CHAPTER 5

5.0 DISCUSSION

5.1 EFFECT OF DOSAGES AND ADMINISTRATION PATTERNS OF oFSH ON OVARIAN RESPONSE IN SUPEROVULATED DONORS GOATS

In this experiment, all does in multiple injection groups were successfully superovulated (more than 2 CL per doe), except 38% of doe in single injection group. The total ovarian response (CL plus anovulatory follicles), mean number of CL, AF and structures recovery rates (embryos plus ova/doe) per doe were significantly higher in multiple administration group than single injection group. However, there was no significant difference between multiple injection groups. There was no significant differences were observed in number of unfertilised ova and viable embryos in all treatment groups.

The results from the current experiment indicate that it is feasible to utilise oFSH as exogenous gonadotrophin in inducing ovarian response in local doe, administrated in multiple injections of equal low total dosage (M_1; 8.8 mg) or decreasing high total dosage (M_2, 14.1 mg). It is indicated that a single administration of 8.8 mg oFSH in saline protocol was not effective in inducing superovulation and *in vivo* embryo production in local does. The ineffectiveness of this treatment was likely to be attributable to the high circulating levels of the gonadotrophin into the bloodstream in short period of time, due to the administration of total dosage (8.8 mg oFSH) all at once, in advance compared to the normal physiological events regulating ovulation rate. The limited exposure time of oFSH short half-life in single injection coincided with the induction of luteolysis by $PGF_{2\alpha}$ injection in this study might also cause saline unable to maintain sufficient oFSH activity to sustain growth of multiple follicles and ovulation (Holtz, 2005; Ammoun *et al*., 2006). This resulted insufficient ovarian stimulation and

poor recruitment and development of ovarian follicles to the ovulatory stage. In unsuccessful superovulation treatment, the inadequate ovarian stimulation usually occurs when dosages of exogenous gonadotrophin administration were not sufficient to overcome the dominant follicle effect present at the time of superovulation initiation, as the dominant follicle is known to suppress growth and recruitment of new ovarian follicles (Driancourt, 2001; Gonzalez-Bulnes *et al*., 2003a, 2004; Senger, 2003). A low ovarian response following superovulation has also been reported in single administration of oFSH in saline of other MOET programme (Knights *et al*., 2003*)*.

In multiple injection groups, no significant differences were observed in total number of ovarian response, ovulation (CL), AF per doe, structure recovery and viable embryo per doe in low and high total dosages of FSH groups. This finding was agreeable with other study in Katjang goat, the obvious reduction in formation of CL and AF were observed in does treated with 20 mg pFSH compared to constant result in 10 and 15 mg of pFSH (Rosnina, 1989). The negatively effect by high dosage of gonadotrophin were also reported in ewes and goat (Martemucci *et al*., 1995; Pendleton *et al*., 1992). This suggested that it may be related to excessive follicle stimulation that inducing high circulating levels of estrogen during the early luteal phase. The accelerated follicular develop may have led to early ovulation of follicles before the granulosa cells acquired maturity required to luteinise properly in response to the ovulatory LH surge (Armstrong *et al.*, 1982). This may explained that although high number of structure recovered, constant in number of viable embryo retrieval in high compared to low dosage in multiple injection group. However, Rosnina (1989) observed increasing number of ovulation and AF with increased in dosage of eCG in Katjang goats. This may indicate that different gonotrophins preparation behave differently in goats superovulation.

In this experiment, the other explanation for constant result in superovulation response in high compared to low total dosages group is the pattern of oFSH administred in superovulation protocols. Torres , Cognie and Colas (1987), who were the first to evaluate a protocol for using decreasing doses of FSH (non-purified porcine FSH) to superovulate ewes, reported greater superovulation rates when a regimen of decreasing doses was used. The lesser amount of LH in commercial batches of oFSH seems to justify the manufacturer recommendation to use a protocol of constant dosages, rather than one of decreasing doses, as used with non-purified preparations. Similarly, Mancheca ewes injected with pFSH in constant-doses had less follicular development and a lesser ovulation rate than did ewes receiving decreasing doses (Gonzalez-Bulnes *et al.*, 2000a, 2002), which is probably because the decreasing-dose protocol mimics better the "wave like pattern" of FSH secretion. In contrast, a study of Berlinguer *et al*. (2004) found that the ovarian response was not influenced by whether FSH was administered in equal or decreasing doses. Furthermore, FSH given in a constant-dose regime tended to produce more embryos. These authors concluded that the initial greater doses of FSH in a decreasing dose protocol can induce rapid and abnormal ovarian follicular development and thereby, the de-synchronisation of oocyte-follicle growth, causing a reduction in embryo quality.

In the current experiment, the mean number of CL per doe in multiple oFSH administration protocol (M_1,11.6+1.0; M_2, 16.9+3.9) recorded in this study is comparable to those previously reported in goats superovulated with either eCG (Amstrong *et al*., 1982, 1983a, 1983b; Pendleton *et al*., 1992), pFSH (Amstrong *et al*., 1982, 1983b, Pendleton *et al*., 1992, Pintado, Gutierrez-Adan and Perez Llano, 1998; Nowshari *et al*., 1995; Greyling *et al*., 2002, Lehloenya and Greyling, 2010) and oFSH (Gootwine *et al*., 1997; Lee *et al*., 1997; Gonzalez-Bulnes *et al*., 2003a, 2004a), which ranged from 10 to 18. Nevertheless, the mean ovulation rate recorded in this study was

higher than the 8.3 ± 1.8 ovulations per doe as reported by Selvaraju, Agarwal, Karche, and Majumdar (2003) in mixed goat breeds as well as the 10.2 ± 3.1 ovulations recorded in Tellicherry goats treated with the pFSH (Senthil Kumar *et al.*, 2003). On the other hand, slightly lower ovulation was observed in the present experiment compared to 21.3 ± 5.9 in Boer goat (Lehloenya *et al*., 2006).

The occurrence of abnormal CL development that indicated premature luteal regression was a phenomenon common in goats following FSH superovulation treatment (38.1% and 12%; Lehloenya *et al*., 2006, 2009; Selvaraju *et al*., 2003). However, this occurrence was absent in this study. This phenomenon is higher in pFSH and eCG treatment groups than oFSH group (McNatty *et al*., 1989; Pendleton *et al.*,1992; Sentil Kumar *et al*., 2003). Saharrea *et al.* (1998) reported that premature luteal regression in 57.5% of does superovulated with eCG. The presence of healthy corpus luteum in oFSH superovulated donor goats in this experiment is reflected by the normal endogenous surge of LH from the pituitary at appropriate time. However, estimation of LH level in local goat is needed to establish this fact.

In the present experiment, high number (86%) of oFSH superovulated does formed at least one AF at embryo recovery, represented 43% of the overall total ovarian response in all treatment groups. Although a low ovarian response, the percent AF for oFSH treatment was significantly higher in single administration groups. In does received multiple administration, the presence of anovulatory follicles was lower in high oFSH dosage group compared to low dosage group. Regardless the pattern of oFSH superovulatory treatment, the mean number of AF remained relatively constant at higher dosages of oFSH, thereby confirming observation for pFSH (D' Alessandro *et al*., 1996). Even though no significant differences were observed, the structure recovery and viable embryo per doe were highest in does without AF. These results indicated that the superovulation yield was affected by the presence of females with ovaries having

combination of ovulations and anovulatory follicles. This is also a common finding in any superovulatory protocols used (Nowshari *et al*., 1995; Gonzalez-Bulnes *et al*., 2005; Veiga-Lopez *et al*., 2006; Simonetti *et al*., 2008). Some follicles grow to preovulatory sizes in response to the administration of exogenous gonadotrophins, but fail to ovulate afterwards. In ewes, the incidence of ovulatory failures can reach up to 60% with the use of non-purified FSH (Gonzalez-Bulnes *et al*., 2000a). Source and purity of the gonadotrophin was identified as causal factors for anovulation (Woollen *et al*., 1985, Lindsell *et al*., 1986), being the presence of LH in the FSH preparation is the major cause (Nowshari *et al*., 1995; Murphy *et al*., 1984). However, with the use of purified oFSH (Ovagen) in current study, the presence of anovulatory follicles is unavoidable (28–60% of the preovulatory follicles depending on superovulatory protocols). This findings support the idea that whatever FSH preparation and protocol of administration is used, superovulation is a highly disturbing process for endocrine and ovarian function (Gonzalez-Bulnes *et al*., 2003, 2004).

The functionality of follicles present in the ovaries at the onset of the superovulatory treatment may be another cause for ovulatory failures (Gonzalez-Bulnes *et al*., 2003). The high supply of exogenous FSH used in a superovulatory treatment induces a large increase in the number of gonadotrophin-responsive follicles growing to ovulatory stages. In goats, FSH stimulated the growth and ovulation of all healthy follicles larger than 2 mm in diameter. On the other hand, some of the follicles stimulated to grow by the exogenous FSH may be in arrest or early stages of atresia and more likely to be those of smaller in diameter (Murphy *et al.*, 1984; Gonzalez-Bulnes *et al*., 2002). However, although atretic follicles can be promoted to grow like normal immature but healthy follicles, further development of these rescued atretic follicles is inadequate, leading to ovulatory failure (Rubianes *et al.*, 1997).

The present results indicate that the mean number of structures (ova plus embryos per doe) retrieved (5.0, 3.2 and 0.5), were found to be lower compared to the 13, 7.1 and 18.0 recorded in Alpine, dairy goats and Boer superovulated does using pFSH (Selgrath *et al.*, 1990; Pendleton *et al.*, 1992; Lehloenya *et al*., 2008) or 11.3 and 10.7 obtained with oFSH in Murciano-Granadina goats (Gonzalez-Bulnes *et al*., 2003, 2004b). These differences may be attributed to breed, age, nutritional status, season, lactation requirements and the physiological condition of the goat differences that have been previously associated with the prolificacy of a doe (Baril and Vallet, 1990). The intrinsic to the superovulatory agents in the embryo industry, due to variation such as the batch and preparation of gonadotrophins, and nutritional status of the donors are factors not to be ignored (Holtz, 2005; Cognie, 1999). Flushing technique could also be the major limiting factor contributing to these differences in structures (embryo plus ova) recovery rate. Since the donor does in this experiment were randomly selected, the other reason for lesser recovery and fertilisation rates would be associated with a tendency of the donor does to produce more large follicles and/or probably with greater oestradiol production.

Previous studies (Jabbour and Evans, 1991b; Mahmood *et al*., 1991; Chagas de Silva *et al*., 2003) shown that a larger total dosage of gonadotrophin may allow the development of large unovulated follicles that persist after ovulation, since long exposure of gonadotrophin. Such follicles, together with their relatively greater individual hormonal production can produce abnormally elevated concentration of oestradiol (Jabbour and Evans, 1991a), which can affect the uterine environment and, thus, interfere with ova captured by the fimbria or with the transport of ova and sperm through the female genital tract (Evans and Armstrong, 1984). This is agreeable with the finding of this experiment where there is no significant increase in the number of viable embryo were observed although high number of structures recovered in multiple

administration of high oFSH dosage compared to low oFSH dosage (2.0 vs. 2.4 embryos, respectively). It is probably that the efficiency of ova captured by the fimbria was impaired in these hormonally superovulated does. This is consistent with the general pattern of an association between a greater ovarian stimulation and a decrease in number of oocyte and/or embryo production (Armstrong and Evans, 1983; Martemucci *et al*., 1995; Thompson *et al*., 1995; D'Alessandro *et al*., 2005). Similar to the present study, previous reports involving FSH-eCG co-treatments also found failures in the rates of fertilisation and recovery (Leoni *et al*., 2001) in high total dosage of gonadotrophin.

5.2 EFFECT OF THE BREED ON OVARIAN RESPONSE IN SUPEROVULATED DONORS GOATS

It is indicated in other ruminants that highly prolific breeds are more sensitive to gonadotrophin stimulation and hence respond better than the less prolific breeds (Kelly *et al*., 1983; Bindon *et al*., 1986). The previous reports on the breed differences in superovulatory response would be mainly explained in terms of response to exogenous oFSH rather than to any differences in the dynamics of FSH absorption and clearance. Highly prolific breeds having a greater response to exogenous stimulation toward high ovulation rates have a greater superovulatory (Ammoun *et al*., 2006).

The results from this experiment showed that although not significantly different in terms of ovarian response and superovulation yield in oFSH superovulated Malaysian goats, the local mixed-breed was observed to produce high ovarian response and viable embryos per doe compared to newly introduced goats, for examples Boer crossbred and Jamnapari does. This would possibly be attributed to the same FSH treatment being given to all donor does, the heavier newly introduced crossbred goats and lighter local mixed-breed. On the other hand, the mean number of viable embryos per donor

recovered from this experiment (2.2, 1.1, 1.5 for local mixed-breed, Boer crossbred and Jamnapari, respectively) were respectively lower than the mean number of viable embryos reported (6.3, 12.3, 13.1), following superovulation in Murciano-Granadina, Tellicherry and Boer goats (Gonzalez-Bulnez *et al*., 2003a; Sentil Kumar *et al*., 2003; Lehloenya *et al*., 2008). This phenomenon may be due to the physiological difference between local goats and other breeds.

5.3 EFFECT OF THE BODY WEIGHT ON OVARIAN RESPONSE IN SUPEROVULATED DONORS GOATS

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 (Ashworth, 1995; Cox, 1998; Butler, 2000; Boland *et al*., 2001; Lucy, 2003; Peura *et al*., 2003; Paula *et al*., 2005). 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 *et al*., 1994). 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 *et al*., 1997; Lozano *et al*., 2003). The result from this experiment however were contradictory to previous findings by other researchers, whereby the present study no differences were observed in ovarian response and superovulation yield for both low and high body weight (30 kg and less vs. 31-50 kg) of donor goats. This insignificance found in this study could be due to confounding effect between the breeds, age and management interactions. For

example, it was frequently observed that the expected high body weight breed at particular age had unexpectedly lower body weight and vice versa, and this situation is beyond the control of the present researcher.

5.4 EFFECT OF THE DAY OF EMBRYO RETRIEVAL ON THE STAGES OF EMBRYO DEVELOPMENT AND THE QUALITY OF THE EMBRYOS

In the present study, the administration of oFSH for superovulation confirms the significant contribution follicular recruitment and subsequent ovulation in goats. However, the effect of exogenous gonadotrophin injections resulted in greater ovarian response (CL plus AF per doe) particularly number of CL signifying ovulation at day 7 compared to day 3 after CIDR withdrawal. This is in agreement with the finding reported by previous researchers (Gootwine *et al*., 1997; Lee *et al*., 2000; Gonzalez Bulnes *et al*., 2002). The reason for this phenomenon is not clear at this time. It could be due to delayed in ovulation after the CL withdrawal, hence resulting in more CL observed at day 7. However, specific study is necessary in the future to elucidate this effect.

Even though ovarian response in oFSH superovulated does was higher at day 7 after CIDR withdrawal than that of at day 3, conversely the rate of recovered structures was lower in the former. This could be due to developmental incompetence of the embryos during the period of development from days 3 to day 7, resulting in degeneration and death of embryos. Hence, the number of viable embryos obtained from both flushing was insignificantly different. The result for structure recovery for oviduct flushing in this study was higher than other reports (Gootwine *et al*., 1997; Lee *et al*., 2000), however, the structure recovery rate for uterine flushing in this study was comparable with those of other researchers (Cervantes *et al*., 2007; Gonzalez-Bulnes *et al.*, 2003a). Previous studies using eCG as superovulatory agent in local Malaysian

goats resulted in 57, 48, 98 and 32% of structure recovered during uterine flushing for 500, 800, 1,000 and 1,500 IU concentration (Rosnina, 1989; Shamsul, 1997). Apparently, increase in the dose of gonadotrophin would increase the rate of structure recovery up to a limit after which the response would be negative. This could be due to suppressive negative feedback of ovarian response and hormonal imbalance caused by abnormal high level of gonadotrophins during superovulation.

In presence of a large number of anovulatory follicles for a prolonged duration before flushing would result in an increase level of oestrogen creating a hostile uterine environment for sperm, oocytes or embryos (Saumande *et al*., 1984; Bevers *et al*., 1989; Gonzalez *et al*., 2004). In other words, the prolonged period of oestrogen stimulation and lower FSH levels could contribute to this phenomenon. Therefore, it could be explained that the oocytes are fertilised, but the viability of the embryo for longer period could not be sustained. Although rate of structure recovery was higher in oviduct flushing, the rate of non-viable embryos (or unfertilised ova) recovered was also higher compared to the uterine flushing. The higher number of unfertilised ova in the oviduct flushing may have been caused by factors related to a poor fertilisation rate (e.g. uterine environment or buck fertility), abnormally earlier and higher level of oestrogen levels released during very early stage of fertilisation and factors related to poor synchronisation (hormonal imbalance) and the unsynchronous timing of ovulation. Other suggested reason that although the oFSH administration can induce follicles to grow and ovulate, the recruited oocytes do not attain complete maturation that are generally recruited from the follicles less than 2-3 mm in diameter size. Furthermore, in goats, it has been found that viable embryos are only associated with the recruitment of follicles of 4-6mm size in diameter (Gonzalez-Bulnes *et al.*, 2003a, 2004a).

The results from oFSH superovulation in Cashmere-Angora and Saanen goats indicated that embryos at the 4-cell, 8 to 16 cell, morula to early blastocyst, expanded blastocyst and hatching blastocyst stages were collected at 2, 4, 5, 6 and 7 days after ovulation, respectively (Rosnina, 1989; Sasada *et al.*, 2001). The results of this study, in which different breeds were used, showed that the time schedule of embryo development is comparatively similar. The mean number of viable embryos of acceptable quality (Grades 1 and 2) in this study was higher other reports involving other goat breeds and somewhat different FSH superovulation regime (McNatty *et al*., 1989; Pendleton *et al*., 1992; Gonzalez-Bulnes *et al*., 2003). This may justify the different responses including embryo quality obtained during synchronisation and superovulation, which may be related to different breeds and individuals of embryo donors used.

Current results support the idea that superovulation treatment generally induced higher ovulation rates than number of viable embryos obtained because of fertilisation failure and early embryonic death (Armstrong, 1983; Callesen *et al*., 1995). There is a need to increase the ovulation rate and at the same time to keep the physiological environment within the reproductive tract as conducive as possible so that embryo development will proceed as normal as possible for long period until recovery for embryo transfer at later stages of development. Previous studies in sheep have shown that the viability of embryos decreased by alteration of the maternal environment during its very early developmental stages in the genital tract of the donor females (Gonzalez-Bulnes *et al*., 2003a). A similar situation probably occurs in Malaysian goats but this needs further investigation.

5.5 EFFECT OF SOURCE OF THE EMBRYO ON THE GOATS EMBRYO TRANSFER

Pregnancy rate and survival rate of embryos following transfer has a major effect on the profitability and success of ET programme (Tervit *et al*., 1986). An acceptable overall early pregnancy rate (80%, ICSI; 62%, *in vivo*, embryo) was diagnosed at 60 days following transfer, which is in line to a range of 27.0 to 79% quoted following laparoscopic, surgical and non-surgical transfer of goat embryos (Kiessling *et al*., 1986; Bessoudo *et al*., 1988; Li *et al*., 1990; Flores-Foxworth *et al*., 1992). These pregnancy rates obtained are also indicating acceptable procedure of embryo transfer used in this trial. The reasons for the differences in pregnancy rates obtained following ICSI and fresh embryo transfer is not easy to explain, as there are too many contributing factors to consider. The differences occurred even when embryos were at the same stage of development, of a similar breed or similar embryos manipulation used. This observation demonstrates the unpredictability of the results following the transfer of manipulation and fresh goat embryos.

The decline in the recipients kidding from the recipients confirmed pregnant occurred following the transfer of ICSI and *in vivo* embryos in the present experiment. Similar tendency following transfer of manipulated embryos have been recorded in goats and sheep (Nowshari and Holtz, 1995a; Baril *et al*., 2001; El-Gayar and Holtz, 2001). Several factors have been quoted as being responsible for embryonic reabsorption and as animals were not monitored 24h a day. The variation recorded in the literature regarding the success rates indicates that there is still much research needed to perfect the ICSI and/or transfer techniques of embryos in goats.

Increased early embryonic loss and failure of implantation in superovulated recipients was a important phenomenon normally related to the hostile maternal endocrine environment (Moon *et al*., 1990). This may be due to defective in foetal

development to the recipients' condition, as the aborted or stillborn foetuses were morphologically normal. It could also be due to malnutrition of the recipients before or during the course of pregnancy. It was reported that maximum kidding rate could be obtained when optimal number and quality of embryos transferred in to healthy recipients.

In this experiment, a single injection of PMSG (500 IU) was administered to prime the recipients for the embryo transfer. This dose was known to be non-superovulatory (Ebert *et al*., 1991), whereby the ovulation rate of induced recipients (1.7) was the same as naturally cycling recipients. The mean kidding rate of Malaysian local goats was reported to be 1.5-1.9 (Devendra, 1983). Therefore, in this experiment, the recipients conceived high number of transferred embryos may be detrimental to the foetal development during pregnancy. In ewes the number of corpus luteum had no significant effect on the overall survival rate of transferred embryos (Bari *et al.,* 2003). Conversely, in goat, significantly higher embryo survival rates were observed in recipients with two or three or more corpus luteum compared to those with only one (Amstrong *et. al*., 1983). In addition, the possibility of the seasonal effect on abortion cannot be ruled out in the hot and rainy summer (Lee *et al*., 2000). It has been also reported that plasma progesterone concentration was correlated to the ovulation rate of recipients (Gootwine *et al*., 1997). Since no relationship between progesterone concentration and pregnancy rate was observed in this experiment, further trial research is needed to clarify the relationship between ovulation rate and pregnancy rate, particularly in Malaysian goats.

In this experiment, the other possible factor that could effect the conception of transferred embryo could be due to stress induce during transportation of the recipient from the farm to laboratory, which is about 0.5 km in distance. The recipients were transported 1-4 hours before embryo collection and transfer was carried out.

Transporting recipient for embryo transfer gave negative effects on the pregnancy and embryo kidding rates (Quan *et al.*, 2010). However, transportation of donor does for embryo flushing does not affect the luteal formation and embryo numbers or quality. Thus, the transportation stress of recipient does may affect the recipient pregnancy. Practically, this stress seems to be unavoidable in many farm and laboratory situations (Dobson *et al*., 2001), unless a new means of overcoming it, for example, to transport the embryos to the recipient site for embryo transfer (Quan *et al*., 2010).

5.6 GENERAL DISCUSSION

The significant findings of the current study had been discussed in detail in previous section. The following is a discussion intended to give a general perspective will focus on the different factors and constraints faced during the course of this study, involving oestrus synchronisation, superovulation, embryo collection and embryo transfer. In addition, future direction of this research is also suggested.

5.6.1 Sychronisation of Oestrus

Oestrous cycle control in MOET programme serves the purpose of synchronising oestrus in groups of donor and recipient to be bred, and subsequent ET to be carried out at a particular time after the synchronisation. Oestrus synchronisation contributed a major influence in the overall efficiencies of ovulation of donor and recipient does. Synchronisation of oestrus is achieved either using prostaglandin $F_{2\alpha}$ and progesterone or one of its synthetic analogues. In this study, oestrus of donors and recipients were successfully synchronised by insertion of Y-shaped silicone-coated devices ("controlled internal drug release", CIDR) impregnated with 30 mg progesterone for period of 10-15 days. As other studies, the incident of CIDR lost during synchronisation treatment is common and may jeopardise its purpose (Holtz, 2005). Regular checking and

immediate replacement of lost CIDR during synchronisation period are vital for successful treatment. In small or nulliparous does, CIDR or subcutaneous implants are preferable in many studies because sponges frequently cause discomfort and may adhere to the vaginal wall causing problems with removal (Shamsul, 1997).

Beside CIDR, other progestagen implants that frequently employed with equal effectives are vaginal pessaries. These are polyurethane sponges impregnated with fluorogestone acetate (FGA) or medroxyprogesterone acetate (MAP). 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 *et al.*, 2000; Oliveira *et al*., 2001; Medan et al., 2002) or on the underside of the tail (East and Rowe, 1989). In another comparison, only 8% of the vaginal sponges and 3% of the subcutaneous ear implants were lost during synchronisation treatment as compared to 19% of the CIDR implant (Holtz, 2005).

There are 3 priming protocols that are normally utilised in does oestrus synchronisation. The use of progestagen in "long protocol" of synchronisation treatments (18–21 days) does not require the use of a luteolytic agent. This period is long enough for corpora lutea to undergo timely regression in all animals no matter at what stage of cycle the animals are at the onset. Low fertility rate was observed, probably due to poor semen transport (Corteel *et al.,* 1988). In this experiment, the does in oestrus was synchronised by implementing "medium protocol" of progesterone treatment with CIDR insertion for the period of 10–15 days followed by one intramuscular injection of luteolytic dosage of prostaglandin (74 µg), two days before sponge removal. In previous study, the reduction of progesterone priming during oestrus synchronisation had improved the kidding rate from 57% for 18-21 days to 61% for 11

days (Corteel *et al.,* 1988). With a treatment length reduced to shorter than the normal luteal phase of 16 days, oestrus and ovulation may be delayed or even inhibited by the presence of a functional corpus luteum at the end of the progestin treatment, prostaglandin or one of its synthetic analogues must be injected to induce luteolysis. In this study, the dosage of prostaglandin was reduced from 125 µg as previously practised in our laboratory to 75 µg, without any decreasing in superovulation response observed. Study on decreasing dosage of cloprostenol from 200 to 0 µg indicated that the best fertility after AI was obtained after one intramuscular injection of 50 µg two days before sponge removal (Corteel and Leboeuf, 1990). In "short priming protocol" the goats are treated with progestagen for the period of 5 days (Rubianes *et al*., 2003). The reason is that progestagen treatment for more than 5 days will result in subluteal concentrations of progesterone that would promote excessive growth and persistence of the largest dominant follicle, leading to lower fertility.

In ET protocol, the oestrus synchronisation treatment of all recipients by rule, is supplemented with one dosage of 250-500 IU of "equine chorionic gonadotrophin" (eCG) and a luteolytic dosage of prostaglandin two days before sponge removal. The eCG promotes follicular development and synchronises ovulation, allowing ET to be performed at fixed times. The eCG dosage is determined according to breed, weight, age and desire for multiple kidding (Leboeuf *et al*., 1996). In this study, the dosage of eCG were standardised at 500 IU for all recipients. This might create a variable in hormonal circulation between recipients and subsequent embryo survival after transferred because the does involved in this experiment were randomly selected from different breeds and body sizes. Other factor that might influence fertility rate of the recipient is the time of eCG injected. Removing implant 48 hours after the eCG injection gave higher fertility (53%) rates than simultaneous implant removal and eCG injection (48%) (Corteel. *et al*., 1988). Repeated use of eCG has been reported to result

in poor fertility in fixed-time AI programs. These results have been attributing to the presence of anti-eCG antibodies developed as an immune response to previous treatments (Roy *et al*., 1999). The presence of such antibodies has been clearly linked to a delay in the occurrence of oestrus, LH peak and ovulation in the synchronised recipients, which may explain the lower fertility if the fixed insemination time is not modified accordingly.

5.6.2 Egg Fertilisation

In this present study, almost half (49%) of the eggs recovered were unfertilised mainly due to the status of the buck used for breeding. Semen evaluation was not carried out on all the bucks selected for breeding. Thus, the soundness of the bucks was unknown. To mitigate this problem, buck were allowed to stay together with the donors in the same pen for extended period of 3 more days after oestrus detected and if necessary the buck was replace every day. The extended staying of the buck and the doe indeed increased the number of fertilised embryos in this experiment. The other aspect related to fertilisation that is worth noting for future study is to test the different insemination techniques and schedules on the fertilisation rate obtained in superovulated does. The results showed that combining natural mating and artificial insemination is able to improve fertilisation rate compared to either mating or artificial insemination alone. Intrauterine insemination is thought to be a valid method for increase ova fertilisation (McKelvey *et al*., 1985; Bari *et al*., 2000). It permits sperm to by-pass the cervix and overcomes the reduction of sperm transport induced by superovulation treatment (Evans and Armstrong, 1984; Baril *et al*., 1993; Cognie, 1999). The combination of artificial insemination to natural mating maximises fertilisation rate probably because there is a compensatory effect between the two insemination systems related to ovulation times in donors. This system might have overcome the possible not optimal insemination time,

which is believed to be critical for the success of fertilisation and subsequent embryo production (Scudamore *et al*., 1993a; McEvoy *et al*., 1997).

5.6.3 Superovulation

The main aim of this study was to focus on developing hormonal treatments to optimise ovarian response in donor does and to improve the quantity and quality of embryo production. In the first experiment the single injection of 8.8 mg oFSH in saline upon CIDR withdrawal showed poor effect on total ovarian response and ovulation rate in donor does. This further reduced the number of does eventually flushed and recovered embryo. The second trial with higher total dosage of 14.1 mg oFSH given in declining dosages of multiple injections resulted instant improvement on ovarian response, ovulation percentage and viable embryo recovered. The variation between donor does was high with respect to these parameters measure, ranging from 5 to 40 responded follicles per does. Two does experienced hyper stimulation with more than 45 responded follicles. Due to shortage of oFSH in our laboratory at that time, the total dosage of oFSH was further reduced to 8.8 mg and adjusted in equal dosages of multiple injections. This attempt promoted good and constant ovarian response, ovulation rate and viable embryos recovered among donor does. The tendencies however, indicated the increase in total dosage of oFSH in multiple administration superovulation protocol increase the structure recovery rate but also increase the unfertilised ova and degenerated embryos and hence reduce the number of viable embryos. As overall, the higher total dosages in the administration regimen of oFSH provided a marginal improvement in these three parameters measured in does superovulated with lower dosages. This suggested that a higher total dosage of oFSH hormonal treatment during the preovulatory period promotes development of a large number of follicles but not embryos yield.

The result from single injection protocol of this experiment is in agreement with other superovulation protocol using single oFSH injection that were previously designed in our laboratory for LOPU (Phua, 2006; Amir, 2007; Rahman, 2008 and Chan, 2008). Originally, the study involved a total dosage of 70 mg oFSH, resulted less numbers of oocytes retrieval compared to 35 mg oFSH in single injection (6.7±1.8 vs. 17.5±1.9; Rahman, 2008). In this current study, further reduction to 8.8 mg oFSH (4 folds) yielded 8.5±1.4 ovarian response per doe (CL plus anovulatory follicles). This response indicated that the increase of oFSH concentration beyond optimum/critical level will suppress the ovarian response of superovulated doe. However, low oFSH in single injection treatment protocol of hormonal administration might be not enough to trigger the recruitment of preovulatory follicles. The short half-life of oFSH is the main reason resulted to poor ovarian response of single injection treatment compared to continuous supply of oFSH during multiple injection treatment (Holtz, 2005; Ammoun *et al*., 2006). Further study for the simplification and less labour-intensive superovulation regime without compromising embryo yield should be carried out. One such attempt is to inject FSH at 24 instead of 12 hours intervals while doubling the dosage (Suyadi *et al*., 2005), or substituting the last three of six FSH-injections by a single dosage of 200 IU eCG, (Pintado *et al*.,1998) obtained acceptable ovulation rate.

Other vehicle for oFSH such as propylene glycol (PGL) and polyvinylpyrrolidone (PVP) need to be tested in Malaysian goats. Simplification of the procedure is to allow the use of this vehicle as a single injection protocol. Lopez-Sebastian *et al.* (1993) observed a three-fold increase in ovulation rate in cyclic ewes with a single injection of FSH dissolved in PGL. A single injection of FSH dissolved in PVP resulted in similar or enhanced ovulation rates and numbers of recoverable embryos compared to the conventional multiple injections in rabbits (Kanayama *et al.,* 1993), ewes (Dattena *et al.,* 2004) and heifers (Takedomi *et al.,* 1995). Additionally,

elevated concentration of FSH was maintained significantly longer in heifers treated with FSH in PVP than in saline (Takedomi *et al*., 1995). Based upon previous studies, PVP and PGL may be effective diluents for FSH.

The random selection of the does involved in this experiment provides no clue on follicular status of each donor. Variation in superovulatory response is believed to reflect the follicular population present at the initiation of gonadotrophin treatment (Gonzalez-Bulnes *et al*., 2003a), which is not controlled by standard superovulation protocols. Further research is however needed for identified the effect of donor follicular status at the initiation of superovulation programme such as the implementation of Day 0 protocol is to be utilised without ovarian inspection. Evident was the high variation in the ovarian response and number of structures recovered for the individual animals in this experiment. This tends to indicate the importance of monitoring the follicular waves when administering oFSH for the purpose of superovulation. The high variation recorded within individual could imply that the donor does within a treatment group being at different stages of their oestrous cycles.

Several strategies have been suggested for increasing the number of small recruit able ovarian follicles at the time of FSH treatment, while avoiding the presence of large (dominant) follicles. A so-called “day 0 protocol” has been proposed recently to avoid the deleterious effects of large dominant follicles and improve results from superovulation treatment (Menchaca *et al*., 2002). This protocol is based on initiating FSH administration immediately after ovulation and resulted in increase in the number of CL following superovulation. However, improvements in terms of number and quality of embryos recovered have not been reported. Poor fertilisation may, partly, be due to poor sperm transport following heat synchronisation (Evans and Armstrong, 1984) as well as to poor synchrony between time of insemination and ovulation. The first of these problems may be avoided by the use of intrauterine (laparoscopic)

insemination. Some of these strategies include the use of GnRH agonist/antagonists and the administration of FSH shortly after an induced oestrus/ovulation. These may be improved by synchronising ovulation by GnRH administration around the time of heat detection and AI. Alternatively, Baril *et al*. (1996) improved the synchrony of ovulation in superovulated goats by administering a GnRH antagonist 12 hours after sponge removal, followed by LH administration 24 hours later. However, other studies showed that pre-treatment with a Buserelin implant one week prior to superovulatory treatment did not improve the ovarian response of superovulated donors (Baldassarre and Karatzas, 2004; Lehloenya and Greyling, 2010). It is possible that GnRH pre-treatment to be administered for more than 1 week to deplete the pituitary of gonadotrophins and allow the ovary to build-up a large number of small follicles. Pre-treatment with Antarelix (GnRH antagonist) for 10 days prior to superovulation resulted in an increased number of small follicles at the time of FSH administration and an increased number of ovulations, however, this improvement in superovulatory response did not yield a larger number of transferable embryos because of poor fertilisation (Cognié *et al.*, 2003).

5.6.4 *In vitro* production of embryos

In vitro production of goat embryos is a rapidly advancing field. It offers an alternative to superovulation as a source of embryos for transfer and manipulation purposes. In this experiment, ICSI embryos were used. Mature oocytes recovered from the ovaries of donor animals by way of laparoscopic ovum pick-up (LOPU) from superovulated does, as described by Baldassarre *et al.* (1994, 2003a) and Graff *et al.* (1999) has of late become the method of choice. Due to the difficulty and impracticality of ovulation detection, as a rule, immature oocytes are aspirated from punctured or slicing follicles either of slaughterhouse ovaries (Pawshe *et al*., 1994; Crozet *et al*., 2000; Han *et al*.,

2001; Reggio *et al*., 2001) or from live animals by way of laparotomy or ultrasound-guided transvaginal aspiration (Graff *et al*., 1999) or in this experiment by laparoscopy. Laparoscopic ovum pick-up (LOPU) from superovulated does, has of late become the method of choice, its less invasive nature and can be exercised repeatedly (e.g. weekly) on the same donor and permits collection from prepubertal, pregnant, puerperal or aged animals (Baldassarre *et al.*, 1994, 2003; Graff *et al*., 2000). Baldassarre *et al.* (2002) found gonadotrophin-primed does of only 2–3 months of age to be particularly efficient donors yielding, on average, 25 oocytes, which is between 50 and 60% more than adult does. The developmental potential of these oocytes, assessed by successfully subjecting them to in vitro fertilisation, was more than 30%, which is comparable to that of oocytes obtained from adult donors (Mogas *et al*., 1997; Izquierdo *et al*., 1999, 2002; Koeman *et al*., 2003). The various steps involved with the *in vitro* production of caprine embryos are quite similar to those employed in the bovine, where the *in vitro* production of embryos is an established procedure. In brief, oocytes of follicular origin need to undergo *in vitro* maturation (IVM) before being exposed to in vitro-capacitated spermatozoa to be *in vitro*-fertilised (IVF). Thereafter, the putative 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 *et al*., 2002; Baldassarre *et al*., 2003a; Cogniè *et al*., 2003; Koeman *et al*., 2003). The developmental potential of *in vitro*-derived embryos can be assessed by their nuclear status using special vital stains and fluorescent microscopy. Caprine embryos, scored morphologically as morulae or blastocysts but, by careful examination, are observed to have fewer nuclei than blastomeres, must be considered pseudo-embryos (Koeman *et al*., 2003). The ultimate proof of the developmental potential of *in vitro*-derived embryos is its development to term after transfer. The first kids born after complete *in vitro* maturation, fertilisation and culture were reported by Keskintepe *et al*. (1994) and

Pereira *et al*. (1998). During the following years there were only haphazard reports on *in vitro*-derived offspring (Traldi *et al*., 1999; Cogniè *et al*., 2003). Only very recently Baldassarre *et al.*, (2003b) reported the birth of an appreciable number of 150 *in vitro*-derived kids.

In this study, 8 ICSI embryos were transferred and none kidded. Amongst other disadvantages and attraction of ICSI embryos production technique lies in the avoidance of potential polyspermia (Palomo *et al*., 1999; Bhatia *et al*., 2002) and the intriguing perspective of being able to predetermine the sex of the offspring by using spermatozoa sex sorted by flow cytometry (Parrilla *et al*., 2004) and 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). At present the efficiency of ICSI is low. However, it is probably only a matter of further sustained research effort until practicable solutions are found.

5.6.5 Embryo Collection

Whereas embryo transfer in cattle proved to be an effective way of increasing the contribution of superior genetic stock to the gene pool, this technology is not widely applied in goats because of the technical difficulties involved in collecting and transferring embryos are considerably larger. Until recently, both embryo collection and transfer involved full-fledged surgery, which is cumbersome, expensive and accompanied by the risk of impairing subsequent fertility, preventing the use of valuable donors. As in this experiment, surgical embryo collection entails exteriorisation of the reproductive tract via mid-ventral laparotomy and lavaging of the uterine horns with flushing medium to retrieve the embryos (Goel and Agrawal, 1990, 2005; Holtz, 2005). Post-operative adhesions are a frequent sequel in this experiment, limiting the number of possible collections. Laparoscopic embryo collection is less

invasive but still entails full anaesthesia and requires sophisticated equipment and considerable technical skill (Baril *et al*., 1993; Flores-Foxworth, 1997). Recently a non-surgical procedure has been put into effect: embryos are flushed from the uterus of goats restrained in a standing position with no sedation required. About 20 hours prior to collection, donors are treated with a luteolytic dosage of prostaglandin $F_{2\alpha}$ to facilitate uterine contractility at the time of flushing. With this approach mean recovery rates ranged from 60 to 80% (Pereira *et al*., 1998; Holtz *et al.*, 2000; Suyadi *et al.*, 2000).

5.6.6 Embryos Transfer

The transfer of embryos to surrogate mothers is most commonly accomplished by surgical means. The required surgical intervention is far less strenuous than that required for collection, in terms of duration of anaesthesia, size of abdominal incision and traumatisation of the genital tract. It rarely causes major post-surgical adhesions and, therefore, may be repeatedly conducted on the same doe. Only the tip of the uterine horn ipsilateral to the ovary displaying an ovulation site needs to be exteriorised. Embryos at an advanced stage of development are deposited in the lumen of the uterine horn by puncturing the uterine wall with a blunt needle close to the uterotubal junction. Early embryonic stages (up to 8 or 16 blastomeres) are to be transferred to the oviduct (Wang *et al*., 2003). Less invasive approaches for the transfer of embryos has been attempted. One consists of laparoscopic determination of the side on which ovulation has occurred, followed by careful exteriorisation of a small loop of the ipsilateral uterine horn with the aid of an Allis forceps through a tiny mid-line incision. An entirely laparoscopic transfer has occasionally been attempted but pregnancy rates were frequently unsatisfactory (Holtz, 2005). Tubal transfer by laparoscopically threading a catheter containing the embryos into the oviduct via the infundibulum has been

executed by Besenfelder *et al.* (1994) leading to pregnancies in five out of nine recipients.

Chances that embryos are carried to term are favourable if the oestrous cycles of donors and the recipients are synchronous, or if the donors were in oestrus 1 day earlier than the recipients (Oppenheim *et al.*, 2000). Age, health and nutritional status of donor and recipient are of paramount importance for assuring satisfactory outcomes (Mani *et al*., 1994). The efficiency of embryo transfer operations may be improved if monitoring systems, such as plasma progesterone determinations as indicator of the presence of functional corpora lutea and ultrasonography for visualising number and size of ovarian follicles or uterine contents, are made use of. The end result is a profound effect on the oocyte, early embryo and their respective microenvironments. Not only are a proportion of non viable ova/embryos on days 6 to 8, but it has further been argued that even though an embryo appears morphologically viable on days 7 or 8, subtle injuries inflicted upon the oocyte or the early embryo may only be seen after transfer of an apparently normal embryo (Moore,1985; Hyttel *et al.*, 1991; Betteridge and Loskutoff, 1993). This would be seen as a reduction in pregnancy rates following transfer. Collectively, these data substantiate that although the superovulatory response may affect the actual recovery of transferable embryos, the pregnancy rate achieved after transfer is independent of the actual response of the donor from which it originates, irrespective of whether the embryos are transferred fresh or after freezing and thawing.

5.6.7 Summary

The results from this experiment suggest that multiple injections of oFSH administration in superovulation protocol performed on common goat breed in Malaysia after synchronisation of follicular growth is a valuable tool to retrieve viable embryos for ET and other embryos manipulation programmes. The total dosage, pattern of

administration, breed and body weight have no effect in production of viable embryos. Low total dosage administered in multiple injections is economically advantageous and convenient for the production of competent *in vivo* embryos. However, the optimum dosage of oFSH and simplified protocol need to be developed in further experiments.

5.6.8 Future Directions

The present research was a preliminary study of oFSH superovulation protocol for local goat and emphasised on the production aspects of ovarian response and *in vivo* embryos production. Therefore, it was beyond the scope to study individual aspects, mechanisms or processes in the greater detail, especially at hormonal levels. Since this study was preliminary in this laboratory, many problems have to be faced and solved. However most of the problems encountered throughout experiment were already overcome. Therefore, the modest results obtained from this study had been influenced by numerous factors, including learning curve, age, breed, health and physiological status of the donor and recipient does, management efficiency in the farm, embryo recovery and ET techniques. However, the findings of the present study could be a basis for more detailed studies of *in vivo* embryos production and ET. The results from the present study offer several aspects for further improvement in future to ensure life birth from ET. The possible aspects identified are: effect of the synchronisation length; dosage of hCG on superovulation response; different insemination schedules; method on the fertilisation rate obtained in superovulated does; vehicles for oFSH (such as propylene glycol, polyvinylpyrrolidone); status of ovarian follicles at the start of superovulation programme; hormonal imbalance resulted from repeated use of eCG; the effect of eCG dosage on embryos survival and kidding.

CHAPTER 6

6.0 CONCLUSIONS

This study presents the ovarian responses and production of *in vivo* embryos obtained from different oFSH superovulated protocols. It can be concluded as follows:

a. High ovarian response and satisfactory number of *in vivo* transferable embryos could be produced from oFSH superovulation protocols.
b. The progesterone synchronisation treatment preceding superovulation performed for the duration of 9-15 days is effective in doe superovulation treatment as high number of treated does on oestrus and ovulated.
c. In same total dosage of oFSH regiment, single injection of oFSH in saline reduced ovarian response, ovulation and production of embryos compared to multiple injections.
d. In multiple injection of oFSH superovulation protocol, high total dosage of oFSH improves ovarian response and ovulation but not production of embryos and their quality.
e. There is economic advantageous of using a low rather than a high level of oFSH in multiple injection protocol in term of embryos production.
f. Lower total dosages of oFSH results in a reduction in costs; the pattern in which the total oFSH dosages are administered does not affect cost but the use of equal dosages may be more convenient.
g. Breed and body weight have no obvious effect on ovarian response, ovulation and embryo production of the superovulated does.
h. The surgical ET procedure is effective to produce high early pregnancy rate of ICSI and *in vivo* embryos.
i. More research is needed for maintaining full term gestation of ET embryos in recipient doe.