In vitro embryo production in cattle

by

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Declaration

I hereby declare that this thesis submitted by me to the University of the Free State for the degree, **Philosophiae Doctor (Ph D)**, is my own independent work and has not previously been submitted by me to any other University. I furthermore cede copyright of the thesis in favour of the University of the Free State.

Johannes Matthias (Jean) Rust
6 March 2007

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ABBREVIATIONS

1. Acepromazine maleate (ACP)
2. Body condition score (BCS)
3. Cumulus oocyte complex (COC)
4. Corpus luteum (CL)
5. Embryo transfer (ET)
6. Epidermal growth factor (EGF)
7. Fetal calf serum (FCS)
8. Follicle stimulating hormone (FSH)
9. In vitro culture (IVC)
10. In vitro fertilization (IVF)
11. In vitro maturation (IVM)
12. In vitro embryo production (IVEP)
13. Insulin like growth factor 1 (IGF 1)
14. Laparoscopic ovum pick-up (L-OPU)
15. Luteinizing hormone (LH)
16. Multiple ovulation and embryo transfer (MOET)
17. New born calf serum (NBCS)
18. Ovum pick up (OPU)
19. Penecillamin, Hypottaurine, Epinephrine (PHE)
20. Phosphate Buffered Saline (PBS)
21. Standard error (SE)
22. Synthetic oviductal fluid (SOF)
23. Tyrode Albumin Lactate Pyruvate (TALP)
24. Tissue culture medium 199 (TCM 199)
25. Ultrasound guided ovum pick-up (U-OPU)
26. Zona pellucida (ZP)

Introduction

Selection pressure is continuously being exerted on the breeding of farm animals to ensure offspring that produce animal protein more efficiently. Worldwide, much research and the refining of techniques have been undertaken as far as the reproduction and the selection of animals for optimum production are concerned. The science of quantitative animal breeding has developed to a stage where the true genetic potential of an animal can be determined without it being masked by the effect of the environment in which the animal is kept. Although this is a huge step forward as far as animal breeding is concerned, the breeding of efficiently producing animals is still a relatively slow process, in which progress is sometimes minimal and can only be observed after a relatively long period of time (Bradfield and Erasmus, 1999).

Fortunately, a physiological science has been developed where certain procedures are performed to artificially accelerate the reproduction rate in farm animals. This science, known as assisted reproduction, and the technologies being developed are in the process of concentrating on the proliferation and the maximum utilization of animals of superior genetic quality. These technologies which were initially developed for bovine were later extended to other farm species and even endangered wildlife species (Baldasarre *et al*., 1994; Nicholas, 1996).

In vitro embryo production was initially developed to produce a large number of embryos for research purposes and to investigate the basic physiological events occurring during early embryonic development. Subsequently researchers realized that this reproductive technology could be utilized for increased production of embryos for use in livestock genetic upgrading programs. Thus one of the first practical uses of *in vitro* embryos in livestock production was the use of slaughterhouse derived beef embryos for transfer to dairy herds (Looney *et al*., 1994). This procedure was performed to produce calves with superior carcass quality for beef production in a dual-purpose beef production system. However, the use of unknown genetic material (slaughterhouse origin) has always been seen as undesirable, as the use of

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embryos derived from this source have no genetic improvement impact in these herds. The actual techniques involved in, *in vitro* embryo production as such, have however undergone many changes over the past number of years to increase the efficiency of the techniques (Lu *et al.*, 1988). A number of different oocyte maturation, insemination and embryo culturing procedures have been tried and tested over time, with mixed success. *In vitro*-derived bovine embryos obtained were initially of poor quality, which was reflected by poor pregnancy rates following transfer and inferior quality embryos not suitable for cryopreservation, when compared to the *in vivo*-derived counterparts. These factors and the fact that the origin of the genetic material is mostly unknown have severe limiting effects on the widespread use of *in vitro*-derived embryos. It has thus taken a number of years for these procedures to be developed to a stage where the quality of *in vitro*-derived embryos is acceptable to have practical application in the animal production industry (Looney *et al.*, 1994). However, the fact that oocytes to be used as a source of genetic material, for *in vitro* procedures, still have to be obtained from slaughterhouse material thus has a severe limiting effect on the widespread use of this *in vitro* technology in genetic improvement programmes. Further sophistication has led to the advent of techniques whereby oocytes could be collected e.g. by means of ultrasound guided oocyte aspiration in live animals i.e. ovum pick up (OPU) (Gibbons *et al.*, 1994). This has offered a solution and meant that oocytes could be collected from a known genetic source on a regular basis. This technique was developed in the mid-1990's by the Dutch and opened a total new field of application for *in vitro* technology, as far as livestock genetic improvement programmes were concerned (Pieterse *et al.*, 1988).

In vitro embryo production (IVEP) from live donors is an extremely versatile and viable technique that can be applied to a wide range of donor species. The technique does not interfere with the physiological status of the animals, as no hormonal intervention is used in the process (Meintjes *et al.*, 1995). In some cases OPU can even be applied as a therapeutic procedure to correct certain disorders in genetically valuable female donors. So for example, animals with ovarian cystic disorders can benefit greatly from these ultrasound oocyte recovery procedures. However, OPU is a complex and demanding technique which requires expensive and diverse equipment with high-tech skills being a prerequisite, especially

regarding the laboratory procedures (Galli *et al.*, 2001).

Superior quality farm animals, in both the dairy and beef industry, could have a great impact in South Africa. The current trade in genetic material is quite vibrant and stud farmers receive high prices for superior stud animals at auctions and sales. South African animal genetics, especially in the beef industry, is also extremely popular overseas, and this factor has contributed to the high unit cost of these animals. Although South Africa is currently a relatively limited user of embryo technology, it is relatively popular with stud breeders and a number of the top quality animals are regularly subjected to these procedures to produce embryos for the local and export market. The main aim of IVEP is, thus, the accelerated genetic improvement of especially the local cattle populations and the export of embryos to other countries (Gibbons *et al.*, 1994).

The conditions in South Africa for cattle farming, both dairy and beef, is extensive and the environmental conditions sometimes quite harsh, and factors such as longevity and reproduction are high on the priority list as regards the criteria for selection. This, however, implies that females must reach a mature age before the true potential of a cow for both reproduction and longevity are realized.

A need has now developed for embryo production technology to be created as an alternative for the conventional multi-ovulation and embryo transfer (MOET) procedures. This has emanated from the fact that the type of animal being selected regarding reproduction and longevity criteria are often not ideal or suitable for conventional MOET procedures. Besides this, the need has also arisen to produce embryos from old or pregnant females and animals that have become infertile due to unnatural causes. In the past there has also been no alternative available to produce embryos from animals that did not respond well to the conventional MOET procedures (Looney *et al.*, 1994). The ultimate was thus to develop a technique that could replace the conventional MOET procedures as a tool for accelerated genetic improvement in the local cattle industry. In short, a cost effective alternative for the current bovine superovulation procedures, with a wider range of application is needed. The

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only way that this could be achieved would be to combine the ultrasound guided oocyte recovery (OPU) technology with effective *in vitro* embryo production (IVEP) technology (Gibbons *et al.,* 1994; Galli and Lazzari, 1996).

This study thus focuses on certain aspects contributing to the development of IVEP procedures for use in different types of cattle not normally suited for conventional *in vivo* embryo production techniques. Certain factors that may affect or enhance the effectivity of the procedures are also addressed, while the efficiency and cost-effectiveness of certain alternatives of approaching IVEP in sub-fertile oocyte donors are evaluated.

Currently the selection of the donor cows and the conditions under which these animals are entered into an IVEP program are dependant solely on the cattle owner. This implies that a number of unpredictable factors and variables were faced by the investigating IVEP team. The main objective of this study was, thus, the development of a practical approach for IVEP in sub-fertile donor cows, even under conditions where the breed, age, body condition, season and reproductive status of the donor cannot be manipulated or controlled.

In this study an opportunity is created to demonstrate the complexity of the reproduction techniques used and the factors playing a role in IVEP. The potential impact on the cattle breeding industry is something not fully realised yet and South Africa with its genetic pool of indigenous and composite cattle breeds which makes it a contender for these reproductive technologies. Emanating from this study an *in vitro* embryo production laboratory was established in South Africa which produces offspring from superior quality donor animals.

Chapter 1

Literature Review

Internal and external factors influencing bovine oocyte competence and *in vitro* embryo production

This literature review intends to highlight important factors that may affect the efficiency of *in vitro* embryo production (IVEP) procedures in cattle. Less emphasis will be placed on the actual processes of *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and the actual embryo culturing procedures, which are complex procedures that have undergone drastic modifications over the last decade. A few standard IVEP procedures are currently being used and different laboratories in South Africa are utilizing methods of their choice. More emphasis will be placed in the review on factors that can affect the practical application of this technology of bovine IVEP. Aspects such as the actual development of ultrasound guided procedures, sourcing and recovery of oocytes, factors affecting the quality of oocytes, follicular dynamics, quality of the embryos and pregnancy rates, cryopreservation as well as the effect of some of these procedures on the health of the donor and offspring will be addressed. All these aspects could play a major role in the development of alternative or more effective embryo production methodology in cattle.

It has been well established that oocyte recovery in live farm animals alone, or in combination with conventional embryo recovery techniques can yield more embryos than conventional embryo recovery practices (Goodhand *et al.*, 1997). Oocyte recovery as such is also a useful tool to produce embryos from animals that are incapable of producing embryos through conventional means (Riddell *et al.*, 1997; Nicholas, 1996).

1.1 Follicular dynamics and internal factors affecting oocyte competency and the embryo production ability

The mature ovarian follicle is a complex structure composed of granulosa and theca interna cell layers which work together in the nutrition and maturation of the ovum and the production of steroid hormones and numerous peptide factors (Sirard and Blondin, 1996; Singh *et al.*, 1998). Earlier follicular studies have indicated that at any given time, 77% of all bovine ovarian follicles are atretic, and small follicles enter the final growth phase throughout the entire oestrous cycle (Choudary *et al.*, 1968; Jaiswal *et al.*, 2004).

Mammalian ovaries contain several hundred thousand primordial follicles at birth, however, less that 0.5 % of these follicles will reach maturity and their ova be released or ovulated. The vast majority of the follicles undergo atresia and regress over the animal's reproductively active lifespan (Irving-Rodgers *et al.*, 2001). The growth and atresia of individual follicles have been monitored by sequential ultrasonography and the growth of a follicle has been categorised into a growing (increasing diameter), static (no change in diameter) or regressing (decreasing diameter) phase (Ginther *et al.*, 1989b).

1.1.1 Development of oocytes within the ovarian follicle

Oocytes grow bigger in diameter in follicles up to a follicular size of approximately 3 mm. Thereafter the diameter of the oocyte does not change before the follicle reaches a diameter of 10 mm. During the period of growth arrest, the development of the follicle is almost complete. Further growth of the follicle and increased RNA transcription activity takes place after the follicle has reached a diameter of 10 mm. After this period, a developmental process of the oocyte occurs which is referred to as a pre-maturation or capacitation phase. This is also the period when the dominant follicle is selected for further development (Arlotto *et al.*, 1996). During this period a number of ultra structural cell changes, such as changes in the Golgi complexes, cortical granules, nuclear membrane, perivitelline space and positioning of the surrounding corona radiata cells take place (Hyttel *et al.*, 1997). This pre-maturation process may also include activities in which the mRNA's and proteins are processed to

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prevent degeneration during storage in the oocyte (Brevini-Gandolfi and Gandolfi, 2001).

During the final stage of oocyte maturation the oocyte nucleus resumes meiosis up to the metaphase stage and several changes such as lipid storage, alterations to the Golgi complexes, mitochondria, cortical granules occur in the cytoplasm, as well as the retraction of the corona radiata cells and cumulus expansion (Brevini-Gandolfi and Gandolfi, 2001). The cumulus-oocyte complex also displays certain time-dependant protein synthesis activities during this period (Kastrop *et al.*, 1992). The presence of the cumulus cells seem to be crucial for the early maturation of the bovine oocytes (Bruynzeel *et al.*, 1997). These processes in the pre-maturation and final maturation phases during follicular development are essential in determining the oocyte's final quality and future developmental capacity (Merton *et al.*, 2003).

 Follicular development during the bovine oestrous cycle is composed of 2 or 3 follicular waves of growth and atresia (Ginther *et al.*, 1989a; Jaiswal *et al.*, 2004). In some cases 4 or more wave cycles have been reported, accompanied by a prolonged interovulatory interval as a result of delayed luteolysis or failure to ovulate (Ko *et al.*, 1991). There seems to be neither a breed- or age specific preference for a one follicular wave pattern over the other patterns, nor is there any apparent difference in fertility (Mapletoft and Adams, 2001). These follicular waves are classified as a wave 1 (emergence of follicles associated with ovulation) and a wave 2 (emergence of follicles during mid-dioestrus). A wave 1 has also been recorded as a cohort or group of 4-5 mm growing follicles on the day of ovulation (Bodensteiner *et al.*, 1996). These follicular wave emergences have been characterised by an increase in the plasma FSH concentrations. Hence, cows with 2-wave follicular cycles have 2 FSH surges and 3-wave cycles have 3 surges respectively. These FSH surges are suppressed by the negative feedback from products of the emerging follicles (primarily estradiol and inhibin). This periodic suppression of FSH preserves the resources of the ovary and prevents a continuous recruitment of large antral follicles (Adams *et al.*, 1994; Bergfelt *et al.*, 1994b).

The first follicular wave in bovine follicular development starts on day 1, with day 0 being taken as the day of oestrus. During the next 3 days of the oestrous cycle these follicles will

grow from 3 mm to a population of follicles of between 4 to 8 mm in diameter (day 3 of the cycle) (Ginther *et al.*, 1996; Hagemann *et al.*, 1998). One of the follicles starts to grow at a faster rate and will become the future dominant follicle, while the other follicles will become subordinate (Ginther *et al.*, 1996). At day 6 of the oestrous cycle this follicle will reach its maximum size and stay functional for another 2 to 4 days. This is called the first dominant phase. The first dominant follicle will then lose its functionality (regression phase) and a new wave of follicular growth will start. In both 2- and 3- wave oestrous cycles, emergence of the second wave occurs on day 9 or 10 for 2-wave cycles, and on day 8 or 9 for 3-wave cycles (1 or 2 days earlier). In 3-wave cycles, a third wave emerges on day 15 or 16 (Mapletoft and Adams, 2001). The regression of the corpus luteum (CL) will stimulate the dominant follicle (of the second or third wave) to stay functional and the final LH surge will lead to the final follicular and oocyte maturation and ovulation of the dominant follicle. All the other follicles will undergo atresia and final elimination (Hsueh *et al.*, 1994). The emergence of the next wave is delayed until the day of the ensuing ovulation. The corpus luteum begins to regress earlier in 2-wave cycles (day 16) than in 3-wave cycles (day 19), resulting in a correspondingly shorter oestrous cycle (20 days vs. 23 days, respectively). Therefore oestrous cycle length may indicate the number of follicular waves that a given cow has within each cycle (Mapletoft and Adams, 2001). Single-wave cycles have been reported in heifers at the time of puberty (Evans *et al.*, 1994) and in mature cows during the first interovulatory interval after calving (Murphy *et al.*, 1990). The elimination process of the follicles can take place for between 1 and 2 weeks and lead to a group of follicles (up to 85%) which may become atretic at any stage of the oestrous cycle (Kruip and Dieleman, 1982).

The production of embryos following *in vitro* maturation, fertilization and culture of oocytes collected directly from the ovaries has been performed for therapeutic reasons in humans, offspring production purposes in domestic animals and for experimental research in laboratory animals (Smith *et al.*, 1996). A large variation in the quality of oocytes aspirated directly from bovine ovaries has been reported (Brackett and Zuelke, 1993) and it has been suggested that the intra-follicular environment to which the oocyte is exposed has a major affect on the variability obtained in the developmental competence of the oocytes (Callesen *et al.*, 1986). Large antral follicles in cattle contain high intra-follicular concentrations of

oestradiol, while the atretic follicles contain higher progesterone and androgen concentrations (Ireland and Roche, 1983; Grimes *et al.*, 1987; Spicer *et al.*, 1987). The atresia of the small follicles is said to be related to the presence of a large dominant follicle (Smith *et al.*, 1996). The development of a dominant follicle is closely related to the regression of the subordinate follicles, and new small follicles will only develop once growth of the dominant follicle has ceased (Savoy *et al.*, 1988).

Oocytes collected from dominant follicles have been reported to have superior developmental capacity, compared to those collected from subordinate follicles (Johnson *et al.*, 2001). However, in other observations by Revah and Butler, (1996) it was suggested that oocytes from prolonged dominant follicles (follicles being dominant for longer than the normal period of time) to demonstrate a marked decrease in developmental capacity, due to the premature *in vivo* maturation of the oocyte. In later studies, Hagemann (1999), indicated factors such as follicle size, the day of the oestrous cycle, level of atresia and other follicles (e.g. dominant follicles), to have an influence on the developmental capacity of the oocytes. In these studies, the blastocyst development was greater in oocytes collected during phases of follicular growth than those collected during phases of follicular dominance. It was thus suggested that different stages of the oestrous cycle and follicle size require different nutrient requirements during the culturing processes of IVEP. Hagemann *et al.*, (1999), recorded significantly higher embryo yields from follicles in the 3-5 mm diameter range, compared to larger follicles.

According to Machatkova *et al.* (1996), the stage of the oestrous cycle has a significant effect on *in vitro* blastocyst production. Oocytes collected during the late luteal phase of the cycle produced a much higher percentage of blastocysts than oocytes collected at any other stage of the oestrous cycle. Hagemann *et al.* (1998), on the other hand, found oocytes collected on day 2 and 10 of the bovine oestrous cycle to produce a higher percentage of blastocysts than oocytes collected on day 7 and 15 of the oestrous cycle.

Contrary to general belief that the developmental capacity of oocytes collected from follicles at different stages may be jeopardized by the stage of the oestrous cycle. Results obtained by Smith *et al.* (1996), demonstrated the stage of the follicle to have no effect on the *in vitro*

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developmental capacity of the oocyte. These results suggest that neither the endocrine milieu, nor the presence of a dominant follicle has any effect on the developmental capacity of an oocyte *in vitro*. This means that the stage of the oestrous cycle plays no major role as far as the collection of oocytes for *in vitro* procedures is concerned, and also paves the way for blind (no time-frame) ultrasound guided oocyte collection protocols in the bovine.

Salamone *et al.* (1999) suggested that oocytes can be reprogrammed after removal from their follicular micro-environment so that even oocytes with an already expanded cumulus complex (sign of maturation) and aged oocytes (early atretic) could be more viable *in vitro*.

The literature thus shows that different researchers have different opinions regarding possible factors influencing oocyte competence in terms of *in vitro* development. These different results and opinions make it even more difficult to develop a standard approach for the collection of oocytes *in vivo* for *in vitro* embryo production purposes. Certain researchers suggest that oocytes can be collected during any stage of development, while others advocate a more systematic controlled approach where all the different factors are considered before *in vivo* oocyte collection is performed.

1.1.2 The effect of the donor type cow on oocyte production and *in vitro* embryo development

A normal embryo, with the capacity to develop to term, is affected by the two parental components which are the spermatozoa (x or y chromosomes) and the oocyte. However, the cytoplasm of the early embryo is composed almost entirely of the cytoplasm of the oocyte. The ooplasm (cytoplasm of the oocyte) contains the necessary elements for early embryonic development and an intrinsic programme that regulates early embryonic development (Waksmundzka *et al.*, 1984). This potential of the oocyte is demonstrated by the phenomenon that the bovine ooplasm can reprogram the nuclei of somatic cells, thus creating a state of totipotency supporting embryonic development, as in cloning (Renard, 1998; Kikyo and Wolffe, 2000).

With the large numbers of oocytes obtained from slaughterhouse material, the paternal

factors influencing *in vitro* bovine embryo production could be demonstrated (Eid *et al.*, 1994; Ward *et al.*, 2001). There have also been a few studies on the possible maternal affect on *in vitro* embryo production in cows. In one of a few studies on this aspect, it was recorded that the individual donor cow with varied genetic background has different abilities to produce oocytes by OPU and the oocytes have different levels of competence for *in vitro* embryo production (Tamassia *et al.*, 2003).

1.2   External factors affecting oocyte viability and embryo production efficiency

1.2.1   The effect of season on oocyte quality and IVEP

Seasonal variation in the reproduction performance of cows does occur. These changes are attributed to changes in temperature, humidity, photoperiod and nutrition (Tucker, 1982). In Holstein cattle there is, for example, a definite seasonal sexual pattern, with peak fertility being recorded in the winter and a marked decrease being observed in summer (Ron *et al.*, 1984). This seasonal pattern is mainly attributed to the effect of high ambient temperatures on the endocrine system (Rosenberg *et al.*, 1982), the ovaries and the uterus (Wolfenson *et al.*, 1995), and the embryo (Putney *et al.*, 1989). High ambient temperatures affect the duration and the intensity of oestrous expression and an increase in the duration of the anoestrous period and the incidence of silent ovulations (Gwazdauskas *et al.*, 1981).

It is further stated that season has a definite effect on the reproductive profiles in Bos Indicus cows, with a marked decrease in fertility being recorded during the autumn and winter months (Kinder *et al.*, 1997). However, season does not seem to affect aspects such as the duration of the oestrous cycle or the number of follicular waves per oestrous cycle. The seasonal decrease in fertility seems to be more related to the interruption of ovulation, luteal formation and luteal function in cows (Zeitoun *et al.*, 1996). Rutledge *et al.* (1999), however showed season to have a definite effect on blastocyst yield from abattoir material. So for example, blastocyst production was reduced during mid- and late summer. This was preceded by an increased variation, starting from mid- to late spring. The winter and autumn periods were

characterized by high and stable yields of embryos. This profile of blastocyst production matched the non-return rate following AI in the same cattle populations. Factors such as breed, climate, age and nutritional status can also modify the affect of the seasonal pattern.

Bovine oestrous cycles are characterized by 2 to 3 waves of follicular development and several reports have shown a definite decrease in the number of small and medium size follicles following the exposure of females to heat stress (Wolfenson *et al.*, 1995). Contrary to this, Badinga *et al.* (1993), found no change in the pattern of follicular growth during the first wave of follicular development. However, differences were observed in the efficiency of follicular selection and dominance in later follicle wave development.

Embryonic losses form a major part of decreased fertility in cattle (Putney *et al.*, 1989) and oocytes subjected to heat stress have demonstrated the increased incidence of embryonic abnormalities in cattle (Payton *et al.*, 2004). Rocha *et al.* (1998) reported a decrease in the quality and developmental competence of oocytes after *in vitro* maturation and fertilization during the warm season. In the Holstein a decrease in viability of both *in vivo* and *in vitro* produced embryos has been reported during the warm summer season. This tendency was not observed during the cold season (Ryan *et al.*, 1992; 1993).

Zeron and Arav (2002), recorded a higher level of saturated fatty acids in the oocytes and granulosa cells during the summer periods, compared to the higher levels of mono-unsaturated and poly-unsaturated fatty acids during the winter months. This fatty acid composition (higher levels of saturated fatty acids) of the membranes can affect membrane functionality and oocyte viability. This finding may explain the lower developmental competence of oocytes during the warmer periods (Payton *et al.*, 2004).

1.2.2 The effect of superovulation, hormonal treatment and timing of oocyte collection on *in vitro* embryo production

It is widely accepted that superovulation causes an interruption or alteration in the oocyte maturation process, thus reducing the number of transferable embryos following the MOET procedures (Callesen *et al.*, 1986). So for example superovulation has been reported to have an effect on the normal physiological processes of final oocyte maturation (Hyttel *et al.*, 1997), follicular steroidogenesis (Assey *et al.*, 1994), fertilization (Hyttel *et al.*, 1991), and embryonic development (Greve *et al.*, 1995). This phenomenon has a direct effect on the use of superovulation for *in vivo* oocyte production and collection.

Superovulation can increase the number of ooyctes produced for *in vitro* culture (Armstrong, 1993). However, the developmental capacity of these oocytes may decrease and similar proportions of embryos have been obtained when using stimulated and non-stimulated oocytes in an *in vitro* culture system (Lonergan *et al.*, 1994; Goodhand et al., 2000; Combelles and Albertini, 2003). This is contrary to the belief that oocytes from larger follicles (stimulated animals) have a higher developmental competence than those from smaller follicles (non-stimulated animals) (Blondin and Sirard, 1995). In post partum beef cows a beneficial effect was reported following superovulation with a higher number of oocytes and embryos being recorded in stimulated, compared to non-stimulated donors (Perez *et al.*, 2000).

Blondin *et al.* (1997b), reported a marked increase in the number of embryos, as well as number of cells per embryo when oocytes were collected 48 h after FSH stimulation, compared to 24 and 72 h and in non-treated cows. It was also suggested that oocytes need time to acquire the developmental capacity with a long or too short period being detrimental. It may be that oocytes from superovulated follicles may not have obtained full nuclear and cytoplasmic maturation and need time for the processes to complete. These factors have a major effect on the timing of oocyte collection following slaughter and the time of ultrasound guided oocyte aspiration after superovulation.

Bousquet *et al.* (1999), after applying a superovulation treatment in a commercial cow enterprise, reported a mean of 4.7 embryos per collection being recorded - which was similar to the mean embryo yield reported in MOET super-stimulation treatments. Goodhand *et al.* (1999; 2000) reported an increase in embryo production per oocytes recovered (22% to 39%) after stimulation for 3 days, using declining dosages of FSH. Although the average embryo yield per session was higher in stimulated animals, the total embryo yield over the same period using a weekly non-stimulated approach was higher (Merton *et al.*, 2003). After FSH stimulation, Dieleman *et al.* (2002) obtained a similar number of embryos from oocytes collected 2 h prior to the LH surge, compared to those collected 24 h after the LH surge. Similarly Van Wagtendonk de Leeuw and De Ruigh (1999) reported a beneficial effect following superovulation in first parity donor cows, where a combined protocol of stimulation and non-stimulation was applied in the same donor animals. The donors produced more embryos when stimulated than over the periods when not stimulated, however, the number of embryos did decline as the number of OPU collections increased.

Guyader Joly *et al.* (1997) recorded the same number of oocytes being collected and embryos produced when OPU was performed twice a week without superovulation, compared to once a week with superovulation. However, in certain individuals the technique of superovulation has resulted in a higher number of embryos being produced (Ptak *et al.*, 2003).

Follicular wave synchronization in conjunction with superovulation treatment can also have an enhancing effect on embryo production. Garcia *et al.* (2000) recorded higher cleavage rates following OPU when the donor's follicular waves were synchronized by dominant follicle removal, and a 17 β estradiol injection, followed by a once-off FSH injection.

Further the induction of a pre-ovulatory FSH and LH surge in female donor calves by means of GnRH administration has been shown to have a beneficial effect on the number of matured oocytes recovered from stimulated animals. However, no significant effect was recorded regarding the overall embryo yields for the stimulated and non-stimulated groups. The conclusion was made that GnRH treatment held no beneficial effects for *in vitro* bovine

embryo production (Fry *et al.*, 1998). The same findings were recorded in mature female donors, where neither GnRH administration alone or in combination with dominant follicle puncture had any beneficial effect on superovulation response for *in vitro* embryo production. Both the number of unfertilised ova and degenerative embryos increased when GnRH treatment was used prior to superovulation (Kohram *et al.*, 1998). In another study, Bordignon *et al.* (1997) recorded a beneficial effect of GnRH treatment prior to superovulation for *in vitro* embryo production in cattle. This study was carried out on heifers and the more satisfactory results obtained could be ascribed to a more synchronous group of oocytes that matured more uniformly *in vitro*.

Bols *et al.* (1998) tested the effect of the long-term use of recombinant BST treatment on oocyte and blastocyst yield in an OPU-IVF program. Although a higher total number of follicles were recorded in the treated group, there was no difference in the total number of oocytes recovered and blastocyst yield per cow per session. Some of the reports on the effect of superovulation and other hormonal interventions to improve embryo yield after OPU, IVM and IVF are quite contradictory, and will have to be repeated in order to confirm certain findings. Most of these differences in findings could probably be ascribed to the multi-factorial nature of the *in vitro* embryo production programme and that no laboratory utilizes exactly the same procedures and protocols.

1.2.3   The effect of body condition and diet of the donor on embryo production

It has been found that the roughage : concentrate ratio has very little effect on *in vitro* embryo yield in beef heifers. However a total restriction of diet does result in significantly higher blastocyst yields (Nolan *et al.*, 1998). Donor animals in an IVEP program with a low body condition score (BCS) can still be utilized for *in vitro* embryo production, although the blastocyst production is relatively low (4.85%). These animals can be utilized and maximum use thus made of the superior genetic material (Aguilar *et al.* 2002).

1.2.4 The effect of the interval between oocyte collections

When evaluating the effect of the interval between oocyte collections heifers subjected to twice weekly transvaginal oocyte aspiration (OPU) developed more follicles per collection than those collected once a week (7.4 ± 0.2 vs. 6.4 ± 0.3 follicles/collection respectively). However, the oocyte yield as such was not significantly different for the twice and weekly collections (2.4 ± 0.1 vs. 2.6 ± 0.2 oocytes/collection respectively). Cleavage rates were also higher in twice weekly aspirated animals, compared to those collected once a week (68.6% vs. 59.7% respectively) and the blastocyst production was higher with twice-weekly aspirations (19.6% vs. 10.4% respectively)(Nolan *et al.*, 1998).

Merton *et al.* (2003) reported the interval between ultrasound guided oocyte retrieval (OPU) to influence both the quality and the quantity of the oocytes obtained. If recovery of oocytes by ultrasound is to be done efficiently, all follicles with a diameter of >2-3 mm are to be aspirated. This will lead to the development of a new cohort of follicles over the next few days. A significantly higher number of cumulus oocyte complexes (COC's) were recovered when using a 7-day interval between oocyte collections, compared to a 3 or 4-day period. However, the quality of the COC's has been recorded to be lower following a 7-day interval and higher following a 3-day interval. The interval between retrievals has also been recorded to have an effect on the blastocyst production (19.7% vs. 13.5% for the 3 and 7 day intervals respectively). This is in agreement with the hypothesis that the dominant follicle that would have developed 3 days after retrieval exerts a negative effect on the subordinate follicles. This in turn leads to impaired developmental competence of the oocytes within these suppressed follicles (Merton *et al.*, 2003). Hanenberg and Van Wagtendonk de Leeuw (1997) recorded a higher embryo production with a 3 day interval between collections, compared to a 4 or 7 day interval between collections - which may be attributed to the formation of the dominant follicle after 3 days.

When a 2 or 5 day interval between aspirations in OPU was used, the best quality oocytes with a subsequent higher blastocyst production were recorded when using a 2-day interval. This again supports the hypothesis of the effect of the dominant follicle on the quality of the

oocytes obtained from subordinate follicles. In theory, a 3 times per week oocyte aspiration interval may yield the best results - as far as the percentage of embryos per oocytes collected is concerned. However, this practice may not be practical and cost-effective (Merton *et al.*, 2003). In a study by Imai *et al.* (2000), it was concluded that a skilled operator will obtain better results, as far as total embryo production is concerned if a 7 day interval for OPU is used. From this it is obvious that there are contradictory reports on the optimum interval between oocyte aspirations *in vivo*.

Petyim *et al.* (2003) tested a different approach whereby a so-called continuous and discontinuous technique of bovine oocyte collection was used. In all cases the animals were aspirated twice weekly, with the difference in the discontinuous group being that the animals were only aspirated between day 0 and 12 of the oestrous cycle. The discontinuous scheme yielded quantitively higher oocyte numbers than the continuous scheme - if it is considered that fewer aspirations were performed, compared to the continuous scheme. This discontinuous protocol was carried out during the period associated with the first follicular wave, which has been proven to yield a higher number of follicles (Sirois and Fortune, 1988). Pieterse *et al.* (1988) also reported a higher number of follicles being aspirated during the early period of the oestrous cycle. The recovery rate of the oocytes was generally also found to be higher during the first period of the oestrous cycle when using the discontinuous system (Takenouchi *et al.*, 2001). A possible explanation could be the higher prevalence of blood filled follicles in the continuous approach, which leads to mistaken follicle identification. Contrary, no differences in the number of oocytes, quality of oocytes and embryo production were recorded between the continuous and discontinuous techniques by Bergfelt *et al.* (1994a).

The effect of frequency, duration of aspiration and subsequent superovulatory response was further evaluated in both primiparous and multiparous donor animals. With an aspiration of once a week, no difference in the number of observed follicles was recorded regarding the parity of the animal. However, the recovery rate decreased when animals were aspirated for a period of 12 weeks, compared to aspirations for a period of 4 or 8 weeks. Increasing the aspiration schedule to twice a week did not reduce the number of follicles observed, follicles

aspirated, oocytes recovered per session or the recovery rate. There was also no difference in the response to superovulatory treatment after aspiration in all groups, including a control group that had undergone no follicular aspiration. It was concluded that by increasing of the number of aspirations to twice a week it should have an effect on the total embryo production, but not on any other parameters of interest in oocyte donors (Broadbent *et al.*, 1997).

1.2.5   The effect of age of the donor cow on *in vitro* embryo production

Many of the constraints (e.g. age at puberty, number of offspring produced, etc.) that previously restricted farm animal reproduction have been eliminated by the use of assisted reproduction technologies. So for example, the use of *in vitro* embryo production systems allow for oocytes to be collected from very young (pre-pubertal) to older animals that are past their effective reproductive lifespan (Armstrong, 2001). In the case of pre-pubertal donors, pre-stimulation is necessary to obtain an acceptable quality and number of oocytes (Armstrong *et al.*, 1992). Kuwer *et al.* (1999), when testing different superovulation regimes, found no increase in the oocyte yield for cows at different ages. There was however an increase in the percentage of embryos from the stimulated group, compared to the non-stimulated group in younger animals.   Oocytes from pre-pubertal animals were also found to have a reduced potential ability to develop to normal embryos (Armstrong, 2001).

The age of the oocyte donor is an important factor influencing the developmental competence of the oocyte. Some age related abnormalities include a) the inability to complete meiosis that could lead to problems with fertilization; b) abnormalities in meiosis that could lead to normal fertilization, but then leads to genetic abnormalities that compromise embryo viability; c) cytoplasmic deficiencies that express itself at different stages before and after fertilization. Oocytes collected from young donors also tend to be less tolerant to handling and *in vitro* culture environments than oocytes obtained from adult donors. As a bonus the use of oocytes from older donors can have a significant beneficial effect as far as the extension of the reproductive lifespan of superior quality females is concerned. This is also applicable to the conservation of rare and endangered breeds and species. It is generally accepted that female fertility decreases with age and this phenomenon is also reflected in, *in vitro* embryo

production procedures with a decreased success rate resulting from aged oocyte donors (Armstrong, 2001).

1.2.6   The effect of the procedure of oocyte harvesting on IVEP

It has been shown that oocytes derived from transvaginal oocyte aspiration have a much lower developmental capacity than selected oocytes from abattoir material. The major difference between differently sourced oocytes being the amount of cumulus cells present - with transvaginally aspirated oocytes having much less cumulus cells surrounding the oocyte. These cumulus cells are known to have a definite effect on the pyruvate production and a role in protecting the oocyte against oxidative stress. Both factors having a marked effect on the *in vitro* embryo production potential (Tervit *et al.*, 2002).

Transvaginal ultrasound oocyte collection (OPU) results in a more homogenous type of oocyte being obtained, compared to the quality of the oocytes obtained following the traditional MOET. This is due to the repetitive nature of the transvaginal oocyte collection technique, which eliminates most of the dominant and atretic follicles (Merton *et al.*, 2003). It has been reported that higher blastocyst rates are achieved with abattoir-derived oocytes, compared to transvaginally collected oocytes - this probably being due to the so-called post mortem effect in which the oocyte becomes less attached to the follicle wall and results in a more complete retrieval, with the whole COC being intact. Incubating the ovaries at 30 $^{\circ}$C for 4 h instead of 2 h can double the embryo production numbers. It has been hypothesized that the post mortem condition causes a certain level of atresia in the follicle, which results in a positive effect on the subsequent embryo production (Blondin *et al.*, 1997a).

Transvaginal ultrasound oocyte pick-up is sometimes performed on animals of high genetic merit, but with an impaired fertility status. Due to the relative scarcity and the value of oocytes from these animals, oocyte selection is sometimes compromised - which results in a lower embryo production success. This phenomenon may also give an incorrect picture of the true developmental capacity of the *in vivo-* derived oocytes (Merton *et al.*, 2003).

Santl *et al*. (1998) evaluated an alternative method of oocyte recovery in cattle by using the laparoscopic ovum pick-up (L-OPU) technique, as an alternative to the ultrasound guided oocyte recovery (U-OPU) procedure. In this study the same group of donor animals were used to eliminate donor variation. The mean number of follicles, oocytes and embryos produced per session were significantly higher following U-OPU, compared to the L-OPU technique. This could probably be ascribed to different follicle populations being aspirated by the two different methods. L-OPU only allowed for superficial follicles to be aspirated, while U-OPU provided for follicles to be aspirated deeper in the cortex ovarii - as a result, more follicles could be punctured by the U-OPU method. The oocyte recovery rate was identical in both techniques, but the total number of oocytes collected was higher with the U-OPU method. This was in agreement with Becker *et al*. (1996) who found the oocyte quality obtained to be better following U-OPU than L-OPU. The fact that different follicle populations were aspirated and the variation in vacuum pressure used with L-OPU could have served as an explanation for this variation experienced.

A difference in oocyte quality was also reflected in embryo production following IVM, IVF and *in vitro* culture (IVC). It is generally accepted that oocytes with more than 7 layers of cumulus cells have a higher developmental competence than those with less intact cumulus cell layers (Palma *et al*., 1996). The fact that fewer oocytes were recovered using L-OPU could also have contributed to the lower embryo numbers produced, as the culturing of embryos in larger numbers result in higher embryo yields (Keefer, 1995). Santl *et al*. (1998) concluded that although the L-OPU method was effective, the U-OPU method was more efficient, less traumatic to the donors and the preferred method.

1.2.7   Bovine oocyte recovery equipment

The vacuum pressure applied when oocytes are aspirated, plays a definite role in both the oocyte recovery rate and the developmental competence of oocytes *in vitro*. Ward *et al*. (2000) found a vacuum pressure of between 30 and 50mm Hg to give the best results and an increase in pressure (above 50mm Hg) to reduce the recovery rate and increase the number of inferior quality oocytes. The highest percentage (49.5%) of grade 3 oocytes (low quality)

was obtained at a vacuum pressure of 90mm Hg. The number of blastocysts recorded was significantly higher on day 7 and 8 of IVC at 30 and 50mm Hg pressure, compared to 70 and 90mm Hg (Ward *et al.*, 2000).

The frequency setting of the ultrasound probe plays a major role in the success of OPU. Hashimoto *et al.* (1999) found the number of oocytes obtained with a 7.5 MHz probe to be greater than with a 5MHz probe (11.2 vs. 4.3 respectively). This was also true for the oocyte recovery rate (81.7 vs. 44.2 % respectively) (Hashimoto *et al.*, 1999).

The diameter of the aspiration needle, combined with the vacuum pressure has been found to have an effect on the oocyte recovery rate. An 18 gauge (G) needle with a pressure of 40 mm Hg gave similar results to a 21 G needle with a pressure of 80mm Hg. Similarly an 18 G needle with a high vacuum pressure was reported to give significantly poorer recovery results, when compared to the same needle with a low vacuum pressure (Hashimoto *et al.*, 1999).

Thus an 18 G needle with the right vacuum pressure (30 - 50mm Hg) gave the best results as far as the recovery rate of oocytes was concerned. However, the proportion of oocytes with better cumulus investments have been said to be higher when using needles with a smaller diameter. Better recovery rates were also reported with long bevel needles, compared with short bevel needles (Bols *et al.*, 1997).

1.2.8 Bovine oocyte classification

In contrast to the worldwide uniform classification system used for embryo evaluation (IETS system), the oocyte classification system for both slaughterhouse and OPU derived oocytes seems to be more laboratory specific. Oocytes are generally classified regarding the morphology of the ooplasm and/or cumulus cell investment (Merton *et al.*, 2003). A number of classification systems for oocyte quality currently exist. This classification may vary from 3 categories to 6 categories. Wurth and Kruip (1992) classified oocytes into 3 different categories, namely; A = compact and bright cumulus; B = slightly expanded and darker cumulus; C = strongly expanded and degenerative cumulus cells. It was found that the class

B oocytes resulted in a higher blastocyst production than the "better" class A oocytes. The system of Wurth and Kruip (1992) is the classification system currently being used extensively by most IVEP laboratories worldwide.

Blondin and Sirard, (1995) (using a more elaborate classification system with 6 criteria), reported the same tendency for class 3 oocytes to result in better blastocyst production, compared to the superior class 1 and 2 oocytes and the inferior classes of 4 to 6. Merton *et al.* (2003), used a classification system of grade 1 to 4 for oocytes. Grade 1 oocytes were oocytes surrounded by a compacted, round shaped cumulus investment and grade 3 oocytes were completely denuded. All oocytes with cumulus investments between these two grades were classified as grade 2 oocytes. Oocytes with an expanded/degenerative cumulus complex were then classified as grade 4 oocytes. Grade 1 oocytes were found to give the best results as far as embryo production was concerned. A surprising finding was the fact that more grade 1 oocytes are generally collected from slaughterhouse derived ovaries than by the technique of OPU (Mullaart *et al.*, 1999).

In practice it is generally accepted that when OPU is used as an oocyte recovery technique, all oocytes (regardless of the grade) are utilized for IVEP programs.

1.2.9   Reproductive status of the oocyte donor

In studies conducted by Ferré *et al.* (2002), it was found that the reproductive status of the donor had no significant effect on embryo production and the subsequent pregnancy rates. So-called problem, pregnant and normally cycling animals were compared and a significant difference in embryo production was only found when the results of individual donor cows were compared. Avelino *et al.* (2002) reported cows with acquired reproductive failure problems (fallopian tube obstructions, ovarian adhesions, uterus problems related to fertilization, etc.) to give results comparable to *in vitro* embryo production from apparently normal donor cows.

In a study where pregnant heifers and cows were compared as oocyte donors, no difference

was found in the number of follicles and oocytes recovered during month 2, 3 and 4 of gestation (Eikelmann *et al.*, 2000). Ultrasound guided oocyte recovery (OPU) was possible until day 102 and 142 of gestation in heifers and cows respectively. The number of follicles and recovered oocytes decreased significantly during the 5th month of gestation in cows. During this period of 142 days in cows, an average of 7.4 follicles was aspirated and 3.9 oocytes recovered per twice weekly OPU sessions. Half of the oocytes were suitable for IVEP and a mean of 0.41 embryos per session was obtained with no difference in embryo yield between heifers and cows following oocyte aspiration during the gestational period (Eikelmann *et al.*, 2000).

1.2.10 Duration of *in vitro* maturation (IVM) on IVEP efficiency

Experimentation with different oocyte maturation times following OPU, showed no clear difference in development following maturation times of between 16 and 28 h. However, it cannot not be excluded that a maturation time longer than 24 h negatively affects embryo production. Results also suggest that maturation still continues during the first few hours of fertilization and this seems to be beneficial to subsequent embryo production (Merton *et al.*, 2003). These results are in agreement with the results obtained using slaughterhouse-derived oocytes. Ward *et al.* (2002) reported a 24 h maturation period to be the optimal period, when compared to 20 and 28 h.

1.2.11 *In vitro* maturation

Various researchers have evaluated the addition of different supplements during *in vitro* maturation of oocytes and several types of hormones and growth factors have been tested. However, none of these supplements has given better results than the conventional fetal calf serum (FCS) which is widely used by different laboratories (Bevers *et al.*, 1997). Dieleman *et al.* (2002) demonstrated no significant difference between *in vivo* and *in vitro* matured oocytes. Merton *et al.* (2003) however, obtained a significantly higher embryo yield when using *in vitro* matured oocytes. This phenomenon may be explained by the use of recombinant hFSH in these experiments. This is also in contrast to reports by Rizos *et al.*

(2002), Hendriksen *et al.* (2000) and Wurth and Kruip (1992). The medium most commonly used for *in vitro* maturation in cattle oocytes is TCM 199 (Tissue Culture Medium 199). Supplements used have included 10% FCS, FSH, LH, estradiol and epidermal growth factor (EGF) (Majerus *et al.*, 1999). The developmental capacity of oocytes were found to improve significantly when granulosa cells (Galli and Lazzari, 1995) or exogenous growth factors such as IGF 1 and EGF (Galli *et al.*, 1997) or heparin were added to the maturation medium (Lazzari and Galli, 1994). The maturation time could vary from 22 to 24 h, but fertilization would start anything from 18 to 26 h post IVM - with maturation taking place in conventional CO_2 incubators in petri dishes, 4 well dishes or micro drops covered with oil (Majerus *et al.*, 1999).

1.2.12 *In vitro* fertilization (IVF)

It has been reported that the duration of sperm-oocyte incubation does not affect embryo development (Rehman *et al.*, 1994a). This however, has been challenged by Sumantri *et al.*, (1997), who claimed that sires from different breeds require different periods of co-incubation to achieve maximum fertility rates. In the case of human oocytes, brief exposure to sperm results in superior quality early cleavage embryos, when compared to the conventional overnight protocol (Gianaroli *et al.*, 1996; Quinn *et al.*, 1998; Dirnfeld *et al.*, 1999), while other studies have shown no difference in the cleavage rate (Lin *et al.*, 2000).

It is known that sperm from different bulls differ in their ability to fertilize oocytes *in vitro* (Marquant-Le Guienne *et al.*, 1990; Ward *et al.*, 2001). Sperm from the same semen batch and even individual straws within a batch have been shown to differ in fertilizing ability (Otoi *et al.*, 1993).

The fertilization of the oocyte in IVEP is usually performed in TALP (Tyrode Albumin Lactate Pyruvate), supplemented with heparin, penicillamine, hypotaurine and epinephrine (PHE) in 5 % CO_2 and 20% O_2 (Galli and Lazzari, 1996). It has been postulated that the reduction of the O_2 level to 5% and the addition of amino acids to the IVF medium may have a positive effect on the fertilization and developmental rate of the embryo. Similarly, synthetic oviductal fluid

(SOF) as a replacement medium with the addition of non-essential and essential amino acids could also have a positive effect on fertilization. Insemination is generally performed using a variation of sperm concentrations varying between 0.1 and 2.0 x 10^6 sperm/ml, substituted with 1µg/ml heparin and separated on a 45 to 90% percoll gradient to ensure maximum recovery of motile sperm (Galli *et al.*, 2001).

1.2.13 *In vitro* culture (IVC)

After *in vitro* fertilization, the presumptive zygotes can either be cultured *in vitro* or *in vivo* in the ligated fallopian tubes of sheep (temporary recipients). The *in vivo* culture is labour intensive, time consuming, expensive and not extensively used. In most embryo production laboratories, the embryos are cultured *in vitro*. The *in vitro* culture of the presumptive embryos can either be done in a co-culture with various cell types or in so-called defined media (Marqaunt-Leguienne and Humblot, 1998). *In vitro* laboratories use a wide variety of different embryo culture systems - the mediums used either being TCM 199 or Menezo B2 with various primary cell types i.e. bovine oviduct epithelial cells (Eyestone and First, 1989; Bousquet *et al.*, 1999), Buffalo Rat liver cells (Voelkel and Hu, 1992; Hasler *et al.*, 1995), vero cells (Carnegie *et al.*, 1997), cumulus cells (Goto *et al.*, 1988; Broussard *et al.*, 1994) or conditioned medium (Eyestone and First, 1989). Other systems being used include cell free cultures with various modifications of the SOF medium (Tervit *et al.*, 1972; Chatot *et al.*, 1989), calorie restricted (CR1a) (Rosenkrans and First, 1994; Donnay *et al.*, 1996), glutamine present, glucose absent (CZB) (Liu and Foote, 1995) or simply defined without serum (KSOM) (Rehman *et al.*, 1994b).

The number of embryos cultured together with the volume of media used is highly variable between IVEP laboratories. In the case of slaughterhouse derived oocytes, embryos are usually cultured in groups of 10 to 30. However, with the advent of OPU where lower numbers of oocytes are recovered and different bulls are used for fertilization, the culture system has been adapted to allow for the culture of small groups and even individual oocytes (Donnay *et al.*, 1996; Peynot *et al*, 1996).

1.2.14   Semen donors

Most trials regarding *in vitro* embryo production procedures are usually carried out with the aid of frozen semen from bulls of proven fertility in an *in vitro* environment. However, the situation drastically changes in the case of *in vivo* derived oocytes from cows of high genetic merit. In these cases farmers usually dictate which bulls should be used in combination with specific cows. This semen is usually untested under *in vitro* conditions and this is a major factor contributing to the variable results obtained in an *in vitro* embryo production system under practical conditions (Leibfried-Rutledge, 1999).

1.3   General factors affecting *in vitro* embryo production success in cattle

1.3.1   The effect of OPU on donor health

Since the OPU technique entails multiple punctures of the vaginal wall, the peritoneum and the ovaries, several questions have been asked regarding the safety of this technique and the long-term effect on the donor's health. The ultrasound OPU sessions are generally performed on a twice weekly basis, which may lead to unnecessary stress on the donor cow. As far as the general health of the donors is concerned, no above normal incidence of associated inflammatory conditions such as vaginitis and mastitis have been observed during repeated OPU procedures (Chastant-Maillard *et al.*, 2003).

1.3.2   The effect of OPU on ovarian function

Pieterse *et al.* (1988), Van der Schans *et al.* (1991), Bergfelt *et al.* (1994a) and Gibbons *et al.* (1994) observed the presence of haemorrhagic follicles and blood accumulations on the ovaries immediately after puncture and for a period as long as 4 days after puncture. These haemorrhagic foci however disappeared fairly rapidly and caused no permanent lesions on the ovaries. No adhesions were observed by any of the above-mentioned researchers.

Some investigators however have reported the formation of a fibrous tissue net around the ovary (Gibbons *et al*., 1994) or local hardening of the ovarian *tunica albuginea* or cortex (Van der Schans *et al*., 1991). The same observations were made by Chastant-Maillard *et al*. (2003). Even with cows subjected to ultrasound OPU procedures as much as 27 times, none of the above-mentioned conditions were reported - bearing in mind that the OPU procedure has been refined over the years, and the puncture needle has also become much thinner (Chastant-Maillard *et al*., 2003).

Some of the later studies confirmed findings that multiple needle punctures caused short term trauma that healed within 10 days after cessation of the procedure and Kruip *et al*. (1994) reported normal fertility after repeated OPU procedures. Repeated needle punctures do not seem to affect the intra-ovarian corpus luteum and normal secretion of progesterone and regression of the corpus luteum occurs naturally during the OPU procedures. During OPU procedures, no new ovulations occurred, no new corpora lutea were formed and the progesterone level remained low during the whole OPU period (Gibbons *et al*., 1994). In some cases luteal structures were formed from follicular remnants, but with abnormal or incomplete luteinization (Petyim *et al*., 2001).

1.3.3 The effect of the OPU technique on blood cortisol levels

The use of the ultrasound OPU procedure does not increase the blood cortisol (stress) levels above that caused by normal veterinary procedures such as constraint, rectal palpation and caudal epidural anaesthesia. Even with repeated OPU procedures, the blood cortisol levels remain half (70 nmol ml^{-1}) of that induced by exogenous ACTH administration and it could be concluded that OPU does not cause unnecessary stress on the donor animals (Chastant-Maillard *et al*., 2003).

1.3.4 Follicular recruitment in donor cows

There exists a large variation in donor animals regarding the recruitment or production of follicles. This is similar to that found regarding the variation in response to superovulation

treatment. Boni *et al.* (1997) found that although a large variation exists between donor cows, there was a relatively high repeatability (r=0.576) for the same donor to constantly recruit a similar number of follicles over an extended period of time.

1.3.5 Embryo quality following *in vitro* fertilization

It is common belief that the use of *in vitro* produced embryos, which are developmentally compromised compared to their *in vivo* derived counterparts, can lead to altered sex ratios, heavier and less vigorous calves at birth, longer gestation periods, increased abortion rates, increased perinatal and neonatal calf losses and higher incidences of fetal abnormalities (Hasler, 1997; Kruip and Den Haas, 1997).

In general, *in vitro* derived embryos have fewer cells (blastomeres) and these blastomeres are more loosely compacted than their *in vivo* derived counterparts. Compaction of the blastomeres is generally a good indicator of embryo quality and embryos that have compacted by days 5 and 6 in culture eventually progress to day 7 blastocysts. This may explain the relatively poor quality of embryos that reached the blastocyst stage only at day 8 of culture (Lazzari *et al.*, 2000)

1.3.6 Sex ratio of *in vitro* produced embryos

Embryos produced via *in vitro* techniques can be sexed without inflicting too much damage to the embryo. A simple biopsy can be taken for DNA analysis without compromising the future developmental capacity of the embryo *in utero*. The pregnancy rate between sexed and non-sexed embryos has been found to be similar and the actual process of sexing may even assist in the hatching process of *in vitro* derived embryos. In a large number of *in vitro*-produced sexed embryos, the total percentage of females was recorded to be 43.7% - this varied between 40 and 47 % for different seasons of production (Galli *et al.*, 2001). Similar sex ratios have been reported by other researchers (45.2 to 49 % female embryos)(Hasler *et al.*, 1995). In another study, a lower proportion of female embryos (37.5 %) were reported (Bousquet *et al.*, 1999), but these percentages are somewhat lower to those obtained

following both *in vivo* embryo transfer and AI (approximately 50%) (King *et al.*, 1985).

1.3.7 Pregnancy rates obtained with OPU produced bovine embryos

There are two different ways of culturing *in vitro* embryos. The first method is a complete *in vitro* system, without making use of any temporary recipient's reproductive tract for incubation. In the second method ligated oviducts of sheep are used for the culturing of *in vitro* embryos. There seems to be no difference in pregnancy rates obtained following the transfer of *in vivo* produced (flushed embryos) and embryos cultured in sheep oviducts - even after freezing and thawing. When the glycerol method of bovine embryo freezing was used, the pregnancy rates obtained were 55.4% and 53.1% for *in vitro* and *in vivo* produced embryos respectively. When the ethylene glycol method (the more general method) was used, pregnancy rates of 47.4% and 57.4% for *in vitro* and *in vivo* respectively, were recorded (Galli *et al.*, 2001).

However, when the complete *in vitro* bovine embryo production system is evaluated, these pregnancy rates obtained with frozen/thawed embryos change dramatically. Pregnancy rates as low as 38.8% for *in vitro* embryos cultured in a cell free environment, using the SOF method and 42.1% for the co-culture method have been reported. A possible explanation for the difference in pregnancy rate could be that the culture of bovine embryos in sheep oviducts is much more favourable than a total *in vitro* culture system (Van Wagtendonk-de Leeuw *et al.*, 2000).

The occurrence of resorption and abortion with *in vitro* produced embryos has been reported to be higher than that reported for *in vivo* produced embryos. A study has indicated the abortion rate for *in vivo* embryos to be 7.3%, compared to 11.1% for *in vitro* produced embryos (Galli *et al.*, 2001).

1.3.8 Gestation and birth data following the transfer of *in vitro* produced embryos

The incidence of a high birth weight, longer gestation periods, higher percentage of male

calves, congenital malformations, Caesarean sections and perinatal mortalities have been reported to be higher in *in vitro* derived offspring, compared to AI or even *in vivo* derived embryos (Hasler *et al.*, 1995; Kruip and Den Haas, 1997; Van Wagtendonk-de Leeuw *et al.*, 2000). This is one of the major reasons why *in vitro* produced embryos cannot be exported to certain countries and it also constitutes a limiting factor for the extensive use and expansion of IVEP techniques. However, taking into consideration all the negative factors, IVEP is still regarded as a viable technology and tool as far as animals of high genetic merit and accelerated genetic progress is concerned (Hasler *et al.*, 1995).

Van Wagtendonk-de Leeuw *et al.* (2000) reported these problems to be less when using a cell free system, based on the SOF protocol, instead of the co-culture systems. Other researchers reported only a slight increase in the incidence of gestation length and perinatal deaths. The latter investigation was performed with embryos cultured in sheep oviducts and it could be expected that fewer problems would be encountered with embryos cultured in a more normal environment (Galli and Lazzari, 1995).

Chapter 2

Methodology

Introduction

In this chapter, all procedures and techniques used during the execution of the study will be described in detail. In the subsequent chapters, reference will be made to the techniques and procedures as described in Chapter 2. Two types of oocyte donors were used during this study, namely fertility-impaired cows and pregnant cows. Regarding the fertility-impaired cows, some were slaughtered (also referred to as abattoir material), but in most of the cases only ultrasound ovum pick up (OPU) was performed on the animals. In the pregnant cows, only OPU was performed as the method of oocyte retrieval. A more detailed description of the specific donor cows used will be set out in each relevant chapter.

The method **(method 1)** for both oocyte retrieval (oocyte aspiration) and *in vitro* embryo production that was followed for fertility-impaired donors (these animals were regarded as fertility -impaired due to factors such as old age, repeated MOET procedures, certain uterine conditions and a general failure to become pregnant after repeated services or inseminations) (Chapter 3 and 5) (ARC Animal Improvement Institute laboratories, Irene, South Africa) and an alternative method **(method 2)** followed for pregnant donors (Chapter 4) (Federal Agricultural Research Centre (FAL), Institute for Animal Breeding, Mariensee, Germany) is specified. None of the results obtained from the different reproductive laboratories were directly compared.

Oocyte retrieval and *In vitro* embryo production procedures for fertility-impaired donor cows

2.1   Harvesting of oocytes from abattoir obtained ovaries (fertility-impaired cows following slaughter)

Bovine ovaries were collected with the aid of sterilized instruments immediately post slaughter and placed in a flask containing a sterile standard phosphate buffered saline (PBS) solution at 38 ^{0}C. The time from collection to laboratory processing was minimized and oocytes were harvested within 2 to 4 h after ovary collection. All oocyte retrieval procedures were performed in a water bath or on warm surfaces at 38 ^{0}C, at an ambient temperature of 30 ^{0}C.

The bovine ovarian surfaces were superficially sliced with a scalpel blade in a checkerboard pattern to release as many as possible of the cumulus oocyte complexes (COC's) from the follicles. During this procedure, the ovaries were regularly rinsed (20ml) with a HEPES buffered, Tyrode lactate collection medium, supplemented with heparin (0.0183 g/l). The released COC's were collected in the same medium. Oocyte collection was limited to 20 minute periods, with the resultant cellular suspension being allowed to precipitate and the pellet stereo-microscopically examined. The COC's were isolated, rinsed twice in the collection medium and once in the maturation medium (see 2.2.6), before incubation in the latter for a 24 h period.

2.2   Harvesting of oocytes from live fertility-impaired donor cows (OPU)

Donor cows were brought to the laboratory on an *ad hoc* basis according to individual owner's preference. These animals were classified as fertility-impaired for different reasons - ranging from old age, repeated MOET procedure treatments, certain abnormal uterine conditions and

general failure to become pregnant following repeated inseminations or natural services. In most cases it was difficult to determine the exact cause of the impaired cow fertility. In short, for animals which failed to produce either embryos following MOET or calves, the only solution left was to harvest oocytes for IVEP. The cows were all of high commercial or genetic merit and the owners agreed to subject them to these experimental procedures in the hope of obtaining some offspring from these valuable animals which would not have been otherwise possible.

All donor cows were subjected to an initial screening period of 3 to 4 weeks at the laboratory to determine whether normal follicular activity was present on the ovaries. The screening was performed with the aid of a twice weekly ultrasound scanning of both ovaries. If acceptable follicular activity (development of at least one follicle per reproductive cycle) was observed, the cows were entered into the ultrasound guided oocyte aspiration (OPU) program. If no ovarian activity was observed or once follicular activity ceased, the cows were put on a superovulation program (see 3.1.7) prior to being slaughtered. After slaughter the ovaries were removed and processed in the same manner as described for abattoir material (see 2.1).

2.2.1 Animal handling and preparation for the OPU procedure

Donor cows were restrained in a standard cattle crush with adjustable sides in order to minimize all animal movement during rectal and transvaginal manipulation. In some cases animals were sedated with acepromazine maleate (ACP; Pfizer, SA) (at a dose of 44 mg/100 kg bodyweight), although in most cases, no sedation was necessary. Caudal epidural anaesthesia was administered (4 ml lidocain hydrochloride; Centaur Labs, Johannesburg, SA.) and after effective epidural anaesthesia, the rectum was emptied of all faeces in order to facilitate free manipulation of the reproductive tract and the ovaries. Following removal of the faeces, the whole area around the vulva and the vulva itself were thoroughly cleaned and disinfected with a diluted (15%) Hibitane solution.

2.2.2 OPU equipment and procedure

An ultrasound apparatus (Pie Medical 200 Vet, Pie Medical, Holland) (Plate 1) with a 5/7.5 MHZ sector scanner was utilized for ovary visualization. This ultrasound apparatus was equipped with a vaginal biopsy device and a dorsally mounted needle guide, specifically designed for the bovine OPU procedure (Plate 2). A 40 mm (20 G) disposable needle was used for follicular puncture and oocyte aspiration. The needle was connected to a disposable IVF set (tube set specifically for oocyte retrieval) (Pie Medical, Holland) connected to a 15 ml Falcon tube which in turn was connected to an aspiration vacuum pump (Cooke, Australia) - set at a constant vacuum pressure of 60mm Hg. The Falcon collection tube was placed in a heating block (Cooke, Australia) set at a constant temperature of 35 ^{0}C (Plate 3). All visible follicles >2 mm on both ovaries were aspirated, until no more follicles of this specific size were visible. The aspiration circuit was flushed at regular intervals with a PBS collection medium (see 2.2.3) and the aspiration needle was replaced during collection whenever effective and smooth penetration of the ovary could no longer be achieved.

2.2.3 Handling of oocytes during aspiration and collection

Oocytes were collected in a PBS medium (Sigma), supplemented with 1% newborn calf serum (NBCS) and Heparin at a rate of 0.0183 g/l.

2.2.4 The ultrasound OPU protocol

All OPU procedures performed on the fertility-impaired cows were executed once a week until no more follicular activity could be detected. Although this is not the preferred method (2X and 3X a week seems to be slightly more effective – see literature review), it was decided to use this schedule to minimize the possible stress of a twice a week aspiration schedule and for practical reasons. The laboratory had a heavy load of other routine work regarding oocyte and semen processing. This OPU schedule was thus designed to develop a practical approach that could be used on farm and not necessarily an approach that would produce the best oocyte retrieval results under experimental conditions or artificial environments.

Plate 1: Ultrasound apparatus – Pie Medical 200 Vet

Plate 2: Biopsy device with 5/7.5 MHz sector scanner

Plate 3: Vacuum pump with heating block and collection tubes

2.2.5   Processing of bovine oocytes following collection

All oocytes collected by OPU and from the ovaries of slaughtered cows were further processed, without prior selection. In the case of the OPU derived oocytes, a classification system was used, but regardless of the result (for practical reasons), all oocytes were matured, fertilized and incubated. The classification system of oocytes used was derived from that described by Leibfried and First (1979):

1.   Grade A: These included all normal size oocytes, with a dense cumulus and an homogenous even-coloured ooplasm
2.   Grade B: The ooplasm of these oocytes were heterogeneous, granular, lightly coloured and did not fill the zona pellucida (ZP)
3.   Grade C: These included all the abnormally small oocytes

The above-mentioned grades are also referred to as Grade 1, 2 and 3 oocytes by many researchers. In the rest of the study the grading of oocytes will also be referred to as Grade A, B or C respectively. Furthermore this grading system was also sub-divided using the following criteria:

1.   Sub-class 1: More than 4 layers of cumulus cells surrounding the whole oocyte
2.   Sub-class 2: Four or less layers of cumulus cells surrounding the whole oocyte
3.   Sub-class 3: Oocyte only partially covered by cumulus cells
4.   Sub-class 4: Oocyte naked – free of cumulus cells
5.   Sub-class 5: Oocyte covered with dark clumps of cumulus cells (typical post mature morphology)

In Plate 4 the different grades of oocytes are illustrated, the main grades and sub-classes are indicated on separate images of individual bovine oocytes (Grade A, B, C and 1, 2, 3, 4, 5 respectively). The above-mentioned sub-classes were not utilised during this study and only the 3 main grades (Grade A, B and C) were used. Of these 3 grades, Grades A and B were regarded as acceptable quality oocytes. However, under these practical conditions all oocytes, regardless of their quality were matured fertilized and cultured
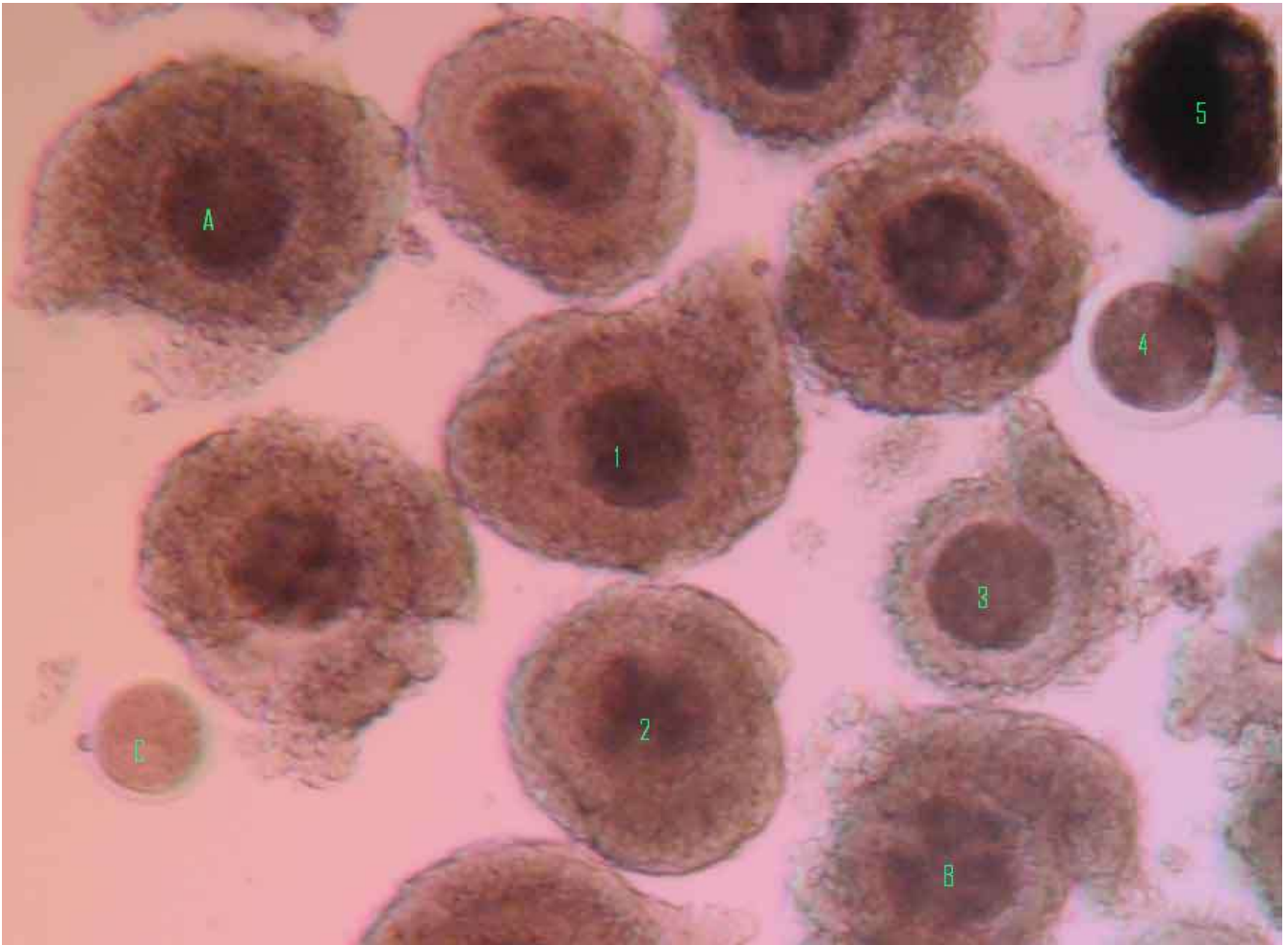
Plate 4: Main oocyte grades and sub-classes indicated on individual bovine oocytes (Grades A,B,C are indicated, with examples of subclasses 1,2,3,4 and 5 also indicated)

2.2.6   *In vitro* maturation of bovine oocytes

The aspirated bovine oocytes were matured for a period of 24 h. The maturation medium consisted of a modified TCM 199 medium (Gibco, Auckland, New Zealand), supplemented with FSH, LH, epidermal growth factor (EGF) and 10% fetal calf serum (FCS). Incubation was performed in a Thermo Forma incubator (Plate 5) at 39 ^{0}C, 5 % CO_2 in air at saturated humidity which was achieved by placing a tray filled with water inside the incubator (DS Visser, 2003; ARC Animal Improvement Institute, personal communication).

Plate 5: Thermo Forma incubators used for all maturation and culturing procedures

2.2.7   Semen processing

The method of bovine semen processing for IVF is extensively set out in 2.2.8.

2.2.8   *In vitro* fertilization (IVF)

After oocyte maturation (24 h incubation), the presumptive metaphase ll oocytes or ova were rinsed vigorously in handling medium (PBS medium, Sigma), supplemented with 1% newborn calf serum (NBCS) and Heparin at a rate of 0.0183 g/l (see 2.2.4) - in order to remove the excessive granulosa cells. This handling medium was used during all phases when ova or embryos were handled under normal atmospheric conditions. The mature ova were then rinsed once in Tyrode's lactate medium, supplemented with amino acids and bovine serum albumin (BSA) and transferred into Nunc multi-wells, containing the same medium, but further supplemented with heparin (0.0183 g/l) (insemination medium)

A dose of bull semen (0.25 or 0.5 ml straw) was thawed at 35 ^{0}C for 30 seconds, before being gently overlaid on a pre-prepared discontinuous Percoll gradient consisting of 90% and 45% isotonic fractions in a test tube. The semen was then centrifuged at 700 G for 30 minutes, the supernatant discarded and the sperm containing pellet re-suspended in 5 ml of the insemination medium. This was followed by a second centrifugation at 300 G for 6 minutes. The supernatant was again discarded and the concentration of the washed live spermatozoa in the resultant pellet was determined with the aid of a Makler counting chamber (DS Visser, 2003; ARC Animal Improvement Institute, unpublished data). After dilution of the sperm pellet with insemination medium, a final insemination dose of 1 x 10^6 per ml live sperm was used for insemination. The insemination dose was standard, regardless of the number of oocytes to be fertilized. Oocytes and sperm were then co-incubated for 24 h to complete the fertilization process (DS Visser, 2003; ARC Animal Improvement Institute, unpublished data).

2.2.9   *In vitro* culture (IVC)

The presumptive zygotes were washed twice in handling medium 24 h after insemination. Two days before this stage, micro droplets (2µl) of embryo culture medium, overlaid with inert mineral oil (Sigma), were prepared and incubated (39 ^{0}C).   Incubation was performed in a Thermo Forma incubator at 39 ^{0}C, 5 % CO_2 in air at saturated humidity which was achieved by placing a tray filled with water inside the incubator (DS Visser, 2003; ARC Animal Improvement Institute, personal communication).

The embryo culture medium consisted of CR2aa (aa = essential and non-essential amino acids) medium (Sigma) supplemented with 10% FCS. Granulosa cells were added to the droplet to form a monolayer of cells in order to establish a co-culture system. Presumptive zygotes were then washed once in the embryo culture medium and then added to the micro droplets. Embryo culture was continued over a period of 7 days, during which 50% of the culture medium was replaced with pre-equilibrated, fresh medium on days 3 and 6. On day 7 of culture, all embryos were evaluated and graded into the 3 main grades according to the IETS guidelines as set out in the 3rd Edition of The Manual of the International Embryo Transfer Society.

2.2.10   Bovine embryo cryopreservation

The freezing of bovine embryos was performed in a cryopreservation apparatus specifically designed for embryo freezing (Freeze Control, Australia) (Plate 6).   Embryos to be cryopreserved were removed from the culture medium and washed in fresh holding medium (AB Technologies, Australia) and after washing, embryos were placed in a 1.5 M Ethylene glycol solution (AB Technologies, Australia) for immediate loading into 0.25 ml French straws. The embryo was placed in the middle of the straw in a column of Ethylene glycol solution surrounded by other columns of holding medium separated by air bubbles (see diagram below). This was achieved by attaching the embryo straw to a 1 ml Tuberculin syringe and aspirating the embryo and different medias into the embryo straw
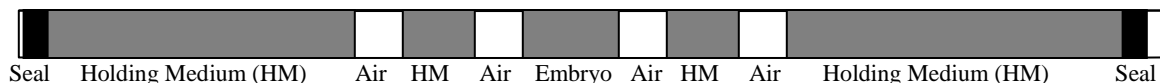
| Seal | Holding Medium (HM) | Air | HM | Air | Embryo | Air | HM | Air | Holding Medium (HM) | Seal |

Straws were loaded into the cryopreservation unit within 10 minutes after placement in the ethylene glycol solution. The following freezing protocol as described by Hasler *et al.*, (1997) and modified by R de la Rey, Embryo Plus, South Africa, 2004, personal communication was used:

Table 2.1 Protocol for embryo cryopreservation

Process	Initial temperature (^{0}C)	End temperature (^{0}C)	Rate (^{0}C/min)	Holding time (minutes)
Start	20	20	-	Max 10
Stage 1	20	-6	5	10
Stage 2	-6	-30	0.6	0
Stage 3	-30	-35	0.1	Immerse in liquid Nitrogen

Plate 6: Embryo cryopreservation unit (Freeze Control, Australia)

2.2.11 Thawing and transfer of cryopreserved bovine embryos

Straws containing the embryos were removed from the liquid nitrogen (-196 ^{o}C) and placed in a water bath at 32 ^{0}C for 20 seconds. After thawing the straws were placed into an IMV embryo transfer pistolette and the entire contents of the straw cervically transferred to a bovine uterine horn, ipsilateral to the side where ovulation took place. All recipient cows were synchronized with the aid of progestagen for embryo transfer 7.5 days after the onset of standing oestrus (see Table 2.2 for the detail regarding the synchronization and embryo transfer techniques).

Table 2.2 The protocol for the synchronization of the bovine embryo recipients

Day	Procedure
0	Insertion of progestagen ear implant (Crestar, Intervet, Holland)
8	Administration of 2 ml prostaglandin F2α intramuscularly (Prosolvin, Intervet, Holland)
10	Removal of progestagen implant with the simultaneous administration of 200 IU FSH (Folligon, Intervet, Holland) intramuscularly
12	Oestrus
19	Embryo transfer (7.5 days after the onset of standing oestrus)

Embryo transfers were routinely performed to establish a baseline pregnancy success rate for all *in vitro* produced embryos (slaughtered donor cows, fertility impaired- and gestating OPU donor cows) (Chapter 3, 4 and 5). This was done to verify the bovine embryo quality, as different laboratories produce *in vitro* embryos of varying quality and to establish the end-impact of this technology. These embryo transfers were continuously performed throughout the total experimental period with both fresh and frozen embryos of all the ad hoc cattle breeds involved being transferred in the *in vitro* embryo production program. A total of 60 embryo transfers were performed to establish these baseline pregnancy rates. The transfer of fresh embryos resulted in a 49.5% pregnancy rate and the frozen/thawed embryos resulted in a 33% pregnancy success rate (these results compared favourably with all others reported in the literature). All the other bovine embryos not transferred were cryopreserved for later

on-farm ET, using the protocol as set out in 2.2.10 (Intervet Product reference for oestrous synchronization – see Table 2.2).

2.2.12   Diagnosis of pregnancy

Pregnancy diagnosis was carried out on a regular basis to determine the pregnancy success rates. This was performed on day 28 to 35 following embryo transfers by ultrasound rectal examination using a Toshiba 32 B ultrasound machine, with a 5 MHz endo-rectal probe. The initial ultrasound rectal examination was followed by regular monthly rectal pregnancy examinations to monitor the gestational progress of the recipient.

Method 2

Oocyte retrieval and *in vitro* embryo production from pregnant donor cows

2.3   Harvesting of oocytes from pregnant donor cows

2.3.1   Animal handling and preparation for the OPU procedure

Animal handling and preparation for ultrasound OPU was performed using the same methods as previously described for fertility-impaired cows (paragraph 2.2.1)

2.3.2   OPU equipment and procedure

In the case of ovum pick-up (OPU) in pregnant dairy cows, different equipment and another set-up was used. Ultrasound OPU was performed using a Picker ultrasound monitor and a 6.5 MHz curved array transducer, using the continuous flushing method (Niemann, 2001; personal communication). The main difference between this method and the one previously

described for fertility-impaired cattle was that the system was flushed continuously with collection medium and not from time to time as described in paragraph 2.2.2.

2.3.3 The bovine OPU procedure and stimulation protocol

In the case of the pregnant donor cows, ultrasound OPU was performed twice weekly, with a 3 and 4 day interval between oocyte aspirations (different from fertility-impaired donors – see 2.2.3). It was decided on a twice a week aspiration protocol to maximize the number of aspirations in the restricted window programme for aspiration. The restricted window for aspiration was caused by the fact that these donor cows were pregnant and the growing size and weight of the fetus would limit the period available for oocyte aspiration.

One group of pregnant donor cows (n=5) was superovulated with a single intramuscular dose of 200 mg FSH (Folltropin, Vetrepharm, Canada) twice weekly at regular intervals of 3 and 4 days. The OPU procedure was performed 72 h after the single FSH administration. The second group (n=5) of pregnant donors did not receive any superovulation treatment and only ovarian follicles ≥2 mm which developed during the natural follicular wave growth patterns were aspirated. The OPU in both groups were continued for a 4 week period (overall 8 OPU sessions in total).

2.3.4 Handling of oocytes during collection

Oocytes were collected in a PBS medium (Sigma), supplemented with 1% newborn calf serum (NBCS) and heparin at a concentration of 0.0183 g/l (see 2.2.4). These oocytes were collected in 15 ml Falcon tubes and kept at a constant temperature of 37 ^{0}C in a water bath, until further processed.

2.3.5   Processing of bovine oocytes following collection

Oocytes were isolated from the collection medium within 30 minutes following aspiration from the ovaries and classified according to the following classification system (Leibfried and First, 1979):

Category 1: Oocytes surrounded by a compact cumulus oophorus, more than 3 layers thick.

Category 2: Oocytes surrounded totally or partially with compact cumulus oophorus, but less than 3 layers thick.

Category 3: Oocytes surrounded by corona radiata cells only.

Category 4: Denuded oocytes without any cellular investment.

Category 5: Small, abnormal and under-developed oocytes.

Category 6: Empty zona pellucida's

Only category 1, 2 and 3 oocytes were further processed and used in the *in vitro* embryo production procedures.

2.3.6   *In vitro* maturation (IVM) of bovine oocytes

TCM medium 199 with Earle's salts (# M2520; SIGMA Chemical CO. St. Louis, USA) was modified by the addition of sodium pyruvate (22 µg/ml), sodium bicarbonate (2.2 mg/ml), penicillin G sodium (0.5 mg/ml) and streptomycin sulphate (0.5 mg/ml) for maturation of the oocytes. Fresh medium was prepared weekly and enriched with estradiol, gonadotrophins (see 2.3.6.1) and 20 % oestrous cow serum before use. Oocytes were matured and incubated for 24 h at 39 ^{0}C in 5 % CO_2.

2.3.6.1   Hormone supplementation for IVM

A stock solution (1mg/ml) of estradiol-17β (SERVA Heidelberg) was prepared in analytical grade absolute ethanol, aliquoted (500 µl) and stored at -20 ^{0}C. The final concentration of the estradiol in the maturation medium was adjusted to 1 µg/ml. Stock solutions of hCG (Ekluton;

Vernie Veterinär Chemie, Kempen, FRG) and FSH (Beckers, Faculté de Medicine Veterinaire, Universite de L Etat a Liége, Belgium) were prepared in saline (0.9% NaCl), aliquotted and stored frozen in Eppindorf tubes. Before use, 1 ml maturation medium was added to each tube to adjust the concentrations to 0.06 IU/ml and 0.05 IU/ml hCG and FSH, respectively. No repeated freeze/thawing of the hormones was permitted and fresh stock solutions were prepared every 2 months.

2.3.6.2   Processing of oestrous cow serum for addition to the maturation medium

Blood was collected from cows during standing oestrus and allowed to stand at 4 ^{0}C overnight after initial clotting at room temperature. The following day, the serum was separated, pooled and centrifuged at 500 G for 20 minutes. Serum was heat inactivated at 56 ^{0}C for 30 minutes and passed through a .22 μm filter for sterilization. The serum was allequoted into 1 ml alliquots and stored in Epindorf tubes at -20 ^{0}C.

2.3.7   Semen processing and *in vitro* fertilization (IVF)

Frozen semen from a Holstein bull of proven IVF-fertility was used in all trials with ooyctes retrieved from the pregnant donor cows. Two semen straws (0.25 ml each) were thawed in a water bath at 37 ^{0}C for 60 seconds. Semen was processed and selected with the so-called swim-up method to separate the motile sperm fraction. The thawed semen was layered under 1 ml of Sp-TALP (see Table 2.3 for details) in sterile glass Pico vials at an angle of 45 ^{0}. After 60 minutes incubation at 39 ^{0}C (5% CO_2 in air), the top 0.85 ml of the medium from each vial was aspirated and pooled into a sterile centrifuge tube. Following the addition of 5 ml Sp-TALP medium, the swim-up separated sperm was centrifuged at 350 G for 10 minutes at room temperature. The soft sperm pellet was resuspended in fresh 5 ml of Sp-TALP medium and centrifuged again after incubation for 5 minutes. The final sperm pellet was suspended in 100 μl of the IVF-TALP medium (containing heparin, hypotaurine and epinephrine) and incubated for 15 minutes at 39 ^{0}C in 5% CO_2 in air. The sperm concentration was determined with the aid of a Coulter counter and/or haemocytometer and adjusted to 50 x 10^6 sperm/ml using the IVF-TALP medium. A 2 μl aliquot of this sperm

suspension was introduced to each 100 µl fertilization droplet to give a final sperm concentration of 1 x 10^6 per ml (Parrish *et al.*, 1986).

2.3.8 *In vitro* fertilization of bovine oocytes

Following the addition of BSA and sodium pyruvate, 100 µl of the Fert-TALP (Table 2.1) medium was laid under silicone oil for washing of the *in vitro* matured oocytes. After washing, the bovine oocytes were placed in a 100 µl droplet of IVF-TALP. The IVF-TALP medium was prepared by adding predetermined amounts of hypotaurine (10 µM), heparin (0.56 µg/ml) and epinephrine (1 µM). Co-incubation of the oocytes and sperm suspension was done for 18-20 h at 39 ^{0}C in 5% CO_2 in air, with a high humidity (Parrish *et al.*, 1986).

2.3.9 *In vitro* culture of *in vitro* produced bovine embryos

All *in vitro* embryo culture procedures were performed in synthetic oviductal fluid (SOF) medium for a period of between 7 and 9 days - until no further embryonic developmental progress (cleavage) could be detected. The composition of the SOF medium is set out in Table 2.4 (Tervit *et al.*, 1972).

2.4 Statistical analyses

To determine significant differences between 2 data sets, the results (i.e. mean number of follicles, mean number of oocytes, oocyte recovery rate, oocyte grading, percentage good quality oocytes, fertilization rate, mean number of embryos, embryo production rate, embryo quality and the number of embryos per OPU session) were analyzed by means of the Student's T test (statistical software for MS Excel). The two-tailed distribution and two sample unequal variance (heteroscedastic) approaches were used. To determine significant differences between 3 or more data sets, the one way analysis of variance ANOVA was used (SAS, 1986). All values are presented as the mean ± standard error (SE). Probability values < 0.05 were considered to be statistically significant.

The correlation coefficient between the two data sets was determined by using the standard formula (statistical software for MS Excel) to determine the correlation coefficient (r) between 2 ranges of values. The nature of the whole investigation (that of technology development for practical use), meant that in some cases, more emphasis was placed on the practical than the statistical significance.

Table 2.3 Composition of media used for IVM, sperm processing and IVF of bovine oocytes (Method 2)

Component*	Unit	Sp-TALP	Fert-TALP	PBS
NaCl	mM	100.00	114.00	130.00
KCl	mM	3.10	3.20	2.38
NaHCO$_3$	mM	25.00	25.00	--
KH$_2$PO$_4$	mM	--	--	1.47
NaH$_2$PO$_4$	mM	0.30	0.30	--
Na$_2$PO$_4$	mM	--	--	8.10
CaCl$_2$	mM	2.00	2.00	0.71
MgCl$_2$	mM	0.40	0.50	--
MgSO$_4$	mM	--	--	0.92
HEPES	mM	10.00	--	--
Na Lactate	mM	21.60	10.00	--
Na Pyruvate	mM	1.00	0.20	--
Penicillamine	μM	--	20.00	--
Phenol Red	μg/ml	0.01	0.01	--
Gentamycin	μg/ml	50.00	50.00	--
Penicillin (Na)	μg/ml	--	--	60.00
Stretomycin-sulphate	μg/ml	--	--	50.00
Bovine serum albumin (fraction V)	μg/ml	6.00	6.00	1.00

*All components were dissolved in SABAX water

Table 2.4 Composition of the SOF culture medium used for *In vitro* culture of bovine embryos (Method 2)

Component*	Unit	Concentration
NaCl	mM	108.00
KCl	mM	7.20
KH_2PO_4	mM	1.20
$NaHCO_3$	mM	25.00
$MgCl_2$	mM	0.50
$CaCl_2$ x 2 H_2O	mM	1.70
Na-Lactate (60%)	mM	3.30
Glucose	mM	1.5
Phenol Red	Mg/100 µl	1.00
Na Pyruvate	mM	0.33
Glutamin	mM	1.00
Gentamycin	Mg/100ml	5.00
Non-essential amino acids aa (Sigma M 7145)	ml/100ml	1.00
Essential aa (Sigma B 6766)	ml/100ml	2.00
BSA (Sigma A 7030)	Mg/ml	8.00

*All components were dissolved in SABAX water

Chapter 3

In vitro **embryo production (IVEP) in beef cattle using fertility-impaired donor cows: The use of oocytes derived from ovaries following slaughter or in combination with ultrasound guided oocyte aspiration (OPU)**

Introduction

A real need exists in South Africa for the development of bovine IVEP technology for application in high producing animals whose fertility has been impaired by age, environmental factors or other unnatural events. The availability of this type of donor could play an important role in the whole concept of genetic improvement, since longevity and reproduction performance are seen as some of the major selection criteria in both dairy and beef cattle. Unfortunately, most of the animals which have been identified as being of high genetic merit for these criteria are usually older animals or animals which have been previously subjected to MOET procedures for long periods of time. Over time it has become a well known fact that both old age and repeated MOET procedures can have a negative impact on the reproduction performance of the donor animals (Pieterse *et al.*, 1988).

In vitro embryo production (IVEP) is the only technique at the disposal of assisted reproduction laboratories which may enable fertility-impaired cows to continue breeding. There are basically two ways of approaching *in vitro* embryo production from fertility-impaired donors. The first method used, would be the slaughter of the animals to retrieve oocytes from the ovaries post slaughter. The other method is to apply ultrasound guided oocyte aspiration (OPU). A combination of the two approaches, OPU for a period of time followed by eventual slaughter, is also an option.

Thus, IVEP is sometimes the only alternative method that can be implemented to produce embryos and offspring from a particular type of donor animal. However, IVEP technology is still relatively new in South Africa and has to be introduced in a responsible way to the animal

breeding industry - so as to avoid resistance due to cost implications by cattle owners and stud breeders. It is, thus, important to investigate the most appropriate and practical approaches and protocols for oocyte harvesting from a unique type of donor animal. The effectiveness and cost-effectiveness of each protocol could lead to either acceptance or rejection by the cattle industry (Pieterse *et al.*, 1988).

There are certain fixed costs associated with the production of *in vitro* embryos – irrespective of the specie. The cost varies according to the different methods (e.g. following slaughter or OPU) of IVEP. Therefore, the cost of producing an embryo can be determined, but the actual value of the offspring in terms of genetic value is more difficult to establish - as it can differ from one donor animal to the next. The question remains from an embryo production point of view, whether it is more effective to keep an animal on a prolonged protocol of ultrasound guided oocyte aspiration or to pre-stimulate and slaughter the donor immediately (Pieterse *et al.*, 1988). No studies have previously been done, especially in South Africa to determine which logistical protocol for IVEP from fertility impaired donors is the best.

The aim of this study was thus to compare the two IVEP approaches and a combination thereof in terms of effectiveness in producing embryos. Additionally, a separate pilot trial was carried out under the same conditions and with the same type of donor animals (different experimental group) to compare the IVEP potential of *in vivo* derived oocytes which were pooled (oocytes from different donors within the same breed, incubated together/same semen donor) and non-pooled (oocytes from different donors incubated separately/different semen donors). This was done in order to establish a more viable approach when confronted by female donors of the same breed and one specific semen donor. Under such circumstances the use of pooled oocytes could be a feasible option in a practical situation.

3.1 Materials and Methods

3.1.1 Experimental animals

3.1.1.1 Experimental animals for pooled and non-pooled oocytes

A group of beef cows (n = 40) (various breeds and parities) of superior genetic quality and with impaired fertility was presented to the reproduction laboratory by individual owners. These cows were from different beef cattle breeds (Simmentaler, Bonsmara, Santa Gertrudis, Romagnola and Brahman) and ranged in age from 2 to 19 years. The average body condition score (BCS) of all these animals was approximately 2.5 on a scale from 1 to 5. The only common denominator or aspect that all animals had in common was the fact that all these animals were classified as being unfit for further reproduction in the cow herd.

The reasons for impaired fertility ranged from old age, repeated MOET procedure treatments, certain abnormal uterine conditions and general failure to conceive and become pregnant following repeated inseminations or natural services, as well as physical injuries. In most of the cases it was difficult to determine the exact cause of the impaired fertility in the cow. In short, animals which failed to produce either embryos (MOET) or calves and thus the only option that remained, was to harvest the oocytes for IVEP.

This group was divided into two sub groups, namely A (n = 10) and B (n = 10). In group A, all oocytes were pooled and in group B, oocytes were processed for each individual donor cow. This trial was performed to compare IVEP efficiency potential following OPU and the use of pooled and non-pooled *in vivo* derived oocytes, respectively.

3.1.1.2 Experimental animals for OPU and slaughter

Donor cows (n=40), (various breeds and parities) of superior genetic quality and with impaired fertility were presented to the reproduction laboratory by individual owners. These cows were from different beef cattle breeds (Simmentaler, Bonsmara, Santa Gertrudis, Romagnola and

Brahman) and ranged in age from 2 to 19 years. The average body condition score (BCS) of all these animals was approximately 2.5 on a scale from 1 to 5. The only common denominator or aspect that all animals had in common was the fact that all these animals were classified as being unfit for further reproduction in the cow herd. The reasons for impaired fertility ranged from old age, repeated MOET procedure treatments, certain abnormal uterine conditions and general failure to conceive and become pregnant following repeated inseminations or natural services, as well as physical injuries. In most of the cases it was difficult to determine the exact cause of the impaired fertility in the cow.

All donor cows were initially rectally screened for a period of 2 to 3 weeks by means of the ultrasound scanning procedure to access and evaluate the follicular activity on the ovaries (De Roover *et al.*, 1997). On request of the owner and if sufficient follicular activity (development of one normal follicle during an oestrous cycle) was observed, certain animals (n=20) were subjected to prolonged ultrasound guided oocyte aspiration protocols in order to obtain oocytes for IVEP procedures. This procedure (OPU) was continued until no more follicles developed or no more embryos could be produced from a specific donor animal.

Once a decision was finally made to withdraw the donor animal from the OPU program, the cow was subjected to superstimulation (see 3.1.7) and slaughtered in a final attempt to retrieve the oocytes for IVEP procedures. The period that cows were subjected to the OPU procedures varied and was only limited and determined by the embryo production performance of the donor animals.

In the other cases the donor animals (n=20) were superstimulated (see 3.1.7) and slaughtered immediately. This was also done either at the request of the owner or in some cases where the animals were not fit to undergo a prolonged OPU procedure. The results were thus recorded for the following groups of animals or combinations thereof: Group 1 (period of OPU) (n = 20); Group 2 (slaughter after OPU – same animals as in Group 1) (n = 20); Group 3 (combination of Group 1 and 2) (n = 20) and Group 4 (slaughter without any OPU) (n = 20).

3.1.2   Husbandry and animal housing

All experimental animals were maintained on natural pastures (Irene Animal Improvement Institute – mixed sweet and sour highveld grazing) with *ad lib* supplementation of a summer or winter lick and eragrostis hay supplementation *ad lib* during the winter months. It was attempted to maintain all cows at a constant BCS of not lower than 2.5, on a scale of 1 to 5. Most animals did, however, lose some body condition during the winter months. This was acceptable due to the fact that there was a constant flow of animals and cows were being transferred from farms where the same pattern of body condition loss during the drier months of the year is experienced. The strategy with this approach was thus to simulate the general BCS profile of animals presented to the laboratory under applied conditions. All animals were kept under constant veterinary supervision for the duration of the trial period.

3.1.3   Ultrasound guided oocyte retrieval protocol

Ultrasound guided oocyte retrieval (OPU) was performed on a weekly basis throughout the year. Although the literature suggests twice weekly retrieval to yield more viable oocytes and embryos, it was decided against a twice-weekly regime of OPU for two reasons. This was due to the fact that the variation within breeds and individual animals would have overloaded the *in vitro* production laboratory with oocyte and semen processing procedures. Secondly, because of the age of certain animals, more than once a week aspiration would put too much stress on the cows. The actual OPU procedures performed were as described in 2.2.1, 2.2.2 and 2.2.3. The general idea was to develop a less labour intensive and practical but effective IVEP technique that could be performed on a large variety of donor cows over an extended period of time.

3.1.4   *In vitro* bovine embryo production (IVEP) procedures

All procedures regarding the total IVEP sequence were performed as described in 2.2.4 to 2.2.9.

3.1.5   Embryo cryopreservation

Cryopreservation of all bovine embryos was performed as described in Chapter 2 (2.2.10).

3.1.6   Semen use for *in vitro* fertilization

In all instances, semen was provided by the individual owners of the oocyte donors, from a variety of bull breeds. All semen was microscopically examined and evaluated and only semen of acceptable quality (% live and progressive sperm movement) was used for the fertilization (IVF) procedures. At no stage was semen pre-tested to determine the capacitation capability with the addition of factors such as heparin and a standard fertilization protocol was used throughout (see Chapter 2; 2.2.8).

3.1.7   Ovarian pre-stimulation of donor cows prior to slaughter

All donor cows which were eventually destined to be slaughtered (n=40) received a standard ovarian pre-stimulation treatment prior to slaughter. This treatment was performed for both donors that finished a period of OPU and donors that were to be slaughtered immediately, without any previous OPU procedures being performed. In this pre-stimulation treatment, cows received a progestagen ear implant (Crestar, Intervet, Holland) for a period of 5 days. On the fifth day a single injection of 2500 IU eCG (Folligon, Intervet, Holland) was administered intramuscularly. Animals were slaughtered 2.5 days after this eCG administration.

3.1.8   Parameters of IVEP recorded

Parameters measured to record the response and efficiency of IVEP included the mean number of oocytes recovered, the number of embryos produced, embryo percentage (total embryos produced as a percentage of the initial number of oocytes retrieved) and the different quality grades of the subsequent embryos. Oocyte quality was not recorded in all

these groups, as the high numbers of oocytes in the slaughtered groups made it impractical to do an accurate laboratory oocyte evaluation.

3.1.9 Embryo transfer and pregnancy rates

Embryo transfers with both fresh and frozen embryos were performed on a regular basis to determine baseline pregnancy rates. See 2.2.11 under Methodology for details regarding the embryo transfer and pregnancy rates recorded.

3.1.10 Statistical analyses

See 2.4 under Methodology for details regarding statistical analyses.

3.2 Results

3.2.1 Pilot trial to establish the IVEP potential of individually cultured or pooled oocytes

The bovine IVEP results obtained from the individual and pooled donor oocyte comparison in the pilot trial are set out in Table 3.1.

Table 3.1 Mean (±SE) individual and pooled bovine oocyte and IVF success rate obtained

Group	Total number of oocytes	% Embryos	Embryo grading (%)		
			Grade 1	Grade 2	Grade 3
Individual	551	17.9 ± 33.3 [a]	46.8 ± 46.5 [a]	38.0 ± 46.6 [a]	15.2 ± 33.9 [a]
Pooled	279	7.4 ± 17.7 [b]	78.9 ± 38.0 [b]	17.3 ± 37.3 [a]	3.9 ± 13.9 [a]

[a-b] Values within a column with different superscripts differ significantly (P<0.05)

The proportion of embryos developing as a percentage of the total number of oocytes processed (matured and fertilized) in the bovine IVEP programme was significantly higher (P<0.05) where oocytes were cultured separately for individual donor cows, compared to the

pooled oocytes (17.9 ± 33.3% vs. 7.4 ± 17.7%, respectively). On the other hand, the percentage of Grade 1 embryos was significantly higher (P<0.05) in the pooled group of oocytes, compared to the individual group (78.9 ± 38.0% vs. 46.8 ± 46.5%, respectively). The large variation (SE) made the interpretation of the data and recording of significant differences difficult.

3.2.2 Results recorded for OPU and slaughtered cows (Groups 1, 2, 3 and 4)

The mean number of oocytes recovered, number of embryos produced, embryo success rate (total number of embryos as a percentage of the initial number of oocytes aspirated) and different embryo grades are set out in Table 3.2.

Table 3.2 Mean (±SE) number of oocytes aspirated, embryos produced, embryo success rate and embryo quality following OPU and slaughtering of donor cows

Group	Number of oocytes recovered	Number of embryos produced	Embryo production (%) (success rate)	Embryo grading (%)		
				Grade 1	Grade 2	Grade 3
1	24.7 ± 9.4[a]	6.6 ± 5.4[a]	22.6 ± 16.8[a]	46.0 ± 36.3[a]	41.5 ± 38.3[a]	12.6 ± 19.0[a]
2	73.0 ± 38.3[b]	10.7 ± 15.7[a]	14.5 ± 15.6[a]	28.5 ± 32.8[a]	51.8 ± 40.5[a]	19.8 ± 37.1[a]
3	97.7 ± 40.2[c]	17.2 ± 16.6[a]	17.4 ± 14.7[a]	46.2 ± 26.8[a]	42.8 ± 23.5[a]	11.1 ± 10.4[a]
4	61.8 ± 40.3[d]	7.2 ± 8.5[a]	10.1 ± 9.6[a]	36.0 ± 42.2[a]	46.8 ± 41.1[a]	17.1 ± 31.4[a]

[a-b-c-d] Values within a column with different superscripts differ significantly (P<0.05)

3.2.2.1 Mean number of oocytes recovered per donor cow

As expected, the number of oocytes recovered per donor was lowest in the group subjected to OPU treatment only (Group 1: 24.7 ± 9.4) and the highest (97.7 ± 40.2) for the OPU/slaughter combination group (Group 3). The number of oocytes in the combination group (Group 3) was also markedly higher than that obtained in Group 4 (61.8 ± 40.3), where cows were slaughtered immediately, following no OPU treatment. The number of oocytes recovered when slaughtered after OPU (Group 2) was higher (73 ± 38.3) than when

slaughtered immediately following stimulation with eCG (61.8 ± 40.3) (Group 4). There were significant differences (P< 0.05) in the number of oocytes recovered between all 4 the groups (Table 3.1), with Group 3 recording the highest and Group 1 the lowest number of oocytes.

3.2.2.2   Mean number of embryos produced per donor cow

The number of embryos produced followed the same pattern as the number of oocytes recovered in the different groups, with a correlation coefficient of r = 0.88 between the number of embryos produced *in vitro* and the number of oocytes retrieved. Again the highest mean number of embryos per group was produced in the OPU/slaughter combination group (Group 3), with no significant difference between the different groups. The overall success rate (percentage embryos produced) was relatively low (± 16%) in all treatment groups.

3.2.2.3   Mean percentage of embryos produced from oocytes recovered per donor cow

Although Group 1 recorded the lowest oocyte recovery rate, the highest mean percentage of embryos produced, as a percentage of the total number of oocytes recovered per donor was recorded in this group (OPU only) (22.6 ± 16.8%). The lowest success rate was recorded in Group 4 (slaughter without any OPU) (10.1 ± 9.6%). No significant difference was recorded between all 4 treatment groups regarding the embryo production success rate, as this success rate was generally very low.

3.2.2.4   Embryo grading

The highest percentage of Grade 1 bovine embryos was recorded in Group 1 and 3, with an occurrence of 46.0 ± 36.3% and 46.2 ± 26.8%, respectively. The lowest percentage of Grade 1 embryos was recorded in Group 2 (28.5 ± 32.8%). The highest (51.8 ± 40.5%) and lowest (41.5 ± 38.3%) percentage Grade 2 embryos was recorded in Group 2 and 1, respectively. Similarly, the highest (19.8 ± 37.1%) and lowest (11.1 ± 10.4%) percentage Grade 3 embryos

were recorded in Group 2 and 3, respectively. There was, however, no significant difference recorded between all the 4 treatment groups regarding the different embryo quality grades.

3.2.2.5 Pregnancy rate following embryo transfer

See condensed results under Methodology (2.2.11) in Chapter 2.

3.3 Discussion

3.3.1 Individual and pooled oocyte comparison

Individual or low numbers of either oocytes or embryos being cultured is the order of the day when OPU is performed on fertility-impaired donor cows and it is of the utmost importance to know how this will affect the overall embryo production. Contrary to other findings, it was found that during this trial the culturing of oocytes from individual donors produced a higher number of embryos than when cultured in bigger groups like a pooled oocyte sample (Keefer, 1995; O'Doherty *et al.*, 1997). It has been shown that in mice (Paria and Dey, 1990); sheep (Gardner *et al.*, 1994) and cattle (De Loos *et al.*, 1989; O'Doherty *et al.*, 1997) oocytes cultured in groups (pooled), showed better developmental competence than oocytes cultured individually. On the other hand, in the canine it has been proven that the stage of the reproductive cycle has a bigger effect on oocyte developmental competence than when the oocytes were cultured in smaller or bigger batches. It has also been proven that oocytes incubated in suitable size groups/batches during IVM performed similar to when cultured individually - when compared to those cultured in groups for the remainder of the culturing process (Otoi *et al.*, 2002).

The fact that in this trial the individually cultured oocytes and zygotes performed better in terms of the eventual embryo production rate than the oocytes and zygotes cultured in groups may be attributed to the fact that relatively low numbers of oocytes were recovered and even when pooled, the number of oocytes were still relatively few. This may negate the positive

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effect of large numbers of co-cultured oocytes/zygotes as reported by other researchers (O'Doherty *et al.*, 1997). Another factor to consider is that most of the studies were carried out on selected abattoir material (Keefer, 1995; O'Doherty *et al.*, 1997).

As the developmental capacity of a smaller number of oocytes is not compromised, it augers well for the way in which donors are usually incorporated into an IVEP programme under practical conditions e.g. where individual cows are used with one specific untested semen donor. The percentage of Grade 1 embryos produced was significantly lower when cultured from individual donors than when the oocytes were pooled. It seems as if the presence of more presumptive zygotes does improve the quality of the embryo at the end of the culture period. This finding is supported by that of Palma *et al.* (1992) and Ferry *et al.* (1994), who found singly cultured embryos to develop more slowly and having fewer blastomeres per blastocyst after an 8 day culture period.

3.3.2   Mean number of oocytes recovered per donor cow

The number of oocytes recovered was lowest following OPU treatment (Group 1) alone. This was to be expected as larger numbers of bovine oocytes are normally recovered when the ovaries are sliced for oocyte retrieval following slaughter (Carolan *et al.*, 1994). The OPU group (Group 1) represented 25.3% of the total number of oocytes retrieved for the OPU/slaughter combination group (Group 3). This constitutes a major contribution towards the number of oocytes available for the IVEP procedures. Similarly Group 3 (OPU/slaughter combination) produced 36.8 % more oocytes than Group 4 - in which the oocytes were retrieved following slaughter only with no ovarian stimulation.

An interesting observation was the fact that Group 2 (slaughter after OPU), produced 15.4% more oocytes than Group 4 (immediate slaughter). This may be indicative of the fact that most animals which were used for OPU procedures initially had a higher follicular activity. This corresponds with the findings of De Roover *et al.* (1997), who found the initial follicular activity, monitored by ultrasound, to be indicative of the number of follicles available for aspiration in a subsequent OPU program for reproductively sound donor cows. There are

currently no reports in the literature of other investigations where similar comparisons were made with the same type of donor cow and similar experimental groupings. All comparisons were made between OPU derived oocytes and slaughterhouse material, but from different donor sources (De Roover *et al.*, 1997).

3.3.3   Number of embryos produced per donor cow

A direct comparison between Group 3 and 4 indicates the potential increase in the mean number of *in vitro* produced embryos per donor cow that can be achieved when OPU is combined with the slaughter procedure. If Group 3 and 4 are directly compared, it is clear that on average there was an increase in favour of Group 3 with 10 embryos produced per donor cow. This represents a significant increase in embryo numbers in practical terms and could have long term implications. Although this difference is not significant in statistical terms, it was significant in practical terms and justifies the use of this advanced and a labour intensive technology procedure of OPU, to be applied on superior donor cows. The same tendency in obtaining a higher embryo output from OPU-derived material, compared to abattoir material has been recorded in Buffalo cows (Neglia *et al.*, 2003). If one considers the fact that a pregnancy rate of approximately 50% can be achieved with frozen/thawed *in vitro* produced embryos, it could relate to 5 more live calves being born if OPU is included in the IVEP protocol (Galli *et al.*, 2001).

The OPU group (Group 1) contributed 38.1 % of the total number of embryos produced in the OPU/slaughter combination group (Group 3). This contribution could justify the extra resources invested in the additional period of OPU treatment before the superior donor animal is eventually slaughtered.

3.3.4   Percentage embryos produced per oocyte

The eventual percentage of embryos produced (success rate) *in vitro* following OPU only, corresponds with the embryos (10 to 30% success rate) obtained by other researchers (Merton *et al.*, 2003). It would seem as if better quality oocytes are recovered by means of

the OPU technique, compared to oocyte retrieval following slaughter – especially when a greater number of oocytes are entered into the IVEP programme (Neglia *et al.*, 2003). This was reflected by the relatively low percentage of embryos produced as a percentage of the total number of oocytes matured and fertilized *in vitro*. This finding is supported by Pavlok *et al.* (1993), Lonergan *et al.* (1994), Blondin *et al.* (1997a), Gandolfi *et al.* (1997) and Kubota and Yang (1998) who found oocytes from the bigger follicles (typical of OPU) to have a better developmental capacity than oocytes derived from smaller follicles (typical of sliced ovaries). The total mean production of transferable embryos from the slaughtered groups (Group 2 and 4) was lower (12.3%) than in the IVM-IVF results from abattoir material (15 to 60%). This may be due to the fact that the oocytes were not previously selected and all available material was used for maximum embryo production (Neglia *et al.*, 2003).

An additional factor that could contribute to the fertilizing capacity of the bovine IVEP programme is the fact that in all the slaughtered groups, the beef cows were pre-stimulated (with eCG) prior to slaughter. It is known that this hormonal stimulation does affect the developmental capacity of the oocytes negatively and this was reflected by the percentage of embryos produced from the initial number of oocytes (Hyttel *et al.*, 1997). The recorded embryo production results in the current trial compares well with those reported in literature where these procedures were evaluated on "normal" cattle donors. It also gives a good indication of the potential for embryo production from this type of donor cow (fertility-impaired) under experimental conditions.

3.3.5 Embryo evaluation and grading

The highest percentage of Grade 1 embryos produced was recorded in Group 1 and 3 - where OPU was used as the only technique or combined with slaughter as the oocyte retrieval method. This was also true for the lowest percentage of Grade 3 embryos recovered within the same groups. An initial better quality oocyte should result in a better embryo grading quality on final evaluation (Wurth and Kruip, 1992). An interesting finding was also the fact that Group 2 (slaughter after OPU) produced the lowest percentage of Grade 1 embryos and the highest percentage of Grade 2 and 3 embryos. If one considers the fact that

these donor animals were kept on an OPU program until either no more good quality oocytes or embryos could be produced, it may reflect on the fact that after stimulation and slaughter the embryo production performance of these cows was still inferior to the rest of the groups. This also indicates that the decision to cease OPU procedures was taken at the right time. No evidence could be found in the available literature where the same approach was evaluated for the *in vitro* production of bovine embryos from this type of donor cow.

3.3.6 General discussion

The question that needs to be answered is whether it is worth the effort to subject fertility-impaired donor cows to a prolonged period of OPU, before eventual slaughter. The answer lies in both the extra number of embryos produced, the percentage of embryos produced from the total number of oocytes recovered, the quality of these embryos and the cost involved. From the current results it can be concluded that the period of OPU produced a high percentage of embryos as well as consequent good quality embryos. Although not all animals performed well when slaughtered after a period of OPU, the combination of the two procedures still effectively produced a higher number of embryos, with acceptable quality. No significant differences could be established following the different procedures and this was mainly due to high standard deviations. This was indicative of the fact that large individual variation existed between individual cows in any type of embryo production procedure used.

In general it can be said that it should be worth the effort, both economically and practically, to subject donor cows that show some form of follicular activity to a period of OPU. This additional window for oocyte retrieval could contribute a significant percentage of embryos, which would otherwise not have been there, to the total number of embryos eventually produced from such a type of donor. This approach could on average produce 5 more calves (10 more embryos with a pregnancy rate of approximately 50%), as opposed to a situation were no OPU was performed (Galli *et al.*, 2001).

Another factor that should be considered and kept in mind is that the decision to slaughter the donor cow is final and if technical problems are encountered during the IVEP process, it may

result in no embryos being produced from a specific superior donor animal. In cows of extremely high genetic merit this can be a disaster and a period of OPU at least affords the opportunity to adjust certain elements of the IVEP procedure, while the donor is still alive.

Animals are usually entered into an IVEP programme as individuals with a specific semen donor. The organization of the *in vitro* procedures should be very precise as to avoid any mixing of the oocytes and embryos due to the commercial value of the animals. It means that in practice the separate culture of oocytes as individual donor groups is preferable. From the results obtained it can also be concluded that the culturing of oocytes from individual donors should not be detrimental to the total IVEP procedure results.

Chapter 4

A novel approach in South Africa to IVEP using pregnant dairy cows

Introduction

The possibility to produce embryos from pregnant cows can be seen as one of the most advantageous developments emanating from the IVEP technologies (Meintjes *et al.*, 1995). IVEP from pregnant cows offer a number of advantages over *in vivo* embryo production and the production of *in vitro* embryos from normally cycling, sub-fertile or infertile donor cows. The fact that donor cows that cycle naturally and show a normal reproduction cycle can be utilized without the disruption of the production cycle, offers added value to the whole drive for genetic improvement in domesticated farm animals (Eikelmann *et al.*, 2000).

The main goal in both dairy and beef cattle production is to manage the animal in such a way as to get the cow to produce a calf as regularly as possible – preferably a calf per year. Any assisted reproduction technique applied to the cows (*in vivo* or *in vitro* embryo production) normally requires the animal to be in a non-pregnant state, while *in vivo* embryo production requires the uterus not to be occupied by a fetus in order to perform embryo collection procedures (Meintjes *et al.*, 1995).

IVEP procedures have previously been attempted on pregnant donor cows with limited success and the window for oocyte retrieval from pregnant animals is limited by time due to the increasing size and weight of the fetus which later makes it difficult to perform the OPU procedure (Galli *et al.*, 2001). The current study was thus performed to evaluate the potential use of IVEP on pregnant cows, to access the feasibility of a simple superovulation protocol to enhance oocyte production and to compare the embryo production yield from stimulated and non-stimulated pregnant dairy cow donors. An overall comparison between the individual embryo production performances of the different donor cows over the experimental period

was also done. It has been clearly demonstrated that individual donor cows have the biggest influence on the eventual IVEP performance results (Twagiramungu *et al*., 1999).

4.1 Materials and methods

4.1.1 Experimental animals

A group of 10 pregnant Friesland dairy cows from the experimental herd of the Institute for Animal Breeding, Mariensee, Germany was used in this experiment. All the cows were either in their first or second lactation and had an average bodyweight of ± 600 kg and an average BCS of 3.5 (on a scale of 1-5). All cows were around peak lactation (90 – 140 days of lactation) and were treated as normally producing (30 - 40 kg milk/day) dairy cows in a dairy herd. The stage of pregnancy in the cows ranged from between 1.5 and 3 months and cows were randomly allocated to 2 experimental groups. Group 1 (n=5) was twice weekly stimulated prior to OPU with a single im FSH treatment (200 mg/treatment) (Folltropin, Vetrapharm, Canada) intensively over a 4 week (28 day) period. Group 2 (n=5) (control group) received no stimulation (see 2.3.3 under the Methodology for stimulation protocol).

4.1.2 Husbandry and housing

All cows were kept indoors in individual stables for the duration (28 days) of the experiment and received a fully balanced dairy diet, with *ad lib* access to hay and water. All animals were kept under constant veterinary supervision. No animal showed any signs of disease or discomfort during the experimental period and formed part of the milking routine and herd on the experimental farm.

4.1.3 OPU equipment, procedure and OPU protocol

The equipment, procedures and protocols used are set out in detail in 2.3.2 and 2.3.3, under the Methodology (Method 2) in Chapter 2.

4.1.4 Stimulation protocol

The superovulation treatment of the cows was performed with a twice weekly single im FSH treatment (200 mg/treatment) (Folltropin, Vetrapharm, Canada) intensively over a 4 week (28 day) period. The superovulation stimulation programme is set out in more detail in 2.3.3 under the Methodology in Chapter 2.

4.1.5 Bovine IVEP procedures

The bovine oocytes were matured in TCM medium 199 with Earle's salts (# M2520; SIGMA Chemical CO. St. Louis, USA) modified by the addition of sodium pyruvate (22 µg/ml), sodium bicarbonate (2.2 mg/ml), penicillin G sodium (0.5 mg/ml) and streptomycin sulphate (0.5 mg/ml) for the maturation of the oocytes. Fresh medium was prepared weekly and enriched with estradiol, gonadotrophins (see 2.3.6.1) and 20 % oestrous cow serum, before use. Oocytes were matured and incubated for 24 h at 39 ^{0}C in 5 % CO_2.

Fertilization was performed using frozen semen from a Holstein bull of proven IVF fertility which was used in the trials with ooyctes retrieved from the pregnant donor cows. Two semen straws (0.25 ml each) were thawed in a water bath at 37 ^{0}C for 60 seconds. Semen was processed by the so-called swim-up method to separate the motile fraction. The thawed semen was layered under 1 ml of Sp-TALP (see Table 2 for details) in sterile glass Pico vials at an angle of 45 ^{0}. After 60 minutes of incubation at 39 ^{0}C (5% CO_2 in air), the top 0.85 ml of the medium from each vial was aspirated and pooled into a sterile centrifuge tube. Following the addition of 5 ml Sp-TALP medium, the swim-up separated sperm was centrifuged at 350 G for 10 minutes at room temperature. The soft sperm pellet was resuspended in 5 ml fresh Sp-TALP medium and centrifuged again after incubation for 5 minutes. The final sperm pellet was suspended in 100 µl IVF-TALP medium (containing heparin, hypotaurine and epinephrine) and incubated for 15 minutes at 39 ^{0}C in 5% CO_2 in air.   The sperm concentration was determined with the aid of a Coulter counter and/or haemocytometer and adjusted to 50 x 10^6 sperm/ml using the IVF-TALP medium. A 2 µl aliquot of this sperm suspension was introduced to each 100 µl fertilization droplet to give a final sperm

concentration of 1 x 10^6 sperm per ml. Co-incubation of the oocytes and the sperm suspension was done for 18-20 h at 39 ^{0}C in 5% CO_2 in air, with a high humidity (Parrish *et al.*, 1986). All *in vitro* embryo culture procedures were performed in synthetic oviductal fluid (SOF) medium for a period of between 7 and 9 days - until no further embryonic developmental progress (cleavage) could be detected. The SOF medium composition is set out in Table 2.4 (Tervit *et al.*, 1972).

A more detailed description of all the IVEP procedures used is set out in paragraphs 2.3.4 to 2.3.9 under the Methodology in Chapter 2.

4.1.6   Parameters of IVEP recorded

Parameters such as the mean follicle populations (number of follicles > 2 mm in diameter), mean number of oocytes recovered, the oocyte recovery rate, oocyte quality, fertilization rate and the number of embryos produced were recorded twice weekly for the stimulated group and the control group for the 28 day period. Individual donor performances for the above mentioned aspects were also recorded.

4.1.7   Embryo transfer and pregnancy rates

Embryo transfers with both fresh and frozen embryos were performed on a regular basis to determine baseline pregnancy rates (see 2.2.11 under Methodology for details regarding embryo transfer and pregnancy rates).

4.1.8   Statistical analyses

To determine significant differences between the 2 data sets, the results (i.e. mean number of follicles, mean number of oocytes, oocyte recovery rate, oocyte grading, percentage good quality oocytes, fertilization rate, mean number of embryos, embryo production rate, embryo quality and the number of embryos per OPU session) were analyzed by means of the Student's T test (statistical software for MS Excel). The two tailed distribution and two sample

unequal variance (heteroscedastic) approaches were used. The values are all presented as the mean ± standard error (SE). Probability values < 0.05 were considered to be statistically significant. The statistical analysis performed for the trial is set out in more detail in 2.4 under the Methodology in Chapter 2.

4.2 Results

4.2.1 Ovarian follicular populations, oocyte numbers and quality, recovery rates and IVEP results for treatment groups

The mean number of follicles, mean number of oocytes collected and the oocyte recovery rate per OPU session for the different treatment groups are set out in Table 4.1. The mean percentage of good quality oocytes, fertilization rates and embryos produced are also indicated in Table 4.2.

Table 4.1 Mean (±SE) follicle populations, number of oocytes and recovery rate in FSH-stimulated and non-stimulated pregnant dairy cows per OPU session over a period of 28 days

Treatment Groups	n	Mean number of follicles	Mean number of oocytes	Oocyte recovery rate (%)
FSH-Stimulated	5	11.8 ± 6.2 [a]	4.4 ± 3.9 [a]	37.2 ± 27.8 [a]
Non-stimulated	5	7.2 ± 2.3 [b]	4.6 ± 2.8 [a]	64.4 ± 33.7 [b]

[a-b] Values within a column with different superscripts differ significantly (P<0.05)

The number of follicles (> 2 mm) per cow was monitored and aspirated at each OPU session twice weekly over the 4 week period. All follicles > 2mm were recorded and aspirated. The mean follicle population (11.8 ± 6.2) per group per OPU session for the stimulated donor group was significantly higher (P<0.05), when compared to the non-stimulated (7.2 ± 2.3) or control group. There was no significant difference in the mean overall number of oocytes recovered (for the 8 OPU sessions) from the stimulated (4.4 ± 3.9) and non-stimulated (4.6 ±

2.8) groups respectively. The standard error (SE), especially in the stimulated group, was abnormally high – an indication of the high individual variation in response.

The oocyte recovery rate was calculated as the percentage of successful oocyte recoveries from the total number of follicles initially observed - serving as an indicator of the aspiration success rate. The oocyte recovery rate (37.2 ± 27.8%) in the stimulated group was significantly ($P<0.05$) lower than in the non-stimulated group (64.4 ± 33.7%). The SE for the recovery rate in both the stimulated and non-stimulated groups was also very high.

Table 4.2 The mean (±SE) percentage of good quality oocytes, fertilization rate and the mean percentage of embryos produced per group per OPU session in FSH-stimulated and non-stimulated dairy cows

Treatment Groups	n	Mean % good quality oocytes	Mean % fertilization	Mean percentage embryos produced
FSH-stimulated	5	48.1 ± 28.7 [a]	53.6 ± 42.5 [a]	13.0 ± 27.1 [a]
Non-stimulated	5	41.3 ± 27.1 [a]	66.3 ± 33.0 [a]	15.8 ± 24.2 [a]

[a] Values with the same superscripts were not significantly different

Although a higher percentage of oocytes and of acceptable quality were produced in the stimulated group of dairy cows (see classification of oocyte categories 1, 2 and 3 in 2.3.5 under Methodology in Chapter 2), there was no significant difference between the FSH-stimulated (48.1 ± 28.7%) and non-stimulated group (41.3 ± 27.1%), with a large variation (SE) between the donors being recorded following OPU procedures.

The mean fertilization rate as indicated by the cleavage rate was higher (66.3 ± 33.0%) in the non-stimulated or control group, compared to the FSH-stimulated group (53.6 ± 42.5%). Although this percentage success was markedly higher, there was no significant difference between the two fertilization rates obtained. There was also a larger variation (SE) recorded in the fertilization rate achieved for the FSH-stimulated group when compared to the non-stimulated group.

The mean percentage of embryos produced per treatment group per OPU session was 13.0 ± 27.1% for the FSH-stimulated group and 15.8 ± 24.2% for the non-stimulated or control group. The variation (SE) was once again high in both groups.

An alternative method of determining the number of embryos was to record the number of embryos produced per donor cow per OPU session over the total 28 day experimental period. The number of embryos produced in this evaluation regime is an indicator of OPU success and gives a more realistic answer on the practicality and reliability of this procedure and the return on embryos per time and money spent on the procedure. When calculated in this way on average 0.38 embryos were produced in the FSH-stimulated group and 0.36 in the non-stimulated group of cows per OPU session.

4.2.2 Ovarian follicular populations for the individual donor dairy cows and the mean for the groups per OPU session

The actual follicle population for the individual Friesland donor cows and the mean follicle population for the treatment group as a whole were monitored twice a week over the total experimental period of 4 weeks. These follicle numbers are illustrated in Figure 4.1, 4.2 and 4.3. The mean follicle population stimulated decreased with time in the stimulated group and remained constant in the non-stimulated or control group. The mean follicle population of the stimulated group reached the same level as the non-stimulated group following 6 OPU sessions.

There tended to be a larger variation in the individual follicular population response in the stimulated group, compared to the non-stimulated group. There was also a general tendency for the variation to decrease with time in the stimulated group and to remain relatively constant in the non-stimulated group.

Figure 4.1 Individual ovarian follicular populations for the FSH-stimulated group of pregnant Friesland cows
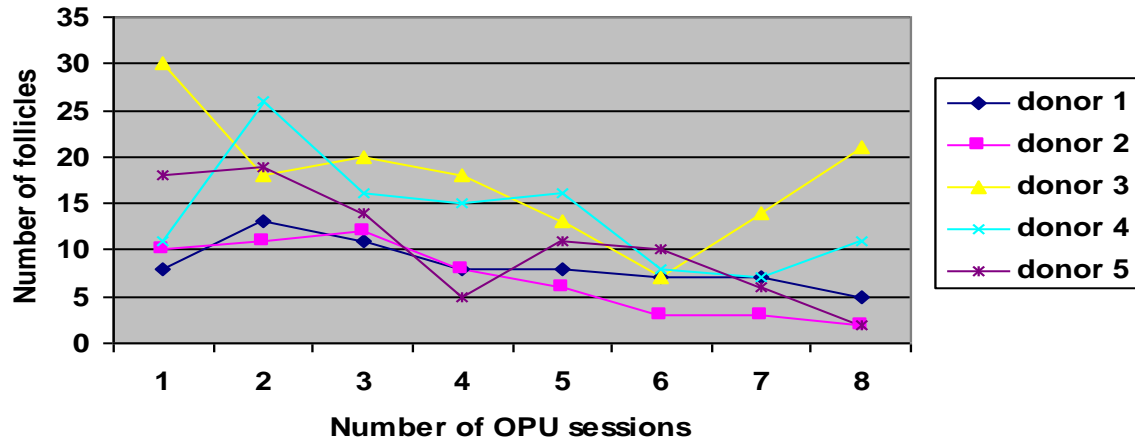
Figure 4.2 Individual ovarian follicular populations for the non-stimulated group of pregnant Friesland cows
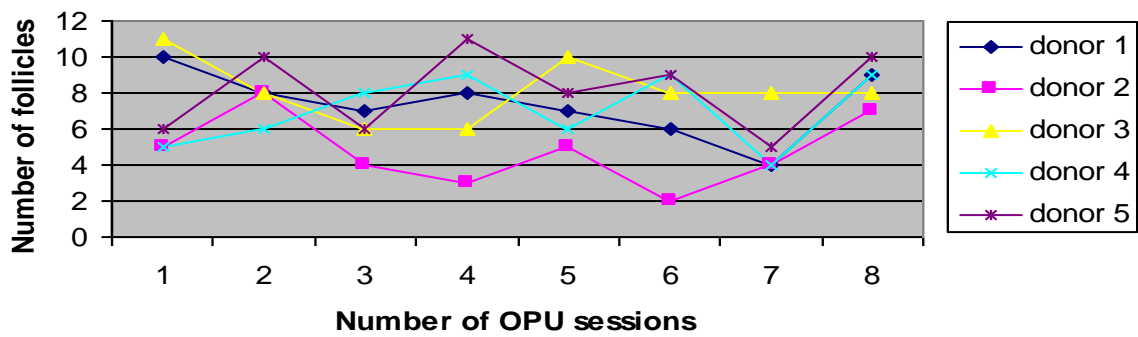
Figure 4.3 Mean (±SE) ovarian follicular populations in both the FSH-stimulated and non-stimulated pregnant Friesland cows
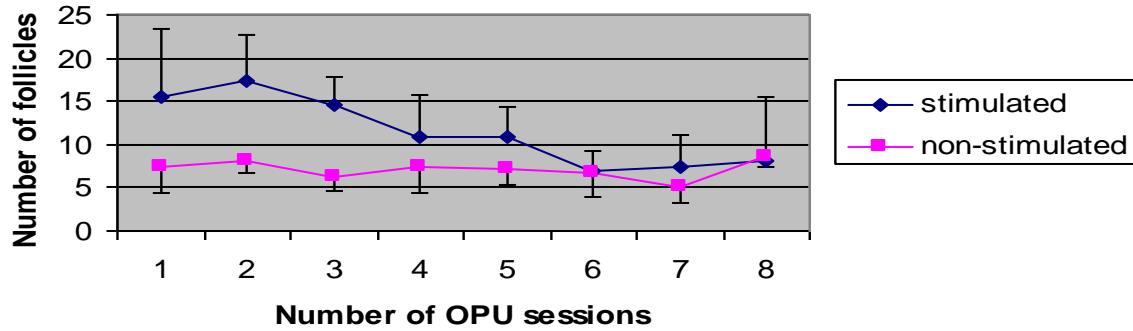
4.2.3   Oocyte numbers recovered from both groups of dairy cows per OPU session

The actual number of oocytes retrieved from the individual pregnant dairy donors and the mean number of oocytes for the treatment groups were monitored over the total experimental period of 4 weeks. The mean number of oocytes produced for both groups is depicted in Figure 4.4. The mean number of oocytes recovered in the FSH-stimulated group was initially higher after which it showed a drastic drop before stabilizing at the same level as the non-stimulated or control group.

Figure 4.4 Mean (±SE) number of oocytes recovered from both FSH-stimulated and non-stimulated pregnant Friesland cows
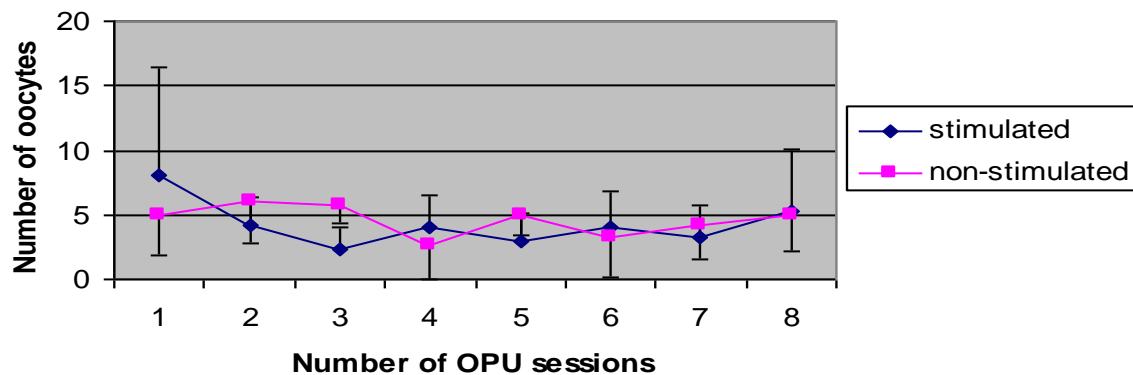
4.2.4 Oocyte recovery rates in both treatment groups per OPU session

The actual bovine oocyte recovery success rate for the individual dairy cows and the mean recovery rate for the treatment groups were recorded over the total experimental period of 4 weeks (28 days). The mean recovery rate for both groups is depicted in Figure 4.5. The oocyte recovery success rate for the non-stimulated group tended to be higher during the initial OPU sessions (OPU session 1 to 3) and then stabilized on more or less the same level of oocyte recovery as the FSH-stimulated group. The oocyte recovery success rate in the stimulated group was low during the initial OPU sessions (OPU session 1 to 3), before stabilizing at the same oocyte level as the non-stimulated group. There was a tendency for the recovery rate to improve with time in the FSH - stimulated group. The overall oocyte recovery rate was significantly higher ($p < 0.05$) in the non-stimulated group, compared to the FSH-stimulated group.

Figure 4.5 Mean (± SE) oocyte recovery rate following OPU in both dairy cow treatment groups
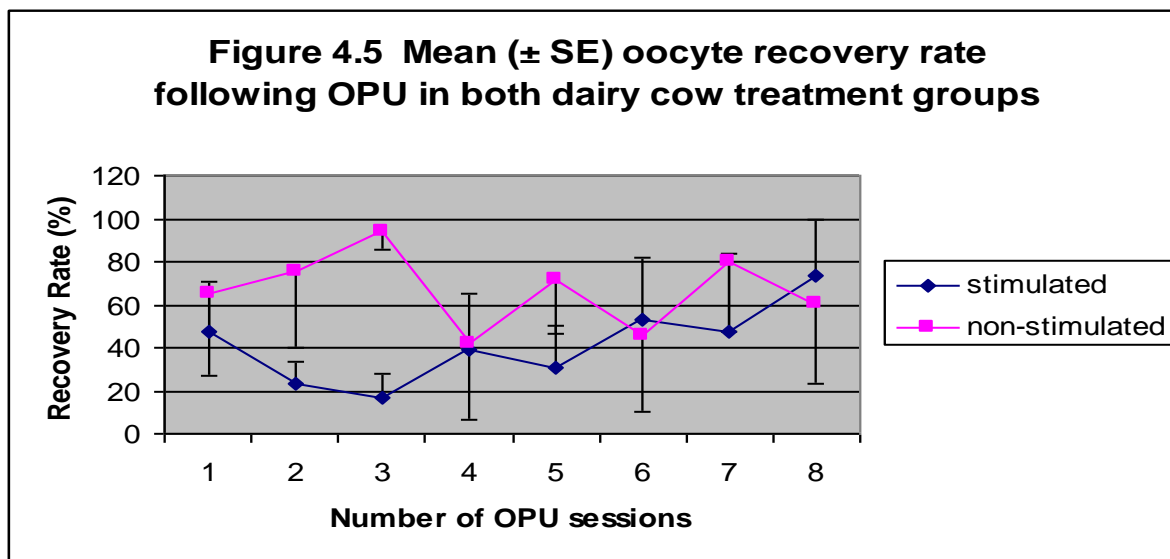
4.2.5 Comparative percentage acceptable quality oocytes per OPU session

The percentage of good/acceptable quality oocytes (see classification of oocyte categories 1, 2 and 3 in 2.3.5 under Methodology in Chapter 2) was recorded in each individual donor cow. However, as no oocytes were recovered during certain OPU sessions, the percentage of

quality oocytes was only calculated as the mean for the treatment group during any given OPU session. The mean percentage good quality oocytes for both groups are illustrated in Figure 4.6. No real trend could be established except for the fact the percentage of good quality oocytes in the non-stimulated group showed a slow decline until OPU session number 6, after which it recovered and remained at relatively the same level as the stimulated group.

Figure 4.6 Percentage (± SE) acceptable quality oocytes retrieved in both dairy cow treatment groups
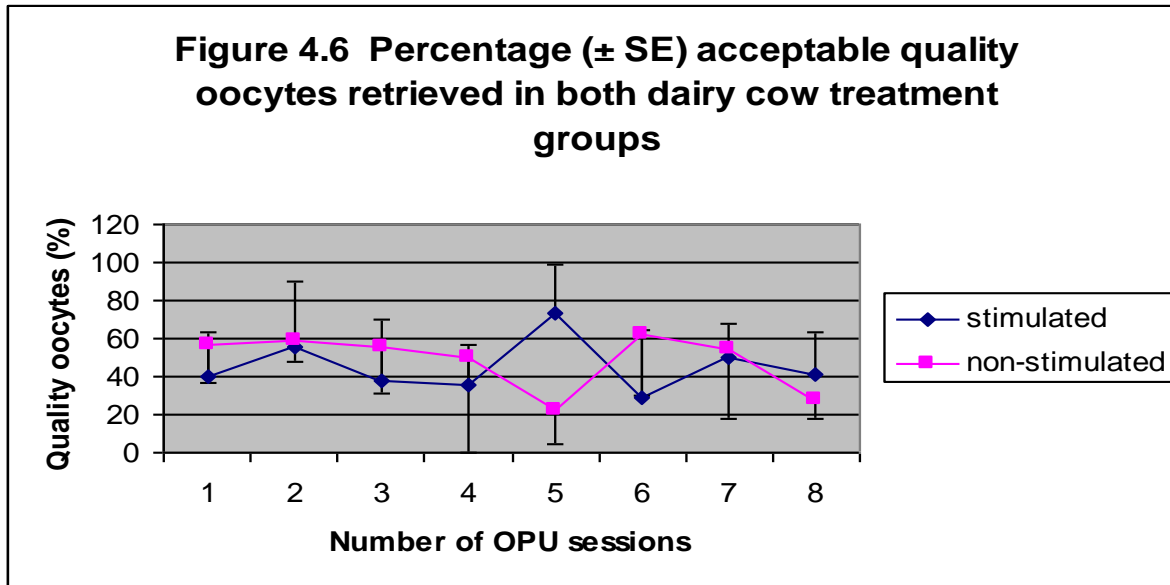
4.2.6 Comparative mean fertilization rates per OPU session

The *in vitro* fertilization rate was recorded for each individual donor cow. However, as no oocytes were recovered during certain OPU sessions, and thus no fertilization attempted, the fertilization rate was only calculated as the mean for the treatment group during any given OPU session. The mean fertilization rate (IVF success rate) in both treatment groups are illustrated in Figure 4.7. No specific pattern was observed. However, the non-stimulated group tended to maintain a higher fertilization rate during the observation period of 4 weeks.

Figure 4.7 Mean *in vitro* fertilization rates for both dairy cow treatment groups

Fertilization rate (%) — y-axis (0–120)
Number of OPU sessions — x-axis (1–8)

Legend: stimulated, non-stimulated
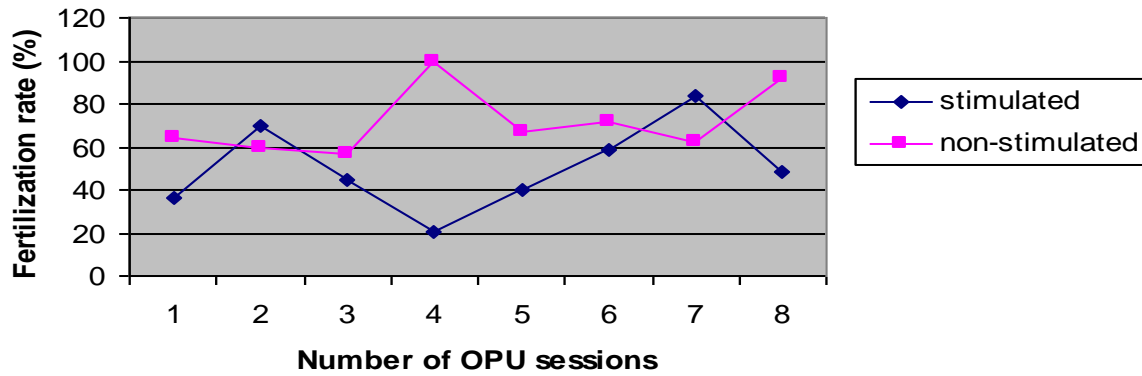
4.2.7   Mean embryo production per OPU session

The percentage of embryos produced was recorded for each individual dairy cow. However, as no oocytes were recovered during certain OPU sessions and thus no fertilization attempted, the embryo production rate was only calculated as the mean for the treatment group during any given OPU session. The mean percentage of embryos produced for both groups are illustrated in Figure 4.8. A better embryo production success rate regarding IVEP was achieved in the non-stimulated, compared to the FSH-stimulated cows.

Figure 4.8 Mean percentage of embryos produced for both dairy cow treatment groups

Embryos produced (%) — y-axis (0–30)
Number of OPU sessions — x-axis (1–8)

Legend: stimulated, non-stimulated
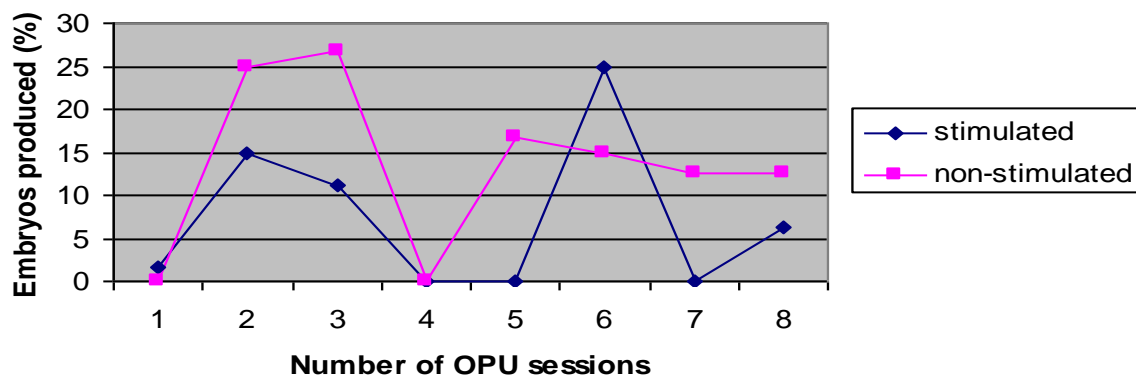
4.2.8   Pregnancy rate following embryo transfer

See summarised results under Methodology (2.2.11).

4.3   Discussion

4.3.1   Ovarian follicular population

The superovulatory FSH treatment in the stimulated group of Friesland cows significantly induced a higher growth of ovarian follicles over the entire experimental period of 28 days. This is in agreement with reports of other researchers who recorded similar results following FSH superstimulation of non-pregnant donor cows (Goodhand *et al*., 2000).   The mean number of follicles recorded in this study (11.8 ± 6.2 per donor) was similar to that (11.9 ± 7.7 per donor) recorded by De Roover *et al. (*2005) in super-stimulated non-lactating cows. However, when one looks at the graphic illustration (Figure 4.3) of the follicular populations recorded over the observation period, it is quite clear that superovulation treatment for increased follicle numbers was only effective during the first 5 OPU sessions. From the sixth OPU session, the number of ovarian follicles > 2 mm was similar to the non-treated or control group.   This same decrease in follicle numbers after repeated stimulation in non-lactating dairy cows has been reported by De Roover *et al. (*2005) and by Guyader Joly *et al.* (1997). This was quite a significant finding as it tends to imply that intensive FSH hormonal superovulation treatment can only be used for a limited period of time in pregnant dairy cattle, before the treatment becomes less effective. The question however that could be asked is whether the donor cows possibly developed immunity following repeated gonadotrophin stimulation.

The follicle numbers per individual donor cow in the stimulated group showed large variation in terms of response. The same trend of a large ovarian response variation between donors was recorded by De Roover *et al. (*2005), for non-lactating dairy cows. This variation was

more evident at the onset of the stimulation period and later the variation became more stable.

In the non-treated or control group, there was also a large variation in the number of ovarian follicles produced per individual donor per OPU session. This phenomenon of the individual donor having the biggest effect on all aspects of IVEP including number of follicles developing has been previously reported by Twagiramungu *et al. (*1999).   However, the mean ovarian follicle numbers recorded remained constant throughout the experimental period and did not show the same decreasing trend as observed in the FSH-stimulated group. This was due to the fact that no superstimulation was used in this group and only the normal or natural cohort of follicles developing per given OPU session were aspirated on a weekly basis.

4.3.2   Number of oocytes collected and oocyte recovery rate

Superovulation treatment with FSH had no enhancing effect on the mean number of oocytes recovered per OPU session in the treated group (4.4 ± 3.9 oocytes in the treated vs. 4.6 ± 2.8 in the non-treated group). This is contrary to the findings of Meintjes *et al*. (1995) who found a higher response in a hormonally treated group of cows, but consistent with the findings of Guyader Joly *et al*. (1997) who recorded a weekly oocyte production of 14 ± 0.9 per session in non-stimulated and 13 ± 0.9 in stimulated beef heifers. In another experiment by Bousquet et *al*. (1999) a mean high oocyte production of 9.5 ± 5.6 per OPU session was recorded in non-pregnant Holstein cows. However, this was a once off superovulation treatment and not repeated intensively over a period of time as was the case with this study.

The low number of oocytes recovered in the FSH-stimulated cows could be related to the fact that the recovery rate was significantly lower in the stimulated, compared to the non-stimulated group. The only possible explanation for this response and lower recovery rate could be the fact that on average the ovarian follicles were larger in the stimulated group of cows. There is the belief that oocyte aspiration from larger follicles is generally less effective due to decreased suction on the larger volume of follicular fluid (Guyader Joly *et al*., 1997; Seneda *et al*., 2001). The recovery rates recorded (37.2 ± 27.8 oocytes for the stimulated

and 64.4 ± 33.7 for the non-stimulated cows) corresponds well to that achieved in previous trials (Rust *et al.*, 1998), where a recovery rate of 43.2 % and 53.2 % was achieved in stimulated and non-stimulated non-pregnant donor cows, respectively.

A possible solution for the low recovery rate following stimulation could be to increase the effective suction power during the OPU procedures (aspiration) in the stimulated donors (Fry *et al.*, 1997). The mean recovery rate (Figure 4.5) demonstrates a very low recovery rate in the FSH-stimulated dairy cows over the first 3 OPU sessions. This could be due to an initial higher number of induced ovarian follicles being present during the OPU sessions. From the fourth OPU session the oocyte recovery rate remained on the same level as that of the non-stimulated or control group. It is important to note that the oocyte recovery rate in the FSH-stimulated group increased as the number of follicles decreased, while in the case of the non-stimulated group, the recovery rate remained fairly constant. This supports the finding that the oocyte recovery rate is lower in stimulated animals (higher number of follicles) than in non-stimulated donor cows (Meintjes *et al.*, 1995; Guyader Joly *et al.*, 1997; Fry *et al.*, 1997; Rust *et al.*, 1988).

4.3.3   Oocyte quality

The mean percentage of acceptable quality oocytes (see classification of oocytes in 2.3.5) was higher in the FSH-stimulated cows, compared to cows in the non-stimulated group - but this difference was not significant. This is supported by the findings of Armstrong (1993), Assey *et al. (*1994), Lonergan *et al. (*1994) and Hyttel *et al.* (1997), who also found the oocytes to be of better quality following oocyte aspiration from FSH-stimulated donor cows. However, the quality of oocytes was not reflected by a lower fertilization rate and the mean percentage of embryos produced when compared to the non-stimulated group. This phenomenon may reflect on the objectiveness of the oocyte classification systems and to the fact that oocyte classification should not be used as the only criteria to determine the outcome of certain treatments in an IVEP system (Goodhand *et al.*, 1999). However, with the oocyte grading system used during this experiment, it would seem as if superovulation using FSH as

such does not have a detrimental effect on the morphology of the oocyte. The dosage, type of FSH, time and interval of administration could however also play a role.

4.3.4   The IVF success rate

Although not significant, the fertilization rate was relatively higher in the non-stimulated or control group (66.3 vs. 53.6%). This finding is supported by that reported by Assey *et al. (*1994)*,* Hyttel *et al. (*1991; 1997) and Lonergan *et al. (*1994).   These investigations were however performed on non-pregnant cows and the same seems to be true for pregnant donor cows. The fertilization rate obtained in the non-stimulated or control group remained constant throughout the experimental period, while the success rate for the FSH-stimulated group showed large variation throughout the experimental period. This large variation in fertilization rate in the stimulated group could be ascribed to the fact that a more varied oocyte quality is achieved after stimulation and this will have a definite effect on the variation in the fertilization rate (Goodhand et al., 1999).

In other IVEP studies by Bousquet *et al.* (1999), a fertilization rate as high as 78.6 ± 22.4% was recorded in FSH-stimulated, non-pregnant Holstein cows. However, this was again a once-off experiment and no repeated stimulations over a period of time were carried out as was the case with the present trial.

4.3.5   Total percentage embryos produced

The total percentage of embryos produced relative to the total number of oocytes entering into the *in vitro* programme was higher (15.8%) in the control than the FSH-stimulated group (13.0%).   This is contrary to the findings of Meintjes *et al.* (1995) in a trial conducted on pregnant beef heifers, while the current results obtained are supported by Calleson *et al.* (1986), Hyttel *et al.* (1991; 1997), Armstrong *et al.* (1993), Assey *et al.* (1994), Lonergan *et al.* (1994) and Greve *et al.* (1995).   All these IVEP studies being performed on non-pregnant beef and dairy cows and the same phenomenon being recorded for pregnant donor cows.

In a study on stimulated non-pregnant Holstein cows by Bousquet *et al.* (1999), the percentage of viable embryos (47.9 ± 27%) following IVEP was much higher than that recorded in this study. Again, the study performed by Bousqeut *et al. (*1999) was a once-off super-stimulation protocol and not repeated over time, as was the case in the present study.

Guyader Joly *et al.* (1997) reported an overall mean weekly blastocyst production of 2.2 ± 0.5 for both stimulated and non-stimulated beef heifers. Superovulation had no beneficial effect in this case and an increased blastocyst production (stimulated vs. non-stimulated) was only recorded in one individual animal. This type of result following superovulation is supported by the findings of Goodhand *et al.* (1999), who recorded a mean embryo production of 2.1 ± 0.4 embryos per week after a once weekly OPU session on stimulated non-pregnant beef heifers - compared to a mean of 2.4 ± 0.4 embryos for twice a week procedures, without FSH stimulation and 1.0 ± 0.3 embryos once a week for a session without stimulation. It is, therefore, evident that the results from the present trial seem to correspond well with those of other trials by other researchers

The mean number of embryos produced per OPU session was 0.36 in the control group, which corresponds well to the results obtained in non-pregnant and non-stimulated Friesland cows (Eikelmann *et al.*, 2000). From the results obtained in the current study it is clear that superovulation of the donors did not show any beneficial effect on the total embryo production performance in pregnant donor cows.

4.3.6   General discussion

The application of OPU during the first trimester of pregnancy does not seem to have any negative effect on both the donor dairy cow and the maintenance of pregnancy (Meintjes *et al.*, 1995). Although no natural ovulation occurs, the natural ovarian follicular wave pattern still continues and the quality of oocytes produced still lends itself to IVEP procedures (Guyader Joly *et al.*, 1997).

The fact that bovine embryos can be produced from donor cows during gestation is of significant importance and has tremendous potential. It could open a whole new field of research and applications and provide a useful source of oocytes for IVEP, without disrupting the production cycle of a superior donor dairy cow. This means that *in vitro* production of embryos from oocytes derived from live donors can now be applied on superior animals that were temporarily lost with regards to embryo production.

The additional window created for embryo production (during the first 3 to 4 months of gestation) can be regarded as a bonus period for IVEP. The obtained results of 0.36 to 0.38 embryos per OPU session per individual translates to a total embryo production of ± 12 embryos in the first trimester of pregnancy. If a pregnancy rate of 50% (Galli *et al.*, 2001) is taken as a norm, the extra period of embryo production could result in an overall additional 6 calves per donor. Although the percentages of embryos produced (efficiency rate) are generally low, it could still have a tremendous impact on the practical application of this technology in the dairy breeding industry.

To use superovulation with the aid of FSH to try and further enhance bovine embryo production during pregnancy does not seem to be practical as no beneficial effect on oocyte numbers, oocyte quality and the number of embryos produced could be established. The low oocyte recovery rate obtained in superovulated donors seems to be a real obstacle. This will have to be addressed by altering the OPU procedures (e.g. different diameter needles and varying vacuum pressures and a possible shorter period between superovulation treatment and oocyte retrieval) in order to obtain higher recovery rates (Hashimoto *et al.*, 1999). The decreased response to superstimulation over a period of time is also one of the major negative factors influencing the overall IVEP result.

Other protocols for the hormonal ovarian stimulation of pregnant dairy cows (multiple injections of equal or decreasing dosages) are an option to be considered for future investigation. De Roover *et al.* (2005) already indicated a more satisfactory embryo production result following multiple dose FSH stimulation in non-lactating dairy cows. However, with all results by other researchers taken into account, it seems as if

superovulation has a more pronounced effect in heifers (especially beef) and non-lactating animals. At present with the obtained results in mind, and with the financial implications of additional hormonal treatment to be considered, the conclusion is that superovulation in pregnant donors should rather be used to stimulate follicular development in cases where the donor cow has naturally low follicle populations.

Chapter 5

The effect of season on different aspects of IVEP in sub-fertile beef cows

Introduction

In this chapter the effect of season on IVEP in sub-fertile cows that would otherwise have been lost for embryo production through conventional MOET procedures will be investigated. The use of assisted reproduction techniques and especially IVEP procedures could be an important tool to be used in an attempt to produce offspring from these genetically superior, but fertility-impaired cows (Riddell *et al.*, 1997; Betteridge, 2006).

Fertility problems or handicaps generally arise from factors such as old age, repeated MOET procedures, certain uterine pathological conditions and a general failure to become pregnant after repeated services or inseminations. In most cases it is difficult to diagnose a specific cause for the infertility problem and the cows are generally just classified as infertile or sub-fertile. It is important to note that this acquired infertility should not be linked to any genetically inherited dysfunction, but rather a phenomenon that has developed over a period of time. The animals used in this study were all normally reproducing animals of high genetic merit before their fertility became impaired.

As longevity and reproductive performances are extremely important selection criteria in both dairy and beef cattle breeds, the *in vitro* production of embryos from older animals play an important role in the South African cattle industry. In most cases, longevity and reproductive rate are regarded as more important criteria than criteria such as growth and feed conversion rates. Although these cows used, already suffered from some form of reproductive dysfunction, the effect of season on the outcome of attempted *in vitro* procedures is an important factor to consider and warrants investigation.

It is a well known fact that season can influence the reproduction performance of both the Bos Indicus and Bos Taurus cattle breeds (Zeitoun *et al.*, 1996). In Bos Indicus cattle it was found that the cows exhibited better fertility during the spring and summer months, compared to the autumn and winter (Harrison et al., 1982; Rhodes et al., 1982; Bastidas and Randel, 1987; Randel, 1994). In Bos Indicus cows this limiting effect seems to be more pronounced during the colder months, whereas in the Bos Taurus, the effect seems more pronounced during the warmer months (Zeitoun *et al.*, 1996).

The effect of season on IVEP from abattoir material and *in vivo* derived oocytes has been previously investigated and many effects of season on embryo production have been recorded. The same fertility pattern normally recorded during conventional reproduction can also be found when either MOET or IVEP results are considered (Rutledge *et al.*, 1999). The effect of season on IVEP in seasonal breeders, like for example sheep have also been demonstrated (Mitchell *et al.,* 2000).

Bovine IVEP is still a relatively new technology in South Africa and the effect of different factors, including season, has not really been tested under local conditions. Almost all the cattle presented for IVEP procedures in South Africa are animals with some kind of impaired fertility. Although these cows already suffer from certain dysfunctions, the effect of season may even further reduce the ability of these animals to produce embryos efficiently via *in vitro* procedures. Limited studies have shown that the production of embryos from so-called problem cows yield lower embryo numbers than with reproductively sound animals (Riddell *et al.*, 1997). Producing bovine embryos from these animals via the use of *in vitro* technology can be a labour intensive and a costly exercise. It is, therefore, important to investigate whether the application of this type of technology should best be avoided or restricted to specific seasons of the year. Even when using reproductively sound animals the effect of season has not been tested specifically - to give some indication regarding more acceptable periods of the year to intensify or focus the application of *in vitro* procedures (Rutledge *et al.*, 1999).

In animals that have some reproductive dysfunction and where the possibilities of producing embryos *in vitro* are limited, it is thus even more important that these procedures should not be attempted during periods where the chances of success are further limited by external factors such as season. The end results of IVEP such as ovarian follicular populations, oocyte recovery rates, oocyte quality and embryo production rates are important aspects in any *in vitro* embryo production system (Rutledge *et al.*, 1999). In this chapter the effect of season on the IVEP parameters carried out over a period of 4 years were investigated.

5.1 Materials and methods

5.1.1 Experimental animals

All cows of various breeds and parities (n=40) used in this trial were beef cattle cows presented to the laboratory for commercial embryo production purposes. These animals were from different beef cattle breeds (Simmental, Bonsmara, Santa Gertrudis, Romagnola and Brahman) and varied in age between 2 and 19 years. The average body condition score (BCS) of these animals was 2.5. The only common denominator for the cows was the fact that all these animals were classified as being unfit for further reproduction in the herd of origin. All cows were of high genetic merit and suffered from some condition preventing the cows from conceiving or normally producing embryos during MOET programs. These infertility conditions ranged from old age (up to 19 years of age), uterine dysfunction, fallopian tube dysfunction, delayed ovulation (hormonal dysfunction), cystic ovarian disease or other undiagnosed conditions hampering conception. These beef cows had the same characteristics as those used in Chapter 3, but were not the same animals.

Prior to acceptance in the laboratory IVEP programme, all cows were rectally and ultrasonically evaluated to determine whether the ovarian structures and functions were normal (development of at least one normal follicle during an oestrous cycle). Being such a diverse group of cows, it was decided to collect data over a 4-year period (2000 to 2003) to try and minimize individual animal differences and generate data as there was a constant flow

of cows to the laboratory over this 4-year period. The diversity (breeds, parity and age) and number of donors (not more than 5 donor cows at a time) was fairly constant at any given time during this observation period.

5.1.2   Husbandry and housing

All cows were maintained on natural pastures (mixed sweet and sour highveld grazing) with *ad lib* supplementation of a summer or winter lick and with *ad lib* eragrostis hay supplementation during the winter months. It was attempted to maintain the BCS of all cows at not lower than 2.5 (on a scale of 1 to 5). All animals, however, lost some body condition (approximately 0.5) during the winter months, but this was acceptable due to the fact that the cows were transferred from farm conditions where the same conditions of weight loss during the drier months of the year were experienced. All these beef cows were under constant veterinary supervision and sick animals received the appropriate treatment and were withdrawn from the *in vivo* oocyte collection procedures, until fully recovered.

5.1.3   Ultrasound guided bovine oocyte retrieval protocol

The ultrasound guided oocyte retrieval (OPU) procedure was performed on a weekly basis on a fixed day of the week. This was done to keep the interval (number of days) between oocyte retrievals constant. Although the literature recommends twice weekly oocyte retrieval to yield more viable oocytes and embryos, it was decided against a twice-weekly regime for two reasons. Firstly due to the large variation in breeds and type of individual animals, twice weekly retrieval would overload the *in vitro* production laboratory with oocyte and semen processing procedures. Secondly the old age of some of the cows would put too much stress on the animals. The actual OPU procedure as it was performed is described in 2.2.1; 2.2.2 and 2.2.3 under the Methodology in Chapter 2.

The general idea was to develop a less labour intensive, but effective OPU technique that could be performed on a large variety of breeds and cows over an extended period of time.

5.1.4   IVEP procedures

All procedures in the total IVEP chain were performed as described under the Methodology (2.2.4 to 2.2.9) in Chapter 2.

Briefly, the IVEP procedures entailed the following:

The oocytes were matured for a period of 24 h and incubation performed in a Thermo Forma incubator at 39 ^{0}C, 5 % CO_2 in air at saturated humidity - achieved by placing a water tray inside the incubator (DS Visser, 2003; ARC Animal Improvement Institute, personal communication).

After maturation (24 h incubation), the presumptive metaphase II ova/oocytes were rinsed vigorously in handling medium (PBS medium; Sigma), supplemented with 1% newborn calf serum (NBCS) and heparin at a rate of 0.0183 g/l (see 2.2.4). The mature ova were then rinsed once in Tyrode's lactate medium, supplemented with amino acids and bovine serum albumin (BSA) and transferred into Nunc multi-wells, containing the same medium, and further supplemented with heparin (0.0183 g/l) (insemination medium).

A dose of specific bull semen (0.25 or 0.5 ml straws) was thawed at 35 ^{0}C for 30 seconds, before gently being overlaid on a pre-prepared discontinuous Percoll gradient, consisting of 90% and 45% isotonic fractions in a test tube. The semen was then centrifuged at 700 G for 30 minutes, the supernatant discarded and the sperm containing pellet re-suspended in 5 ml insemination medium. This was followed by a second centrifugation at 300 G for 6 minutes. The supernatant was again discarded and the concentration of washed live spermatozoa in the resultant pellet was determined with the aid of a Makler counting chamber. After dilution of the sperm pellet with insemination medium, a final insemination dose of 1×10^6 per ml live sperm was used for insemination (DS Visser, 2003, ARC Animal Improvement Institute, unpublished data). The insemination dose was standard, regardless of the number of oocytes to be fertilized. Oocytes and sperm were co-incubated for 24 h to complete the *in vitro* fertilization process.

The presumptive zygotes were then washed twice in handling medium, 24 h after insemination. The embryo culture medium consisted of CR2aa (aa = essential and non-essential amino acids) medium (Sigma) supplemented with 10% FCS. Granulosa cells were added to the droplet to form a monolayer of cells in order to establish a co-culture system. Presumptive zygotes were then washed once in an embryo culture medium and then added to the micro droplets.

Embryo culture was continued over a period of 7 days, during which 50% of the culture medium was replaced with pre-equilibrated, fresh medium on days 3 and 6 of culture. On day 7 of culture, all embryos were evaluated and graded into the 3 main grades according to the IETS guidelines as set out in the 3rd Edition of The Manual of the International Embryo Transfer Society (1990).

5.1.5   Embryo cryopreservation

Cryopreservation of all embryos was performed as described in 2.2.10 under the Methodology in Chapter 2. The embryo cryopreservation briefly entailed the following:

The freezing of bovine embryos was performed in a cryopreservation apparatus specifically designed for embryo freezing (Freeze Control, Australia). Embryos to be cryopreserved were removed from the culture medium and washed in fresh holding medium (AB Technologies, Australia) and after washing, embryos were placed in a 1.5 M ethylene glycol solution (AB Technologies, Australia) for immediate loading into 0.25 ml French straws. Straws were loaded into the cryopreservation unit within 10 minutes, after placement in the ethylene glycol solution.

The following freezing protocol (Table 5.1) as described by Hasler *et al.* (1997) and modified by De la Rey (2004), Embryo Plus, South Africa, (personal communication) was used:

Table 5.1 Bovine embryo freezing protocol

Process	Initial temperature	End temperature	Rate	Holding time
Start	20 ^{0}C	20 ^{0}C	-	Max 10 minutes
Stage 1	20 ^{0}C	-6 ^{0}C	5 ^{0}C/min	10 minutes
Stage 2	-6 ^{0}C	-30 ^{0}C	0.6 ^{0}C/min	0 minutes
Stage 3	-30 ^{0}C	-35 ^{0}C	0.1 ^{0}C/min	Immersing in liquid Nitrogen

5.1.6   Semen for *in vitro* fertilization

In all instances, the bull semen for IVF was provided by the individual owners of the oocyte donor cows. Semen was morphologically microscopically (X100) examined and only semen of acceptable quality (final concentration of 1 x 10^6 per ml after processing – see 2.2.8) was used during the IVF procedures. At no stage however was the semen from bulls of proven fertility in an *in vitro* situation used – in other words, bulls were not selected according to their fertilization history when using IVF procedures, but solely on historic genetic merit. This was done so as to obtain a better picture of fertility results under the real or practical farming conditions.

Generally, in other IVEP experiments and field trials by other researchers, semen of bulls of proven *in vitro* fertilization capacity have been used. This often gives a wrong impression of what results could be achieved under routine applied local conditions.

5.1.7   Parameters recorded during IVEP

The following parameters that could have an effect on the bovine IVEP procedures were recorded: parameters included the ovarian follicular populations, oocyte recovery rates, oocyte quality, the number of embryos produced and the quality of the embryos produced on a monthly basis over the 4 year period. All these parameters were not analyzed for the individual animals, but the data was pooled over the total trial period to get a better picture of the efficiency of the bovine IVEP procedures. Criteria for evaluation were designed so as to

obtain information on the possible effect of season on IVEP performance for a practical set-up and not only individual animals.

5.1.8   Data on ambient temperature and photoperiod changes

All data regarding seasonal changes (maximum and minimum monthly temperatures and photoperiod) were obtained from the historical data of the South African Weather Service Station at Irene.   This is set out, together with the production data following the OPU and IVEP sessions in Table 5.2.

5.1.9   Embryo transfer and pregnancy rates

Embryo transfers using both fresh and frozen embryos were performed on a regular basis to determine baseline pregnancy rates (see 2.2.11 under the Methodology for details regarding embryo transfer and pregnancy rates).

5.1.10   Statistical analyses

To determine significant differences between the 3 or more data sets, a one way ANOVA analysis of variance was used (SAS, 1986).   The values are all presented as the mean ± standard error (SE). Probability values < 0.05 were considered to be statistically significant. The correlation coefficient between the two data sets was determined by using the standard formula (statistical software for MS Excel) to determine the correlation coefficient (r) between the two ranges of values. The statistical analyses used are set out in more detail in 2.4 under the Methodology in Chapter 2.

5.2 Results

The results obtained following the total number of OPU and IVEP sessions per month are summarized in Tables 5.2, 5.3 and 5.4 and graphically illustrated in Figure 5.1, 5.2, 5.3, 5.4, 5.5 and 5.6.

Table 5.2   The mean (±SE) ovarian follicular development, number of oocytes recovered and recovery rate following OPU from fertility impaired cows over a 4-year period

Month of the year	Average daily maximum and minimum temp (^{0}C)		Daylight Length (Hours)	OPU sessions (n)	Number of folliclles	Number of oocytes recovered	Oocyte recovery rate (%)
	Maximum	Minimum					
January	29	18	13.5	39	2.7 ± 1.3 [a]	1.4 ± 1.2 [a]	50.9 ± 37.9 [a]
February	28	17	13.0	64	3.3 ± 2.4 [b]	1.4 ± 1.6 [a]	39.5 ± 37.2 [a]
March	27	16	12.2	44	3.0 ± 1.7 [b]	1.4 ± 1.4 [a]	45.0 ± 38.5 [a]
April	24	12	11.5	22	2.6 ± 1.8 [a]	0.8 ± 1.0 [a]	33.7 ± 38.0 [a]
May	22	08	10.9	23	4.1 ± 2.7 [b]	1.8 ± 1.9 [a]	44.4 ± 36.1 [a]
June	19	05	10.5	64	3.2 ± 1.6 [b]	1.3 ± 1.5 [a]	36.4 ± 35.6 [a]
July	20	05	10.7	42	3.4 ± 1.7 [b]	1.5 ± 1.5 [a]	41.8 ± 35.6 [a]
August	22	08	11.2	29	3.5 ± 1.6 [b]	1.6 ± 1.4 [a]	46.8 ± 36.2 [a]
September	26	12	11.9	27	4.3 ± 2.2 [c]	1.9 ± 1.7 [a]	41.0 ± 33.4 [a]
October	27	14	12.7	60	3.6 ± 2.2 [b]	2.1 ± 2.0 [a]	53.4 ± 35.7 [a]
November	27	16	13.4	66	3.3 ± 1.8 [b]	1.7 ± 1.8 [a]	46.1 ± 36.6 [a]
December	28	17	13.7	36	3.1 ± 1.9 [b]	1.6 ± 1.5 [a]	55.7 ± 38.5 [a]
Total Mean				**44**	**3.3 ± 1.9**	**1.5 ± 1.5**	**44.6 ± 36.6**

[a-b-c] Values within a column with different superscripts differ significantly (P<0.05)

5.2.1   Ovarian follicular populations

The mean (± SE) follicular populations recorded per OPU session is set out in Table 5.2. This was determined as per weekly OPU session on a monthly basis over the 4-year period. The general tendency was for a lower ovarian follicle density (growth) during the warmer

(summer) months and after that a higher follicular population during the colder (winter) months. The highest ovarian follicle populations were recorded in the months (May and September) associated with seasonal (temperature and photoperiod) changes. A significantly lower ($P<0.05$) number of follicles was recorded in the months of January (mid-summer) and April (autumn) with mean follicular populations of 2.7 ± 1.3 and 2.5 ± 1.8 respectively. In September (onset of spring) a significantly higher ($P<0.05$) mean follicular population (4.3 ± 2.2) was recorded following bovine OPU. A large variation between OPU sessions regarding the mean number of follicles on a weekly basis was recorded and thus the relatively high standard deviation (variation) recorded.

5.2.2   Number of bovine oocytes recovered

The mean number of oocytes recovered per OPU session over the seasons is set out in Table 5.2. As with the follicular population, the number of oocytes was calculated per weekly OPU session on a monthly basis, over the 4-year period. The number of oocytes recovered, followed the same trend as the follicular population, with the same general tendency of higher numbers of bovine oocytes aspirated during the colder months and lower numbers during the hotter months. There was however no significant difference in the number of oocytes aspirated in the warmer and colder months.

The correlation between ovarian follicular population and the number oocytes recovered was r = 0.79. The highest number of oocytes recovered being in mid-spring (2.1 ± 2.0) and the lowest recorded in mid-autumn (0.8 ± 1.0). There was no significant difference between any of the months of the year. The lowest number of oocytes was aspirated in the month of April, preceding the month of May with the highest recorded number of follicles. On the other hand, the highest number of bovine oocytes was recorded in the month of October following the month (September) with the highest follicular population. As with the ovarian follicular activity, there seemed to be peak periods during the year associated with seasonal changes (temperature and photoperiod). The large variation in ovarian activity recorded between animals and between months complicates the making of predictions regarding the effect of season.

Figure 5.1 Mean (±SE) follicular populations and number of oocytes recovered in beef cattle over the 4-year period
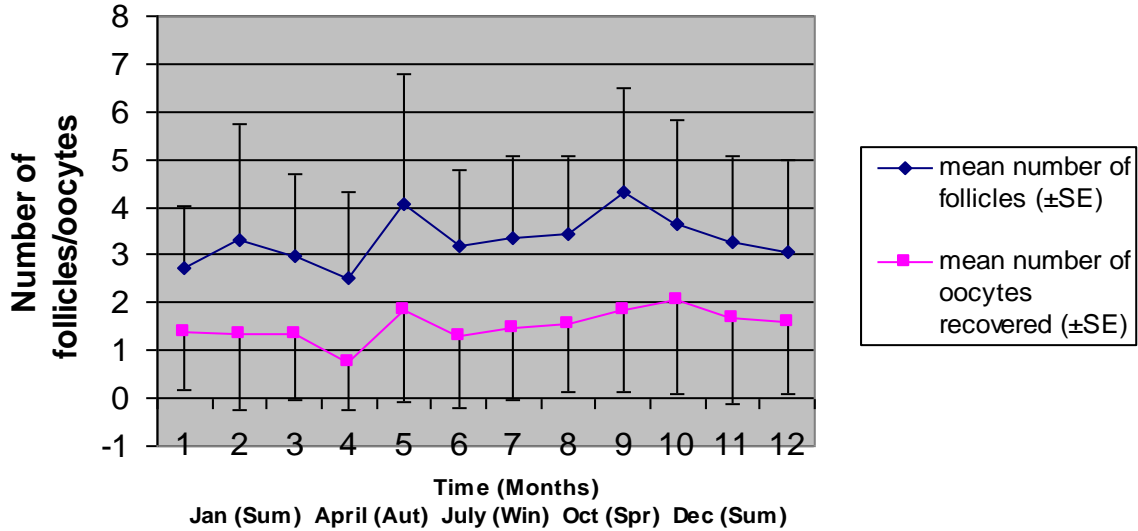
Figure 5.2 Mean annual oocyte recovery rate in beef cattle over the 4-year period
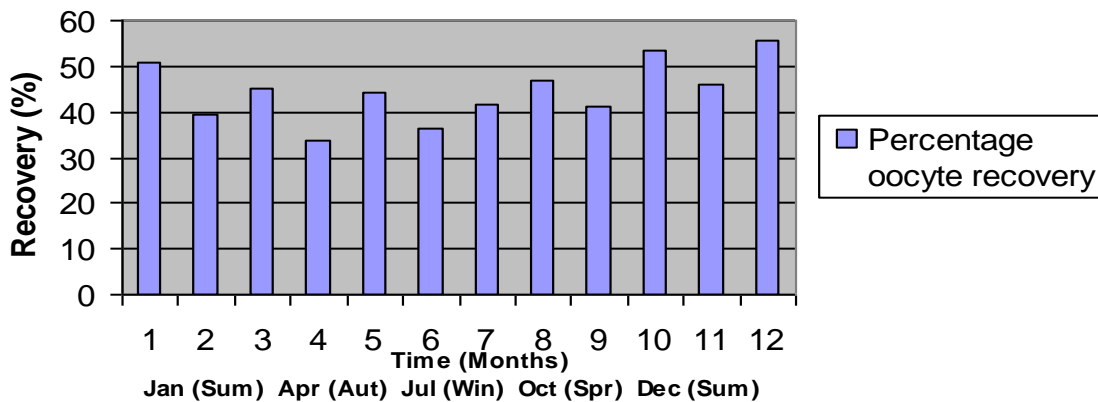
5.2.3   Bovine oocyte recovery rate

The highest recovery of bovine oocytes (55.7 ± 38.5%) was recorded during the month of December (onset of summer) and the lowest (33.7 ± 37.9%) during April (autumn) (Figure 5.2). There were, however, no significant differences in recovery rate between the different

months of the year. The correlation coefficient recorded between the oocyte recovery rate and follicular population was r = 0.07. This correlation coefficient was extremely low and it was evident that the oocyte recovery rate decreased with an increase in the follicular population.

Table 5.3 The mean (±SE) annual oocyte quality recorded in sub-fertile beef cows over a 4-year period

Month	Number of oocytes	Oocyte quality (% of total)		
		Grade A	Grade B	Grade C
January	57	47.5 ± 44.6 [a]	49.7 ± 46.5 [a]	2.9 ± 11.0 [a]
February	92	42.2 ± 43.6 [a]	48.5 ± 44.3 [a]	6.9 ± 25.8 [a]
March	61	49.9 ± 46.4 [a]	46.8 ± 46.3 [a]	3.5 ± 18.6 [a]
April	19	75.0 ± 39.9 [a]	16.7 ± 32.6 [a]	8.3 ± 28.9 [a]
May	44	63.3 ± 45.0 [a]	30.8 ± 42.7 [a]	5.9 ± 24.5 [a]
June	85	58.2 ± 40.9 [a]	34.7 ± 41.1 [a]	9.8 ± 23.8 [a]
July	65	61.9 ± 39.8 [a]	32.6 ± 38.7 [a]	5.8 ± 22.0 [a]
August	45	63.4 ± 41.0 [a]	27.9 ± 38.2 [a]	8.7 ± 23.0 [a]
September	50	64.3 ± 45.4 [a]	28.6 ± 43.8 [a]	7.1 ± 23.9 [a]
October	126	60.3 ± 44.5 [a]	39.4 ± 44.3 [a]	0.4 ± 2.3 [a]
November	117	62.3 ± 42.3 [a]	35.8 ± 41.3 [a]	3.1 ± 11.7 [a]
December	60	66.1 ± 41.8 [a]	32.2 ± 42.2 [a]	1.7 ± 9.3 [a]

[a-b] Values within a column with different superscripts differ significantly (P<0.05)

5.2.4 Bovine oocyte quality

There was no significant difference between the different grades of oocytes classified over the 4-year period. The different Grade A, B, and C classes were categorized as follows:

The classification system used was from that as described by Leibfried and First (1979):

1. Grade A: Normal size oocytes, dense cumulus and homogenous even-coloured ooplasm

2. Grade B: Ooplasm of oocytes are heterogeneous, granular, lightly coloured and

not filling the zona pellucida (ZP)

3.　　　Grade C: Abnormally small oocytes

The standard deviation or variation in Grade A oocytes was less pronounced than for the Grade B oocytes and even less than for the Grade C oocytes. There was a tendency for the number of Grade A oocytes to be higher during the cooler months (June to October) and lower during the hotter months (January to March). An inverse tendency was recorded for the Grade B type oocytes (Table 5.2). As for the case with the follicle numbers and number of oocytes recovered, the highest Grade A number of oocytes was recorded in April (autumn). The same observation was made for Grade B oocytes - in which case the lowest occurrence was recorded during April.   A significant high negative correlation (r=-0.95) was recorded between Grade A and B oocytes.

Figure 5.3 Annual average oocyte quality in beef cattle recorded over a 4-year period

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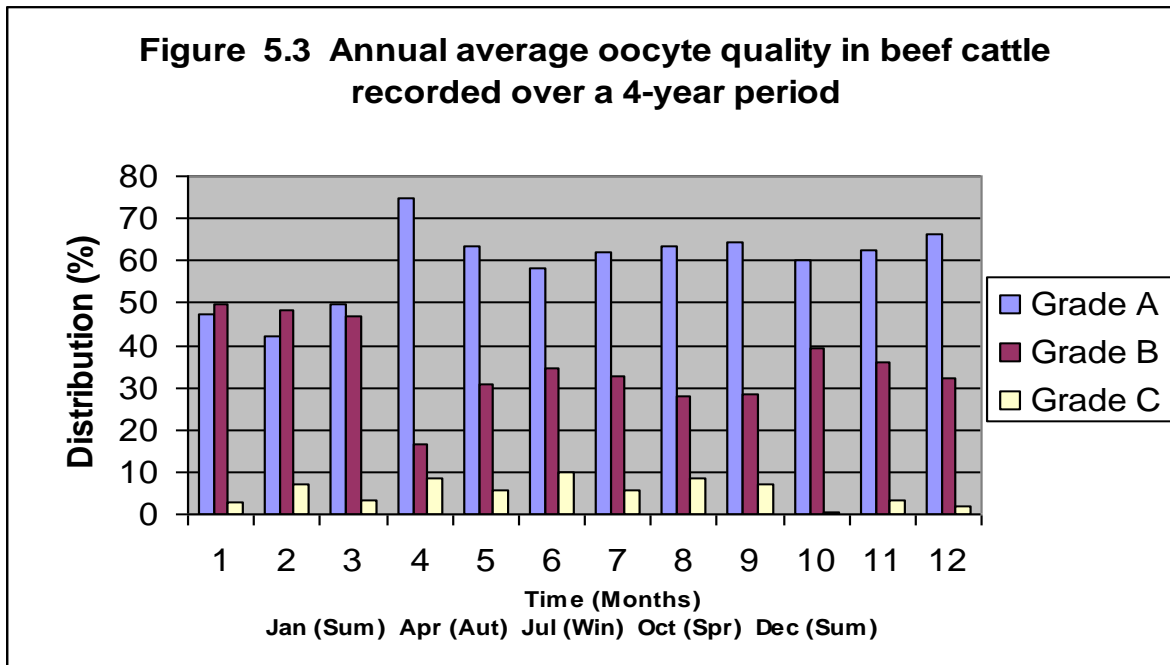
Table 5.4 The mean (±SE) annual percentage of embryos, number of embryos per OPU session and embryo quality produced over a 4-year period in beef cattle

Month	Number of embryos	Embryos/oocytes produced (%)	Number of embryos per OPU session	Embryo quality (% of total)		
				Grade A	Grade B	Grade C
January	5	8.8	0.1 ± 0.5 [a]	83.3 ± 28.9 [a]	0.0 ± 0.0 [a]	16.7 ± 28.9 [a]
February	5	5.4	0.1 ± 0.3 [a]	37.5 ± 47.9 [a]	37.5 ± 47.9 [a]	25.0 ± 50.0 [a]
March	0	0.0	0.0 ± 0.0 [a]	-	-	-
April	5	26.3	0.2 ± 0.5 [a]	75.0 ± 50.0 [a]	25.0 ± 50.0 [a]	0.0 ± 0.0 [a]
May	5	11.4	0.2 ± 0.5 [a]	0.0 ± 0.0 [b]	75.0 ± 50.0 [a]	25.0 ± 50.0 [a]
June	10	11.8	0.2 ± 0.6 [a]	37.5 ± 49.4 [a]	29.2 ± 45.9 [a]	33.3 ± 51.6 [a]
July	12	18.5	0.3 ± 0.7 [a]	68.8 ± 45.8 [a]	25.0 ± 46.3 [a]	6.3 ± 17.7 [a]
August	9	20.0	0.3 ± 0.8 [a]	83.3 ± 40.8 [a]	16.7 ± 40.8 [a]	0.0 ± 0.0 [a]
September	13	26.0	0.5 ± 0.9 [a]	78.6 ± 39.3 [a]	14.3 ± 37.8 [a]	7.1 ± 18.9 [a]
October	28	22.2	0.5 ± 0.9 [a]	49.4 ± 45.0 [a]	40.6 ± 46.0 [a]	10.0 ± 28.0 [a]
November	19	16.2	0.3 ± 0.7 [a]	30.8 ± 48.0 [a]	46.2 ± 51.9 [a]	23.1 ± 43.9 [a]
December	9	15.0	0.2 ± 0.6 [a]	50.0 ± 44.7 [a]	41.7 ± 49.2 [a]	8.3 ± 20.4 [a]

[a-b] Values within a column with different superscripts differ significantly (P<0.05)

5.2.5   Number of bovine embryos produced

The number of embryos produced was evaluated and recorded in two different ways. The first method was to determine it as a percentage of the total number of oocytes recovered (Figure 5.4). The pattern of efficiency observed using this method of recording was low with ever decreasing success rates recorded during the middle and late summer months (January to March) with a high (26.3%) percentage being obtained in April (Table 5.4). This trend was followed by a steady increase in embryo production during the winter months (June to August) with a peak during spring and early summer (September to October) and again a steady decrease towards middle and late summer (November to March). In the month of March (onset of autumn) no embryos could be produced *in vitro*.

The second method of recording the efficiency of embryo production was by calculating the mean number of embryos produced per OPU session (Figure 5.5). This was considered a better indication of the true embryo production potential for a specific period. It also reflected on the labour intensity and cost-effectiveness of the embryo production process. There was no significant difference in the number of embryos produced per OPU session for the different months of the year. The highest mean number of embryos per OPU session was recorded in September (0.5 ± 0.9) (spring) and the lowest (zero) in March (autumn). The general tendency as judged by this method was a low, with decreasing embryo production during the warm months, with a steady increase during the cooler months (June to August) - with peak embryo production during spring (September to October). During the whole 4-year period no embryos were produced during the month of March. Although these differences were not significant in statistical terms, it has a big impact on the practical feasibility of embryo production during this specific month of the year. No influence of breed could be detected in terms of embryo production. The correlation recorded between embryos produced per OPU session and Grade A, B and C oocytes was r = 0.56; r = -0.47 and r = –0.11 respectively (not significant).

Figure 5.4 Mean annual percentage of embryos produced per total number of oocytes over the 4-year period in beef cattle

Figure 5.5 Mean annual number of embryos produced per OPU session in beef cattle
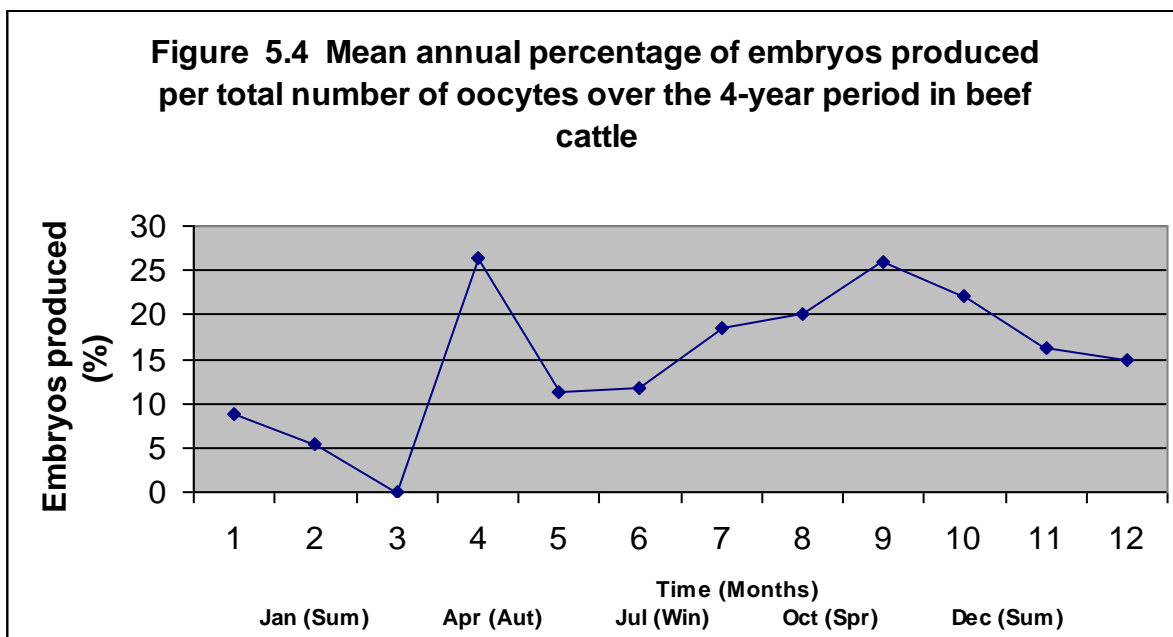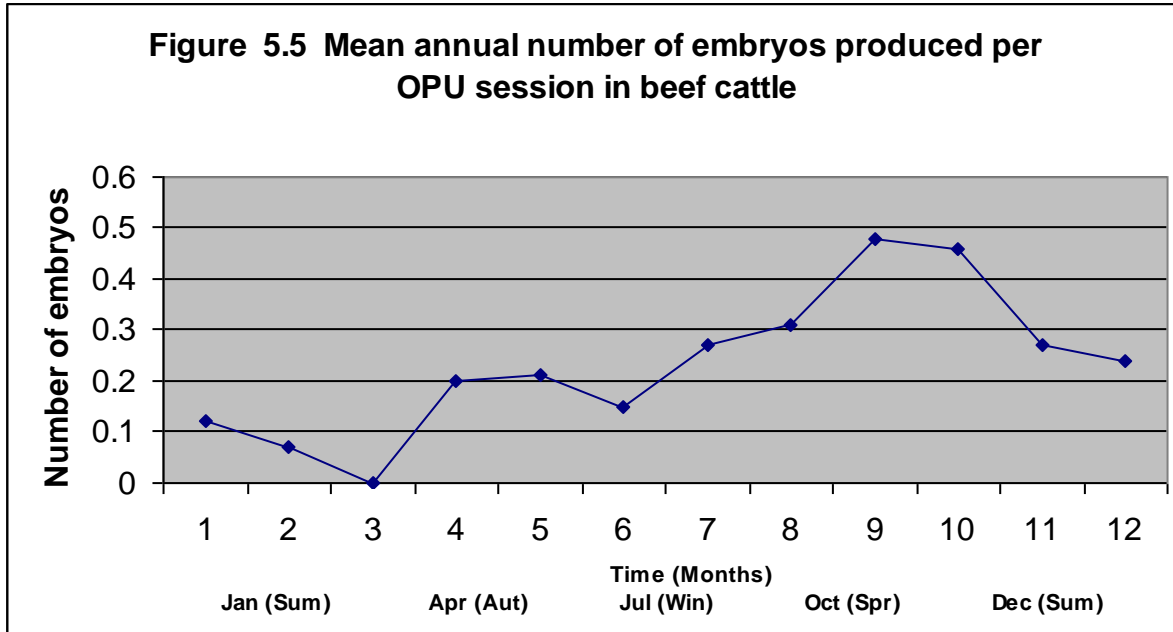
5.2.6   Quality of bovine embryos produced *in vitro*

There was no significant difference between the different months of the year (except May; $p < 0.05$) regarding Grade A embryo production. The highest percentage of Grade A embryos were recorded in January (83.3 ± 28.9 %) and August (83.3 ± 40.8 %). A markedly lower percentage (no embryos) of Grade B embryos were produced in January and in the case of Grade C embryos, markedly lower percentages (no embryos) were produced in April and August (autumn and late winter).

Figure 5.6 Mean annual embryo quality recorded over a 4-year period in beef cattle
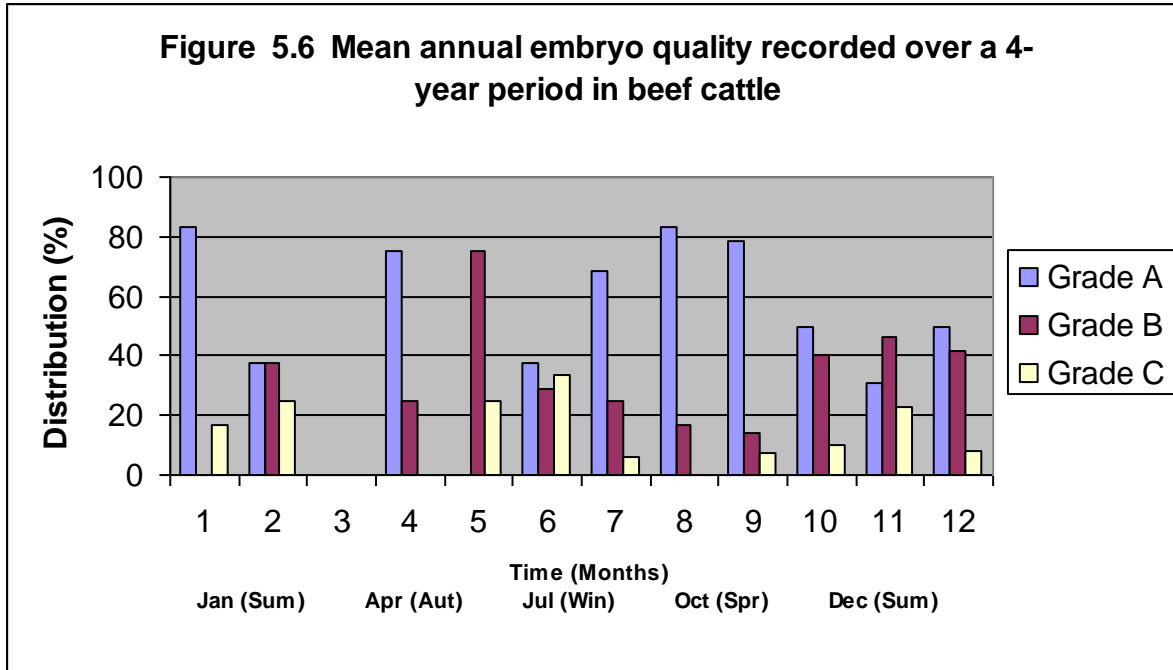
5.2.7 Pregnancy rate after embryo transfer

See summarised results under Methodology (2.2.11).

5.3 Discussion

The effect of season on the different facets of IVEP programmes in fertility impaired donor beef cows have yielded some interesting results. The only negative aspect was the fact that a large variation exists in results and reflects the large individual variation in response, which makes it difficult to establish any significant differences between months or seasons. This individual variation is supported by the findings of Twagiramungu *et al.* (1999) and Tamassia *et al.* (2003) who clearly indicated the individual donor to have the biggest influence on the IVEP results obtained.

Although the effect of season in both *in vivo* embryo production and IVEP is mentioned in the literature, all reported studies only considered "normal" donor animals and no effect of season on fertility-impaired donor cows was reported – a fact that has certain practical

implications as most animals presented for IVEP in South Africa are usually fertility-impaired donors.

5.3.1   Ovarian follicular populations

There seemed to be a definite trend in the follicular population growth pattern. This trend was characterized by two definite activity peaks over seasons during the year.   The general tendency (although not significant) was for lower follicle numbers to be recorded during the warmer months and higher follicle numbers during the colder months. This phenomenon coincides with the normal sexual activity as experienced in South African beef cows. A finding of note is the fact that the lowest ovarian follicle number (April – mid autumn) was recorded just after the warm period and the highest number just after the cold period (September - spring).   This may imply some delayed effect of ambient temperature on follicular growth. This corresponds with the findings of Wolfenson *et al.* (1995), who indicated a decrease in small and medium sized follicles in normal donor cows after being exposed to heat stress. This decrease in follicular activity may be attributed to a decrease in hormonal activity especially the decrease in gonadotropin releasing hormone secretion which consequently influences all other gonadotrophin hormone secretion (Wolfenson *et al.*, 1995).

The peaks in follicle numbers (indicative of ovarian activity) were recorded during the months of May (end autumn) and September (onset of spring) in the beef cows. A more detailed investigation is needed to further determine whether these ovarian follicular peaks are associated with either ambient temperature or photoperiod changes or even both. The effect of photoperiod has been described for seasonal breeders but, not in detail for the bovine (Chemineau *et al.*, 1996).   Some limited reports indicate season to influence the concentration of LH secretion in both beef and dairy cows with the highest concentration being recorded during spring (Stumpf *et al.,* 1993). This correlates with the recording of higher follicle populations and higher embryo production during the spring period in this trial. Di Palo *et al.* (2001), recorded a pattern of low follicular activity in Mediterranean buffalo cows during the hotter months.   According to these researchers this confirmed the buffalo's

sensitivity to heat stress and photoperiod - which may also still be evident in the domesticated cow.

5.3.2 Number of oocytes aspirated and the oocyte recovery rate

The number of bovine oocytes recovered (also being indicative of the effectiveness of the technique used) followed the same pattern (lower during warmer months and higher during colder months) corresponds to the number of follicles - with a correlation coefficient of r = 0.79 between these two parameters. This is similar to the finding of Di Palo *et al.* (2001), in Mediterranean buffalo cows and is generally to be expected as the more follicles are available for aspiration, the more oocytes could be aspirated – if it is accepted that the technique of aspiration is satisfactory. The lowest number of oocytes aspirated was recorded in the month with the lowest follicle number. However, this was not true for the highest number of oocytes which was recorded in the month after the month with the highest follicle number. This recording corresponds with the fact that the oocyte recovery rate decreases with increased follicle populations (Pieterse *et al.*, 1988). More or less the same pattern of oocyte recovery peaks were recorded as was the case in follicle numbers in the current IVEP trial.

The fact that there was no significant difference in the oocyte recovery rate is supported by the high correlation between follicle numbers and oocytes recovered and could thus also point to an acceptable technique of aspiration used for this trial.

5.3.3 Quality of bovine oocytes recovered following OPU

It was difficult to establish a specific pattern in the oocyte quality for the oocytes aspirated by means of OPU over the different seasons. However, there was a trend for the percentage of Grade A (superior) oocytes to be lower during and after the warmer months and higher during and after the colder periods of the year. A surprising fact was that the highest percentage of Grade A oocytes was recovered in the month of April (mid autumn) (mean ambient temperature of 24 ^{0}C) after a period between January and March (mean ambient temperature of 28 ^{0}C) - during which relatively low numbers of Grade A oocytes were recovered. After

April (mid autumn) there seemed to be a stabilizing effect, with the percentage of Grade A oocytes recovered being similar for the subsequent months. This phenomenon corresponds with the findings of Rocha *et al.* (1998), where the quality and developmental capacity of oocytes were compromised during the warmer periods of the year.

Seasonal studies have shown the quality of the bovine oocytes and the rate of embryo development to deteriorate during the summer, compared with the winter months (Rocha *et al.*, 1998; Rutledge *et al.*, 1999) and the lipid composition of oocyte membranes to differ between seasons (Zeron *et a*l., 1999).

The decrease in oocyte quality may be attributed to aspects such as decreased steroidgenesis in the thecal and granulosa cells (Roth *et al.*, 2001), an effect on the RNA synthesis (Fair *et al.*, 1995), impairment of cell-cell communication between the granulosa and cumulus cells and between the cumulus and oocyte. Another possibility is an alteration in the follicular fluid content such as insulin-like growth factor binding protein (IGFBP) (Ambrose *et al.,* 1999) or steroids (Wolfenson *et al.*, 1997).

Ambient temperature modulates the physical properties of the lipids in biological membranes and changes the lipid composition of the membrane (Quinn, 1985). Ambient temperature also regulates the transition from liquid crystalline to the gel phase (lipid phase transition) (Crowe *et al.*, 1989). Unsaturation of the acyl chains of the membrane phospholipids increases during cold temperatures, which supposedly depresses (decreases) the midpoint (TM) of the lipid phase transition (Cossins and Raynard, 1987). Injury to the oocytes occurs during the lipid phase transition (Arav *et al.*, 1996) and is maximal at a TM of 16 ^{0}C (Zeron *et al.,* 1999). All these findings have led to the belief that changes in ambient temperature are the main signal for the regulation of fatty acid composition of membranes, which in turn affects the membrane functionality and oocyte viability.

Currently there have been no published reports on the delayed effect of heat stress on oocyte quality. Roth *et al.* (2000) showed that by exposing ovarian follicles to hyperthermia in summer, during the early stages of development, oocyte quality may be impaired together

with the subsequent embryo development during autumn. Fertility data sets have indicated that a period of about 2 months (3-4 cycles) (developmental cycle of an oocyte) is needed for low autumn fertility to be restored to the level prevailing in winter (Ron *et al.*, 1984).

Roth *et al.* (2001) hypothesized that the normal growth and atresia of ovarian follicles within the follicular wave dynamics, causes only a slow depletion of the heat stressed impaired follicles from the ovaries. Enhanced removal of these ovarian follicles by means of OPU may actually enhance the recruitment of healthy follicles and improve autumn fertility. This is supported by the hypothesis that long term repeated removal of all follicles > 2 mm could increase the number of follicular waves in cattle (Boni *et al.*, 1997; Garcia and Salaheddine, 1998).

The Grade B oocytes aspirated showed an opposite tendency - with higher percentages of Grade B oocytes being recorded during the hot months and less during the colder months. This may be an indication that the effect of heat stress manifests itself in an increase in the number of Grade B oocytes at the cost of the number of Grade A oocytes. This pattern is well supported by the negative correlation of $r = -0.95$ between Grade A and Grade B oocytes. The pattern for the occurrence of Grade C oocytes was completely at random and no real trend could be established throughout the observation period of 4 years and it does not seem to have any real effect on embryo production efficiency. The Grade A (superior quality) oocytes seemed to be the determining factor in efficient embryo production (see 5.2.5). The month (April) in which the highest percentage of Grade A oocytes occurred also produced the highest percentage of embryos per oocyte. It may be indicative that this classification system used for oocytes is a useful tool in predicting the developmental competence of the oocytes.

5.3.4   Number of embryos produced following IVEP

As the objective of this study was to develop a practical approach to IVEP in fertility-impaired donor cows, the number of embryos produced per OPU session seems to be a more appropriate way to express embryo production potential and the success and efficiency of the operation. The number of embryos produced per OPU session when illustrated graphically,

shows a definite seasonal pattern regarding effective *in vitro* bovine embryo production. The number of embryos produced during the months of September and October (spring) was much higher than during the rest of the year. The IVEP pattern exhibited a low embryo success rate, with a decrease during the summer months and a higher percentage of embryos produced with an increase during the winter months (Rocha *et al.*, 1998). Graphically, the bovine embryo production success rate seemed to follow the same pattern as the follicle population, the number of oocytes recovered and the percentage of Grade A oocytes obtained. It seems evident that the percentage of Grade A oocytes (depending on the grading system) is indicative of the success rate that can be expected in bovine embryo production.

This seasonal bovine embryo production pattern obtained in this trial is supported by the findings of Ryan *et al.* (1992; 1993); Rocha *et al.* (1998); Rutledge *et al.* (1999) and Zeron and Arav (2002). However, all these researchers worked on either abattoir material or normal (naturally cycling, with a satisfactory reproduction history) donor cows and similar trials have not been reported for sub-fertile cows.

An evident finding (although not significant) was the fact that during the observation period of 4 years, no embryos could be produced during the month of March (onset of autumn). This could not have happened by chance, as a relatively high number of bovine donors of different beef breeds were used over an extended period of time. The trend in the number of embryos produced indicates that when planning an IVEP programme for fertility-impaired donors, certain months of the year (January to March) should best be avoided. Any attempt to produce *in vitro* embryos during this period will negatively influence the total efficiency of the procedure. This is a significant finding which can impact greatly on the development on a practical level, of this relatively new technology in accelerated animal breeding.

5.3.5   Quality of bovine embryos produced

It was difficult to establish a seasonal pattern regarding embryo quality. There seems to be a more constant high percentage of Grade A embryos produced during the winter months,

whereas the other grades are more or less randomly distributed throughout the year. This tendency regarding embryo quality corresponds with the finding of Ryan *et al.* (1992; 1993), who found a decrease in bovine embryo quality for both *in vivo* and *in vitro* produced embryos during the warmer periods of the year. The embryo quality pattern followed the oocyte quality pattern - which is to be expected (Rutledge *et al.*, 1999).

5.3.6   General discussion

The production of *in vitro* bovine embryos from genetically superior fertility-impaired donor cows is a potential powerful tool for use in the genetic improvement of cattle. It will ensure the continuation of offspring production from quality animals which would otherwise have been lost to the cattle industry under normal circumstances. The South African beef and dairy industry rely heavily on the genetic make-up of the animals with a proven "stay ability" (longevity) component. However, the production of *in vitro* embryos from these types of animals still remains a costly and labour intensive process.

Ultrasound guided oocyte aspiration (OPU) is already becoming a routine procedure for use in normally cycling reproductively sound donor cows. However, there are many unanswered questions as far as the same *in vitro* embryo production procedure for fertility-impaired cows is concerned. South Africa is a country with a large variation regarding climatic conditions between seasons and this has also led to the question whether season could have an effect on certain facets of IVEP in fertility-impaired cows.

From the obtained results it is evident that there are possible seasonal trends in aspects such as ovarian follicular populations, the number of oocytes recovered and the number of embryos produced per OPU session. It would seem as if the period between January and March should best be avoided for the application of IVEP procedures in cattle. Although the number of embryos produced is low (15%), compared to those generated following the use of MOET and IVEP from normally reproducing donors (± 40%) - it is acceptable if one takes into account the fact that these sub-fertile donors cannot produce embryos or offspring by any other means than IVEP. The effect of season is a very important finding that can have a

significant effect on the efficiency of this relatively new technology in the cattle industry. It provides important information which can be used to plan future IVEP programmes for sub-fertile and possibly even reproductively sound donor cows. This strategic planning should also enhance the results obtained in these programs and to make it more effective for the technology supplier and more cost-effective for the end-user, which is the livestock producer.

Chapter 6

General conclusions and recommendations

The South African cattle industry can be regarded as a limited user of conventional bovine embryo technology (e.g. MOET), and the introduction of an already more advanced reproductive technology (e.g. IVEP) will be a difficult task. *In vitro* embryo production from live donor cows is a relatively new technology that has only been introduced in the South African cattle industry over the past number of years since 2000, but the potential in terms of accelerated genetic progress and the storage of superior genetic material for future use is priceless.

In vitro embryo production (IVEP) in cattle is, thus, a versatile assisted reproduction technique that lends itself to the enhancement of several aspects of animal breeding and the conservation and utilization of superior and rare genetic material e.g.:

- The recovery and utilization of superior genetic material that would have been lost to the dairy and beef industry. As in the case of this study where animals of superior genetic quality, but with impaired fertility can still be utilized for IVEP.
- Substantial genetic improvement through the identification of superior genetics and by using IVEP to induce an accelerated reproduction rate with an accompanied shortening of the generation interval when applying this technology on young and even pre-pubertal animals. This specific aspect is even more important in the dairy industry where a small genetic improvement in animal performance can translate to a substantial economic benefit to the farmer.
- The application of IVEP technology on rare and endangered wildlife species where conventional *in vivo* embryo production techniques e.g. MOET cannot be applied.
- The whole issue of cost-effectiveness is addressed by the IVEP procedures as it is a less expensive and a more controllable technique than the other conventional embryo production procedures like e.g. MOET.

Although *in vitro* embryo production from live donor cows can be seen as a possible future alternative or extension for conventional embryo technology, the South African application of IVEP focuses more on the application of this technology in sub-fertile superior donor cows. This IVEP is currently seen as an area in South African cattle industry where this technology could have the biggest short term impact.

Substantial research has been done locally on actual *in vitro* embryo production procedures, but in most cases the input genetic material (oocytes) have been derived from slaughterhouse material and only semen from bulls of proven fertility in an *in vitro* fertilization environment have been used. However, the practical application of this technology presents the industry with a different set of factors to consider in order to achieve acceptable results for the end user – normally the cattle stud breeder. Oocytes are usually derived from a varied group of fertility-impaired superior donor cows and a variety of semen donors are used. The conditions under which the oocytes are harvested also vary from country to country and could also vary within a given environment.

Many factors need to be taken into consideration when one is presented by the challenging task such as producing *in vitro* embryos from sub-fertile donor cows. Many of these factors may have a substantial influence on the eventual outcome and acceptance of this technology in the cattle industry. Aspects such as efficiency and cost-effectiveness of embryo production remain paramount in any *in vitro* embryo production programme and needs to be evaluated.

In this study the following aspects regarding *in vitro* embryo production from fertility-impaired cows were investigated: (i) which is the most appropriate logistical approach for *in vitro* embryo production when confronted with a sub-fertile donor cow; (ii) the maximizing of embryo production in pregnant cows and (iii) the effect of season on *in vitro* embryo production efficiency from fertility impaired superior donor cows.

From the results obtained it could be concluded that an initial period of OPU prior to slaughter has a definite beneficial effect on the total effectivity of maximizing the number of oocytes available for *in vitro* embryo production and possible genetic progress that could be acquired

from this type of donor cow. The best approach would be a period of OPU (until no more oocytes are obtained), followed by the eventual slaughter and final oocyte harvesting. This approach seems to be by far the most effective and reliable procedure.

The logistical fact that oocytes and embryos have to be cultured in small numbers from individual donor cows does not seem to affect the final outcome of the embryo production results. This is a positive result if one considers that the situation in practice dictates that the oocytes from individual donors should be cultured separately to avoid the unnecessary blending of genetic material.

The fact that embryos can be produced from pregnant cow oocyte donors is a potential positive outcome of this technology, which re-emphasizes the advantages of *in vitro* embryo production from live donors. To be able to produce embryos and offspring from pregnant cows can be seen as a real bonus in terms of a source of oocytes and time saving. What makes IVEP even more attractive is the fact that the embryo production process does not interfere with the actual reproduction cycle of the donor cow. It would seem as if additional exogenous FSH superstimulation of the pregnant donor cow did not yield any more positive results in terms of higher numbers of oocytes and embryos and that it may actually have a negative effect on the cost-effectiveness of the procedure. An additional negative effect may be the possible development of a resistance to repeated FSH stimulation (immune response) and a long term negative influence on the general fertility of the donor animal.

The effect of season is a very important aspect for *in vitro* bovine embryo production from fertility-impaired donors in the South African context. Laboratories are presented with these types of donors throughout the year. There is a definite need to establish the effectiveness of this procedure during different times of the year. From the obtained results, it is clear that the period from January to March (mid-summer to the onset of autumn) should best be avoided when implementing *in vitro* bovine embryo production programmes. The bovine IVEP success rate increased during autumn, stabilized during the winter and peaked during spring and early summer. This information is of utmost importance when planning an *in vitro* embryo production programme and will help to avoid unsatisfactory results that may influence the

acceptance of this technology by the cattle stud breeder. Another important aspect to be kept in mind is the fact that the period (from April onwards) of improved *in vitro* embryo production coincides with the period of pregnancy diagnosis in beef cattle production systems which makes it easier for the farmer to select cows to be used as oocyte donors. In practice it means that these animals do not have to stay on as "passengers" on the farm after the breeding season, and the cows can immediately enter an *in vitro* embryo production programme, when culled due to old age or for impaired fertility reasons.

Although the recorded results and analyses give a good perspective on the expected outcome when bovine IVEP is applied on fertility-impaired animals, there are still some facets that need to be investigated and improved - especially technical issues regarding the actual recovery of oocytes and the subsequent laboratory (IVEP) procedures that will have to be refined in future.

In conclusion, it can be said that *in vitro* embryo production from fertility-impaired donor cows (as an alternative embryo production technology) is something that has practical implication when applied in the correct manner and if certain problem areas, as indicated in this study, are avoided. This technology is a useful tool for the cattle industry which will greatly enhance genetic improvement and conservation, if applied correctly in combination with the objective selection criteria of genetically superior donor cows.

ABSTRACT

IN VITRO EMBRYO PRODUCTION IN CATTLE

by

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The objective of this study was the development of efficient *in vitro* embryo production (IVEP) technology for application on fertility-impaired beef and dairy cows. Fertility-impaired implying animals that could neither reproduce nor produce embryos through conventional MOET procedures due to old age, pregnancy, repeated MOET procedure treatments, certain abnormal uterine conditions and general failure to conceive and become pregnant following repeated inseminations or natural services, as well as due to physical injuries.

The investigation concentrated on whether it was possible to produce embryos from a donor cow with this profile and additionally other aspects such as - the best logistical approach for IVEP when presented with a fertility-impaired donor cow - whether superovulation could be used as an enhancing tool when presented with a pregnant donor - and the affect of season on all aspects of IVEP from fertility-impaired donor cows.

It was established that a period of OPU could enhance the production of higher numbers of embryos additional to eventual slaughter of the donor cow (10 more embryos on average per donor) and that the culturing of a small number of oocytes and embryos does not negatively affect the eventual IVEP result (10 % more embryos were produced from individual donors).

This result was a good indicator of how to plan the logistical approach when confronted with these types of donor cows and it also suits the practical situation faced when producing *in vitro* embryos from small numbers of oocytes collected from these donor cows.

It was evident that additional superovulation does not enhance the IVEP potential of pregnant dairy cows (0.02 more embryos per donor per OPU session), unless more refined research is undertaken to optimize the actual superovulation protocols and oocyte recovery procedures. The additional cost implication of added hormonal treatment for superovulation may actually act as a deterrent for cattle stud breeders to embrace this technology. However, the mere fact that it was possible to produce embryos from pregnant dairy cows could be utilized as a useful tool for accelerated genetic improvement in the dairy and beef cattle industry in South Africa.

It was established that season (recorded as ambient temperature and photoperiod) had a definite effect on all aspects of IVEP in fertility-impaired beef cattle cows, a trend that was evident from literature in some laboratory IVEP trials on slaughterhouse material and also in the normal reproduction pattern of beef cattle cows in the South African context. This trend manifested itself in the IVEP pattern of fertility-impaired cows. There was a definite decrease in IVEP during the period of late summer (5.4 %) and early autumn (0 %) with a definite delayed effect of high temperatures and possibly photoperiod evident. Alternatively, the optimum period for IVEP seemed to be during winter (between 11 % and 20 %) and spring (between 22 % and 26 %) which also indicated a possible temperature and photoperiod affect. This effect on oocyte quality and eventual embryo production results could also serve as an indicator for other management decisions regarding normal reproduction management in beef cattle as well as the planning of conventional superovulation and embryo transfer programmes.

From the obtained results it was clear that IVEP is possible from fertility-impaired and pregnant donor cows. However, there are certain aspects that need to be focussed on to ensure that optimum results are obtained when applying IVEP on these animals. Optimum results are a prerequisite for this technology to be accepted by the South African cattle

breeding industry. IVEP is still in a preliminary phase of application in the South African cattle industry and is not within the ability of all breeders. This will, however, become a powerful tool for use in accelerated genetic improvement programmes and will also ensure that superior genetic material from animals that would have been lost for breeding will continue to be utilized in the cattle breeding industry.

OPSOMMING

IN VITRO EMBRIO PRODUKSIE IN BEESTE

deur

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Die doel van die studie was die ontwikkeling van 'n effektiewe *in vitro* embrioproduksie (IVEP) tegnologie vir toepassing op reproduksie-gestremde vleis- en melkbeeste. Reproduksie-gestremheid verwys na diere wat nie normaal kan reproduseer of embrios produseer as gevolg van ouderdom, dragtigheid, herhaaldelike superovulasie en embriokolleksie prosedures, abnormale uterus toestande, algemene onvermoë om dragtig te raak na herhaalde inseminasies of natuurlike dekkings, asook as gevolg van fisiese beserings.

Die studie het gekonsentreer op die feit of dit wel moontlik is om beesembrios van diere met die spesifieke profiel te produseer en addisionele aspekte wat ondersoek is, was – die beste logistiese benadering vir IVEP wanneer gekonfronteer met 'n reproduksie-gestremde skenkerkoei – of superovulasie wel gebruik kan word as 'n hulpmiddel met IVEP in dragtige melkkoeiskenkers - die effek van seisoen (gemeet as omgewingstemperatuur en dagliglengte) op alle aspekte van *in vitro* embrioproduksie in reproduksie-gestremde skenkerkoeie.

Daar was vasgestel dat 'n periode van ultraklank geleide oosiet aspirasie (OPU) wel verhoogde embrio produksie (gemiddeld 10 meer embrios per donor) tot gevolg kan hê,

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addisioneel tot die uiteindelik slagting (kolleksie van oosiete vanaf die eierstokke van die dooie koei en IVEP). Daar is ook gevind dat die kweking van oosiete en embrios in klein hoeveelhede nie enige nadelige effek op die uiteindelik IVEP prestasie het nie (10 % meer embrios was geproduseer wanneer gekweek as groepe oosiete vanaf individuele skenkers). Hierdie resultaat was 'n goeie aanduiding van die logistiese beplanning wanneer gekonfronteer met die tipe skenkerkoei en dit pas ook goed in met die praktiese Suid-Afrikaanse situasie wat ondervind word tydens IVEP waar die tipe dier as oosietskenker gebruik word.

Dit was duidelik dat addisionele superovulasie nie enige positief-bydraende effek op die IVEP potensiaal van dragtige melkkoeie het nie (0.02 meer embrios per skenker per OPU sessie). Meer verfynde ondesoeke sal gedoen moet word na alternatiewe superovulasie - asook oosiet herwinningsmetodes. Die bykomstige koste implikasie van hormoonbehandeling kan ook verhinder dat stoettelers die tipe tegnologie sal aanvaar. Nieteenstaande, is dit belangrik dat dit wel moonlik blyk te wees om embrios van dragtige melkkoeie te produseer. Hierdie feit kan gebruik word as 'n belangrike instrument vir versnelde genetiese vordering in melkbeeste in Suid Afrika, sonder enige ontwrigting van die reproduksie en produksiesiklus van die skenker diere.

Daar is ook vasgestel dat seisoen (gemeet as omgewingstemperatuur en dagliglengte) 'n besliste effek op alle aspekte van IVEP in reproduksie-gestremde vleisbeeste het. Hierdie tendens is alreeds bepaal tydens sekere laboratorium embrioproduksie eksperimente waar daar van slagpale materiaal gebruik gemaak is. Dit is ook 'n tendens wat aanwesig is in die normale reproduksiepatroon van vleisbeeste in Suid Afrika. Dieselfde tendens het homself gemanifesteer in die IVEP patroon van reproduksie-gestremde vleisbeeste. Daar was 'n besliste daling in embrioproduksie tydens die laat somer (5.4 %) en vroeë herfs (0 %) periodes wat dui op 'n vertraagde negatiewe effek van hetsy hoë temperature en of dagliglengte. Alternatiewelik, was daar verhoogde embioproduksie tydens die winter (tussen 11 % en 20 %) en lente periodes (tussen 22 % en 26 %) wat ook dui op 'n temperatuur sowel as 'n moontlik dagliglengte effek in lente. Hierdie tendens is 'n goeie aanduiding vir ander

bestuursbesluite aangaande normale reproduksiesiklusse asook die beplanning van superovulasie en embriokolleksieprogramme vir vleisbeeste.

Vanaf die behaalde resultate is dit duidelik dat dit wel moontlik is om *in vitro* embrios te produseer van reproduksie-gestremde asook dragtige skenkerkoeie. Daar is egter sekere aspekte waarop gekonsentreer moet word indien optimale resultate behaal wil word met die tipe diere as oosiet skenkers. Optimum resultate is van kardinale belang indien die nuwe tegnologie deur die Suid Afrikaanse beesteler aanvaar sou word. Nieteenstaande die negatiewe aspekte sal IVEP 'n nuttige instrument word vir gebruik in versnelde genetiese opgraderingsprogramme en dit sal verseker dat superieure genetiese materiaal van diere wat andersins verlore sou wees vir teling wel nog gebruik kan word.

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