of Embryo Transfer

Embryos have been normally transferred by surgical, laparoscopic and transcervical methods (Bessoudo *et al*., 1988; Flores-Foxworth *et al*., 1992; Besenfelder *et al*., 1994). In goats, most embryos are transferred surgically into the oviduct or uterine horn. The reproductive tract being exteriorised via a mid-ventral laparotomy, which allows for the inspection of the ovaries for presence of corpora luteum (Moore, 1974; Armstrong and

Evans, 1983a; Kiessling, Hughes and Blankevoort, 1986; Bessoudo *et al*., 1988; Li, Cameron, Batt and Trounson, 1990; Selgrath *et al.*, 1990; Yuswiati and Holtz*et*, 1990; Wallace, 1992; Holtz, 2005; Guignot *et al*., 2006). However, the laparoscopic method of embryo transfer has lately gained popularity in small ruminants, the reasons being that it is safe, easy and quick to perform and it creates the opportunity to visually confirm the presence and quality of corpora luteum before transfer. This leads to survival rates similar to surgical embryo transfer. In laparoscopic embryo transfer a small incision is made as opposed to surgical Transfer which involves the exterioration Of the tip of the uterine horn in which embryo transfer is going to be made (Kraemer, 1989; Stefani *et al*., 1990; Brebion *et al*., 1992; Flores-Foxworth *et al*., 1992; Besenfelder *et al*., 1994; Ishwar and Memon, 1996; Cognie, 1999). Transcervical embryo transfer in goats has been tried with success. When comparing the pregnancy rates following transcervical and laparoscopic embryo transfer no significant differences were found. However, the transcervical embryo transfer method is not popular due to the low embryo survival rates generally obtained. This is because this method does not allow for the confirmation of the presence of corpora lutea. Moreover, trancervical transfers have been reported to induce contractions of the cervix and the uterus, which result in the expulsion of the transferred embryos. In cattle, embryos were found in the vagina a few hours following transfer (Rowson, 1971; Flores-Foxworth *et al*., 1992; Wallace, 1992; Cognie, 1999).

#### 2.5.2.3 Stage of Development and Quality of Embryos

The stage of development and quality of the embryos have a significant effects on the subsequent survival rates after ET. Selections of the best embryos at proper stage of development for transfer improve the pregnancy rates. In Katjang, Cashmere-Angora and Saanen goats that received superovulation treatment, embryos at the 4-cell, 8- to 16-

cell, morula to early, expended and hatching blastocyst stages were collected at 48, 96, 120,144 and 168 hours after ovulation, respectively (Rosnina, 1989; Sasada *et al.*, 2001). Non-invasive embryo assessment is normally used in evaluation of the recovered embryos. This assessment based on simple methods of observation, focused on morphological appearance and dynamics of embryo development. Several parameters corresponding to embryo qualities are examine under contrast-phase microscope to predicting embryo quality. Generally, the appearance of cytoplasm (pitting, vacuoles and halo effects) and zona pellucida; cleavage; number of blastomeres in particular days of recover; size, symmetry and fragmentation of blastomeres; compaction and expansion of blastomeres are the parameters influence the selection of the good quality embryos.

The high number of blastomeres in the embryo correlated with the higher success of implantation rates. This is because the frequency of mitotic divisions has related to developmental potential of the embryo (Baczkowski *et al*., 2004). In goats, embryos are generally collected on days 7 to 8 following ovulation and are expected to be at the morula to blastocyst stage. However, goat embryos recovered at this time are always variable in development stages and quality, even following the synchronisation of the onset of oestrus and even for the same individual (Baril *et al*., 1996; Yang, Tan and Qin, 1991; Gordon, 1997). Eight or less blastomeres on the 7 or 8 days of recovered indicates the extremely low developmental potential and subsequently very small chance for development after transfer. This embryo is often associated with the arrest of divisions, also called developmental block. The transfer of blastocysts always leads to a higher survival rate compared to the morula stage in ruminants. No significant differences in embryo survival rate between the transfers of morula or blastocyst embryos were reported. However, when advanced blastocysts collected on day 5 were transferred, a higher survival rate was obtained compared to the transfer of arrested

morula embryos collected on day 6. For this reason, the transfer of embryos with arrested growth must be avoided in order to have good embryo survival rates (Donaldson, 1985; Breuel *et al*., 1993; Wallace, 1992; Bari *et al*., 2003).

Beside the number of blastomeres in particular days of collection, embryos are divided into classes, depending on several morphological parameters, the appearance of blastomeres and the presence of cytoplasm defects or fragmentation are the most frequent criteria used. The good quality embryos have many blastomeres and no, or negligible fragmentation. Although most of the accounted for variation in developmental stages of embryos recovered were associated with the donor animals, the grading of embryo quality is more subjective and limited by evaluator bias (Callesen, Lovendahl, Bak and Greve, 1995). It is difficult to explain the differences in survival rate between grades 1 and 2 embryos. However, in small ruminant, a lower pregnancy rate has been reported following the transfer of grades 3 and 4 embryos compare to 63–75.6% pregnancy rate of grades 1 and 2 embryos (Geyling *et al*., 2002; Bari *et al*., 2003; Angela, Prefac, Paul, Alexandru and Dana, 2006).

#### 2.5.2.4 Number of Embryos Transfered

The number of embryos transferred has an effect on the survival rate of embryos in goats. It has been reported that the survivability of the embryos is higher when two embryos are transferred compared to the transfer of 1 or 3 embryos (Moore, 1974; Armstrong and Evans, 1983; Tervit *et al*., 1986; Ishwar and Memon, 1996; Gootwine *et al*., 1997; El-Gayar and Holtz, 2001, 2005). The survival rates following a twin embryo transfer has been reported to even improve when the unilateral transfer, where 2 embryos are transferred to the same oviduct compared to bilateral transfer (one embryo transfer to each oviduct). This may suggest that unilateral embryo transfer induces a stronger maternal signal of recognition of pregnancy to the endometrium (Armstrong

and Evans, 1983; Ishwar and Memon, 1996). Although there is a general agreement regarding a higher embryo survival rate following 2 embryos being transferred, other studies have shown no difference with respect to the survival rate following a single or twin embryo transfer. The survivability of embryos has also been shown to decline, when 3 or more embryos were transferred per recipient (Moore, 1974; Bessoudo *et al*., 1988; Stefani *et al*., 1990; Ishwar and Memon, 1996).

### 2.5.3 Recipient Factors

#### 2.5.3.1 Superovulation Rate

Superovulation rate had little or no effect on survival of transferred embryos, which developed into normal embryos or lambs (Moore, Rowson and Short, 1960). Embryo survival as a function of recipient ovulation rate has been studied by these authors. This is confirmed by Bari *et al*. (2003) and found that the there was no difference in embryo survival rate following transfer to recipients with different number of corpora lutea. In contrast, Armstrong and Evans (1983) observed increased ($P < 0.01$) survival of embryos number of ovulations in recipients increased. In goats, embryos are mostly transferred into the oviduct or uterine horn ipsilateral to the ovary, with at least 1 normal CL (Moore and Eppleston, 1979; Bessoudo *et al*., 1988; Stefani *et al*., 1990; Besenfelder *et al*., 1994; El-Gayer and Holtz, 2001; Holtz, 2005; Guignot *et al*., 2006). In goats, the embryo survival rate ares not affected when transferring 1embryo to each uterine horn or by transferring both embryos to the uterine horn ipsilateral to the ovulation point in unilaterally ovulated goats (Rowson, 1971; Tervit, 1987). Besides transferring embryos to the uterine horn ipsilateral to the ovary that ovulated, the number of corpora lutea at transfer can also influence the embryo survival rate. In goats, the embryo survival rate has been positively correlated with the number of normal corpora lutea present in the recipient during transfer. This has been demonstrated where

the embryo survival rate in a recipient with one ovulation was lower when compared to a doe recipient with 2 or 3 corpora lutea. However, the embryo survival rate between recipients with 2 or 3 ovulations did not differ. The utilisation of rectal ultrasonography could help in evaluating the ovulation site of the recipient and giving a clear indication of the development of the corpus luteum (by the measuring of the diameter). This technique could also help to evaluate the state of the uterus, especially for pathological symptoms (Cox *et al*., 1998; Santiago-Moreno *et al*., 2001; Gonzalez-Bulnes *et al.*, 2004a).

#### 2.5.3.2 Health

Just as in the other species, health of donor and recipient does are of paramount importance for assuring satisfactory outcomes of embryo transfer programme (Mani *et al*., 1994). Failures of early interspecific and intraspecific pregnancies following ET were explained as an immunological rejection as evidenced histologically by the infiltration of large numbers of leucocytes into the placenta and uterus (Dent, McGowen and Hancock, 1971; McGovern, 1973). The occurance of pregnancy failure was also due to lack of trophoblastic stimulation at the decidua with subsequent foetal death due to failure of suppression of maternal cytotoxic lymphocyte activation (Clark, Croy, Rossant and Chaouat, 1986). However, when immune-mediated failures have been suspected, most pregnancies are reported lost in the first half of gestation (McGovern, 1973; Clark *et al.*, 1986, Buckell *et al.*, 1990). Other possible reason for poor pregnancy rate that worth to be considered are the stress. These stress will either due to transportation, feed, animal handling and farm management. The stress of recipient doe due to transportation greatly affected the recipient pregnancy. This stress was unavoidable and led to negative effects on the efficiency of synchronised oestrous treatment and the pregnancy rates ( Dobson *et al*., 2000; Quan *et al*., 2010). With goats

being seasonal breeds in temperate country, poor conception rates were also observed with recipients induced to come into oestrus out-of-season.

#### 2.5.3.3 Site of Transfer

Embryos are normally transferred into the oviduct or uterine horn, depending on the age or stage of development of the embryo being transferred. Embryos at early embryonic stages (8-16 cell stage) or embryos collected from day 3 to 5 following mating are usually transferred to the oviduct (Armstrong *et al.*, 1983c; Bessoudo *et al.*, 1988; Wallace, 1992; Holtz, 2005). When the site of transfer was evaluated it was found that a higher the survival rate was obtained if 4 to 8-cell embryos are transferred into the oviduct than when the embryo is transferred into the uterine horn. The poor survival rate of an early stage embryo transferred into the uterine horn is indicative of an unfavourable environment for the development and survival of the embryo (Moore and Shelton, 1964; Armstrong *et al*., 1983b; Ishwar and Memon, 1996). Embryos at a more advanced stage of development, i.e. from day 5 and later after mating are generally transferred into the lumen of the uterine horn at uterotubal junction (Wallace, 1992; Ishwar and Memon, 1996; Holtz, 2005). In goat MOET programmes, embryos are generally transferred into the uterine horn of recipients, at days 6 to 7 following oestrus. This is to accommodate the transfer of frozen-thawed embryos normally at the morula to hatched blastocyst stage and also because of a higher survival rate being obtained at this embryonic stage (Traldi *et al.*, 1999; El-Gayer and Holtz, 2001; Guignot *et al.*, 2006).