ULTRASONIC EVALUATION OF SUPEROVULATION IN CATTLE

BY

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DECLARATION

I, Javier Cattoni, do hereby declare that the work presented in this thesis is original, was carried out by me and has not been presented for an award of a degree in any other University.

Signature:

Date:

Summary

Embryo transfer is now a standard management technique. Effective embryo transfer is dependent upon the success of superovulation used to increase the yield of viable, transferable embryos. Numerous studies have identified problems with repeatability and predictability of response to superovulation. Given these problems, an effective method of monitoring follicular growth, ovulation and formation of corpora lutea would be a useful adjunct to the embryo transfer procedure. This study evaluated ultrasound for monitoring follicular growth, ovulation and formation of the corpora lutea in superovulated cows and correlated findings with embryo recovery and post mortem examination of the ovaries. Eleven cows were studied through a control and superovulated cycle; superovulation was achieved using pregnant mare serum gonadotrophin (PMSG, 3000 or 1500 i.u.). Ovarian response was monitored using a real time B-mode scanner equipped with a linear 7.5 Mhz rectal transducer. In the control cycle, which was similar in all cows, luteolysis was followed by growth of a single follicle to 1.4 $\pm$ 0.2 cm. Ovulation was identified by collapse of the follicle and appearance of a corpus haemorrhagicum and was confirmed by measuring plasma LH and progesterone concentration. By day 5 the mature corpus luteum was visible. At the time of PMSG injection the majority of follicles were $<$ 6 mm. Two days later, 6-10 follicles $>$ 8 mm were identified on most ovaries. Ovulation, in the superovulated cycle was identified as either disappearance of large follicles or obvious reduction in the size of the ovary. It was possible to identify but not to quantify corpora haemorrhagica. It was considered possible to quantitate corpora lutea on day 6 after oestrus. However, this estimate of corpora lutea number correlated poorly with the number counted at post mortem.

Plasma progesterone concentration was monitored throughout the cycles, until embryo flushing. Very high progesterone concentrations were measured at embryo flushing; these levels correlated poorly with either the number of corpora lutea on the ovaries post mortem, or the number of embryos recovered. Histological study of the superovulated ovaries revealed many follicles showing differing degrees of luteinisation. It was suspected that these luteinised follicles contributed to the high progesterone levels. It was impossible to categorise some fully luteinised structures as either corpora lutea or anovulatory follicles at post mortem. However, ovulation failure would have explained the poor embryo yield.

In conclusion, ultrasound examination was useful for monitoring follicular growth in response to superovulation. It proved difficult to accurately quantify ovulation or numbers of corpora lutea. As an aid to predicting embryo recovery ultrasound monitoring was inadequate for the same reasons that rectal palpation, laparoscopy, laparotomy or progesterone concentrations have proven inaccurate, namely, abnormal luteinised structures on the ovary and aberrant progesterone production. This problem is inherant to the superovulatory treatment.

Chapter 1

Review of the Literature

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1.1 Introduction

The technique of embryo transfer consists of recovering embryos from donor cows of outstanding genetic potential and transferring them to surrogate recipient cows of lower genetic value thus increasing production of offspring from valuable breeding stock.

The first embryo transfer was carried out in 1890 by Walter Heape with the transfer of rabbit embryos from donor to surrogate mother (Heape, 1890). But application of this technique really came to the fore with the introduction of gonadotrophin hormones for superovulation (Smith and Engle, 1927), allowing the production of multiple embryos. Work done by Willett and colleagues marked the beginning of embryo transfer in cattle, with the first calf born in 1951 (Willett, Black, Casida, Stone and Buckner, 1951). A significant improvement in pregnancy rates was achieved by the late 1960s (Rowson, Moor and Lawson, 1969).

The last two decades have seen important advances in the technology of both embryo recovery and transfer, with surgical transfer being replaced by nonsurgical procedures. In the 1970s methods for deep freezing cattle embryos were developed, which brought further advances to the industry by allowing a greater synchrony of donor and recipient, and the long term preservation of genetic material (Wilmut and Rowson, 1973). Nowadays embryo transfer is a tool used for reproductive management in conjunction with other reproductive technologies such as *in vitro* fertilization, cloning, production of transgenic embryos and MOET programs (Smith, 1988). In spite of great progress there are still unpredictable areas of the procedure which need further study. One such area is the response of cattle ovaries to superovulation.

1.2 Oestrous Cycle of the Bovine

The cow is a non-seasonal breeder with periods of receptivity or oestrus recurring at 21 day intervals, range 18-24 days. In every cycle generally only one mature follicle ovulates

1.2.1 Anatomy of the Non-Pregnant Bovine Reproductive Tract

In the bovine the uterus is divided into cervix, uterine body and uterine horns. The cervix is a collagenous structure composed of 3-4 cartilaginous rings, 6-10 cm in length and 2-4 cm wide. The body of the uterus is short, about 2 cm; the uterine horns are 35-40 cm, shorter in heifers. The horns are initially joined by the dorsal and ventral intercornual ligaments then bifurcate downwards and laterally. The oviduct is a narrow tube of around 25 cm running between the uterotubal junction and the ovary, divided into three areas namely the isthmus, the ampulla and ending in the fimbrae (Sissons and Grossman, 1975).

In cattle the ovaries are almond-shaped, measuring 2-5 cm along the attached border, 1.5-4 cm in height from the attached border to the free border and 1.5-3 cm wide from the anterior to the posterior side (Morrow, 1980). In an ovary there are many thousands of follicles of different sizes. Just before ovulation a follicle reaches a maximum size of 1.9 cm. At ovulation the follicle ruptures and the oocyte is released into the oviduct. The corpus luteum is the structure which is formed from the collapsed follicle after ovulation. It is a glandular structure which secretes progesterone and attains a maximum size of 2-2.5 cm during the first eight day of the oestrous cycle (Arthur, Noakes and Pearson, 1989).

On rectal palpation the cervix is identified as a hard cylindrical tube. On palpation, the uterus is softer than the cervix and the horns form a semi-circle. The oviduct is not usually palpable except in some pathological conditions. The ovaries are readily palpable and the ovarian structures that can be felt upon rectal palpation are follicles and the corpus luteum. On palpation the corpus luteum has a much firmer consistency than that of a follicle.

1.2.2 Histology of the Bovine Reproductive Tract

Histologically the cervix of the cow is composed of 3-5 fibrous annular folds and lined with mucosa containing mucous secreting cervical glands (Roberts, 1971). The wall of the uterus is composed of a serous membrane layer or mesometrium; a muscular layer or myometrium and a mucous membrane layer or endometrium. The mesometrium is the external layer and consists of loose connective tissue covered by peritoneal mesothelium. The myometrium is the intermediate layer which consists of a broad inner circular and an outer longitudinal layer of smooth muscle. The endometrium in ruminants comprises caruncular and intercaruncular areas. The oviduct has longitudinal and smooth muscle layers with a mucosal lining covered in ciliated epithelium and thrown into folds which provide a suitable environment for fertilisation.

The ovaries of the bovine consist of a thick peripheral zone or cortex and the medulla or zona vasculosa. Situated in the cortex, which is formed by connective tissue, are follicles and the corpus luteum. Follicles are present in a wide range of sizes representing various stages of development. There are two different types of follicles namely primordial and growing. Primordial follicles consist of a single layer of epithelial-like granulosa cells and an immature oocyte. Growing follicles can be subdivided into primary, characterised by multiple layers of cuboidal granulosa cells surrounding an immature oocyte enclosed in a zona pellucida; secondary, where the follicular antrum has started to form, and mature follicles. The mature follicle wall consists of the theca externa, theca interna and granulosa cells. The fluid filled antrum has formed and the oocyte is surrounded by the cumulus oophorus. The majority of follicles become atretic with only a tiny minority going on to ovulate. The corpus luteum is formed by large luteal cells derived from the granulosa and small luteal cells derived from the theca interna. Histology of the normal ovary is discussed in greater detail in Chapter 3, section 3.

1.2.3 Hormones of the Bovine Reproductive System

The hormones regulating the reproductive cycles of the bovine are mainly derived from the hypothalamus, the pituitary and the gonads. The principal hypothalamic hormone is the decapeptide, gonadotrophin-releasing hormone GnRH (Matsuo, Baba, Nair, Arimurs and Schally, 1971). Its function is to stimulate the release of both LH and FSH from the anterior pituitary in domestic animals. In recent studies it has been shown that GnRH is released in a pulsatile manner and that each pulse of GnRH coincides with release of a pulse of LH (Clarke and Cummins, 1982). The main gonadotrophins in the cow are the glycoproteins follicle stimulating hormone (FSH) and luteinising hormone (LH). FSH stimulates the growth and maturation of the follicles, binding mainly to receptors on the granulosa cells. It is synthesized by the basophilic cells of the anterior pituitary gland and has a half life of 300 minutes (Laster, 1972). LH is also synthesized by the basophil cells of the anterior pituitary, although its half-life is only 35 minutes. The LH stimulates maturation and ovulation of the Graafian follicle, and the formation and maintenance of the corpus luteum. It has been reported that LH is released in episodes or pulses throughout the oestrous cycle (Rahe, Owens, Fleeger, Newton and Harms, 1980).

The female gonad produces two chemically different groups of hormones specifically steroids and non-steroids. The main steroid hormones are oestrogens and progesterone. Oestradiol-17B is the major biologically-active oestrogen. It is synthesized jointly by the theca interna and the granulosa cells of the follicle (Hansel and Convey, 1983). One function of this hormone is to stimulate the LH and FSH preovulatory surge (positive feed back). The other major gonadal steroid is progesterone which is produced by the corpus luteum. Progesterone exerts a negative feedback effect on LH by reducing LH pulse frequency (Ireland and Roche, 1982). The non-steroidal hormones are oxytocin, inhibin and prostaglandins. Prostaglandins are produced by the endometrium and provoke luteolysis.

1.2.4 Regulation of the Oestrous Cycle

The oestrous cycle is divided into stages, namely proestrus, oestrus, metoestrus and dioestrus.

Proestrus is the 24-48 hour period prior to oestrus when the follicle which has been selected to ovulate grows rapidly. At the endocrinological level a decrease of progesterone follows the regression of the corpus luteum. This process of luteolysis is not well understood but it is suggested that prostaglandin is secreted by the endometrium and transfered to the ovary through the arterial-venous pathway (Ginder, 1974). As progesterone levels fall, LH pulses increase both in amplitude and frequency. Through each cycle 2 or 3 waves of follicular growth occur. The ovulatory follicle resulting from the last wave has more receptors on the granulosa cells for LH and greater capacity to secrete oestradiol (Ireland and Roche, 1982); high oestradiol levels lead to the gradual relaxation of the cervix, increased secretion of a clear, transparent mucus and more uterine tone (Hunter, 1980).

Oestrus is the period when the female is receptive to the male. Characteristic behavioural signs are readily recognised. Oestrus in the cow lasts about 18 hours with ovulation occurring 10 to 12 hours after the end of oestrus or 25 to 30 hours after the preovulatory surge of LH (Schams, Schallenberger, Hoffman and Karg, 1977). The ovulatory follicle rapidly increases its size to approximately 1.9-2 cm secreting more oestrogens which result in an increased frequency of GnRH release and an augmentation of the ability of the anterior pituitary gland to secrete LH and FSH (positive feedback) (Quirk, Hickey and Fortune, 1986). Therefore more LH is secreted leading to a surge in LH which in turn triggers ovulation. Follicular rupture is produced by the hormonal influence of LH, and local production of prostaglandins with an increase in intrafollicular pressure and friability of the wall of the mature follicle. On the wall of the ovulatory follicle an avascular spot develops called the "stigma" through which the rupture takes place. Ovulation occurs and the oocyte is discharged into the fimbria of the oviduct. The follicle collapses and formation of the corpus haemorrhagicum commences.

Metoestrus is the period immediately after oestrus when the corpus luteum is forming from the corpus haemorrhagicum. Dioestrus runs from the formation of the corpus luteum until its regression. During metoestrus the dominant feature is the formation of the corpus luteum from the theca and

granulosa cells of the former follicle under the influence of LH. The corpus luteum takes about 7 days to reach its mature size of 2-2.5 cm (Hunter, 1980). During metoestrus the production of progesterone is lower than 0.5 ng/ml, increasing thereafter to 6.6-10 ng/ml at mid dioestrus (Stabenfeldt, Ewing and Donald, 1969). In spite of the fact that progesterone has a negative feedback effect on the hypothalamic gonadotrophins two or three waves of follicular growth have been observed during the luteal phase. The pattern of LH secretion is high amplitude, low frequency (Walters, Schams and Schallenberger, 1984)).

During the metoestrous and dioestrous period the cervix is tightly closed to prevent infection from entering the reproductive tract. The muscular layer of the uterus loses its tone in absence of high levels of oestrogens. The corpus luteum functions till day 17-18 of the cycle when its regression starts with a steady decline in progesterone production: the cycle then repeats.

The gonadal hormones act on the nervous system to promote behavioural signs which are useful to visually identify proestrus and oestrus. During proestrus, under the influence of oestrogens secreted by the ovulatory follicle, the cow shows particular behaviour like sniffing, mounting other cows from the front and flehmen. In general, the animal is restless and active. A cow in oestrus shows the unique sign of standing to be mounted by other cows, which is a definite sign of oestrus. It is generally agreed that the animal should be standing for no less than 10 seconds to be considered in oestrus. Just before oestrus and immediately after, the animal will not stand to be mounted.

1.3 Embryo Transfer

The embryo transfer procedure includes the steps of selection of donor and recipients, superovulation of donor, synchronization of donor and recipients, artificial insemination and embryo flushing of donor, evaluation or grading of the embryos and transfer to the recipients.

1.3.1 Selection and Management of the Donor Cow

Selection and management of the donor cow and recipients has been reviewed extensively. Helpful review papers are Mapletoft (1975), Greve and Callesen (1989), and Coulthard (1991).

Selection Characteristics

The donor cow is selected primarily for outstanding production characteristics, for sound physiological characteristics and for market value of the progeny.

Age of the Donor

The optimum age for a donor is said to be after three to four calvings (Holm, Greve and Willeberg, 1987). Performance in other age groups is said to be poor, possibly due to low follicle numbers (Erickson, Reynolds and Murphy 1976).

Cyclicity of the Donor

It is generally felt that the donor should have displayed at least three normal oestrous cycles prior to superovulation. This ensures that the donor has displayed normal ovarian function and secondly that the donor is at least 90-120 days post-partum. Animals which display weak signs of oestrus are said to be poorer donors (Greve and Callesen, 1989).

Nutrition

It has been shown that overfeeding with concentrates reduces the number of viable embryos collected. It was recommended to control nutrition of the donor from breeding until embryo collection, and of the recipients from breeding until 40 days after breeding, avoiding either low or high levels of nutrition (Dunn, 1987).

The Effect of Infertility

Although cows with various kind of infertility have been used as donors the success rate is only one - third of that achieved using healthy donors without a history of infertility (Elsden, Nelson and Seidel, 1979; Johnson, 1986). Retained foetal membranes and cystic ovarian disease are specifically cited; cows with cystic ovarian disease were reported to give significantly fewer embryos than control animals (Greve and Callesen, 1989). Kweon and co-workers reported that febrile disease or anorexia was detrimental to the donor (Kweon, Kanagawa, Takahashi, Miyamoto, Masaki, Umezu, Kagabu, Iwazumi and Aoyagi, 1987). Fatty liver conditions are said to reduce the production of viable embryos (Kweon et al, 1987). Obviously disease problems such as endometritis and other infections or adhesions in portions of the tract will affect embryo recovery. Infertile heifers are considered particularly poor candidates for embryo recovery (Mapletoft, 1975).

Interherd Variation

Greve and Callesen (1989) report interherd variation in dairy herds. This variation is attributed to the various factors causing problems with the individual cow.

Clinical Examination of the Donor prior to Embryo Transfer

The reproductive soundness of a potential donor is naturally of paramount importance. A history should be taken and a thorough clinical examination performed. By stringently demanding a donor cow show three normal oestrous cycles prior to superovulation, Greve and Callesen (1989) delayed superovulation post-calving and claim to have recruited more normal donor animals into their embryo transfer program. Rectal and vaginal examination are necessary to eliminate pathological conditions. In addition to examining the ovaries upon rectal examination, the uterus and oviducts should be carefully palpated for abnormalities or adhesions. Coulthard (1991) recommends an oviduct patency test using the dye phenolsulphonphthalein for repeat breeders. On the day of commencement of superovulation a mature corpus luteum should be present. This can be confirmed by using milk or plasma progesterone assay or by ultrasound examination. Donor cows are then synchronised with recipients using one of the methods described in the section on synchronization of oestrus.

1.3.2 Superovulation

Superovulation can be defined as procedure by which the number of ova released in a given ovarian cycle is above the normal ovulation rate for that species. For cattle embryo transfer, superovulation is an economic necessity since it has been calculated that the unstimulated bovine produces 0.8 ± 0.4 calves per donor compared to eight times this yield after FSH or PMSG stimulation (Slenning and Wheeler, 1989).

Superovulation requires administration of a gonadotrophin preparation which is either rich in FSH or has FSH properties. The first superovulation was performed by Smith and Engle in 1927 using anterior pituitary extracts in mice (Smith and Engle, 1927). Cole and Hart demonstrated the ability of serum from pregnant mares to cause superovulation in immature rats (Cole and Hart, 1930). Since then, pituitary gonadotrophins from equine, porcine and bovine have become available for use in cattle.

1.3.3 Physiological Mechanism of Superovulation.

At birth a cow has approximately 1,000,000 follicles; this number is reduced to about 400,000 by puberty. Some of these will be recruited and grow and the rest will undergo atresia or degeneration (Fortune, Sirois, Turzillo and Lavoir, 1991). There are estimated to be some 200 follicles in the growing pool at any time (Erikson, 1966). The potential ovulatory follicles are then selected from the growing pool (Armstrong, 1991).

Studies carried out mainly in rats suggest that exogenous gonadotrophins increased the ovulation rate in two ways. The first is by lowering the size at which gonadotrophins can trigger follicular recruitment. The second is by reducing or preventing atresia in the population of follicles recruited by their administration (Monniaux, Chupin and Saumande, 1983).

Folliculogenesis in the cow develops in two or three waves. During each wave a dominant follicle emerges from the cohort of potential ovulatory follicles. In the last wave this is the follicle which goes on to ovulate while the others become atretic (Savio, Keenan, Boland and Roche, 1988; Sirois and Fortune, 1988; Knopf, Kastelic, Schallenberger and Ginther, 1989). There are contradictory opinions on whether the presence of a dominant follicle affects the superovulatory response. Grasso and co-workers suggested that the dominant follicle reduced the number of ovulations

(Grasso, Guilbault, Roy, Matton and Lussier, 1989). In contrast, Wilson and co-workers found no effect on the superovulatory response (Wilson, Jones and Miller, 1990).

1.3.4 Pharmacological Preparations used for Superovulation in Cattle

Among the different hormones used for superovulation the most commonly used are PMSG (also called equine chorionic gonadotrophin) and FSH preparations.

Pregnant Mare Serum Gonadotrophin (PMSG) or Equine Chorionic Gonadotrophin (eCG)

PMSG is a glycoprotein found in high concentrations in the blood of pregnant mares between 46-130 days of gestation (Cole and Hart, 1930) which exhibits both FSH and LH-like activity (Papkoff, 1978). PMSG is lyophilized to allow storage and its potency is expressed in International Units (IU). It is composed of an Á and a ß subunit, the latter being responsible for both FSH and LH activity. The half life of PMSG is longer than either FSH or LH alone, (40 to 123 hours compared with 2-3 hours), because of its high sialic acid content (Schams, Menzer, Schallenberger, Hoffman, Hahn and Hahn, 1978). This allows one single injection per cow to be effective in inducing superovulation.

There is said to be variability in both FSH and LH activity of PMSG with experiments suggesting that PMSG preparations with high FSH activity relative to LH produced better superovulatory response than those with a low FSH/LH ratio (Humphrey, Murphy, Reiger, Mapletoft, Manns and Fretz, 1979; Murphy, Mapletoft, Manns and Humphrey, 1984). Studies of the mechanism of action of PMSG suggest that this is prevention or reversal of the process of atresia in follicles around 1.7 mm in size thus increasing the number of follicles capable of responding to its action (Monniaux et al., 1983). PMSG is generally administered as a single intramuscular injection of 2000-3000 i.u. (Hunter, 1980) Large doses of PMSG (5000-7500 i.u) have been clearly shown to inhibit ovulation in comparison to a dose of 2500 i.u. (Saumande and Chupin, 1986).

Workers have found FSH a more productive method of superovulation than PMSG (Elsden, Nelson and Seidel, 1978). It has been suggested firstly, that the LH component of PMSG causes preovulatory luteinisation of large follicles and that secondly, by stimulating a second wave

of follicles after ovulation PMSG creates a highly oestrogenic environment which is detrimental to oocyte maturation and embryonic development. Improved embryo yield has been effected by injecting an anti-PMSG monoclonal antibody (Neutra-PMSG) shortly after the LH peak to neutralise any further effects of PMSG (Dieleman, Bevers, Wurth, Gielen and Willemse, 1989). On the same principal antisera to PMSG raised in other animals has also been used to inactivate PMSG.

Follicle Stimulating Hormone (FSH)

Generally FSH preparations are made from purified pituitary extracts (Beckers, 1987) (eg. porcine FSH, FSH-P) or biosynthetically by recombinant DNA technology (Looney, Bondioli, Hill and Massey, 1988). Chemically it is a glycoprotein, with lower sialic acid content than PMSG. FSH has to be injected more frequently, because its half life is 110 minutes approximately (Humphrey et al., 1979). Commercially available FSH has been shown to vary between batches in contents of both LH and FSH activity. Studies carried out by Murphy and co-workers suggest that the best ovulatory response is obtained when the FSH-LH ratio is maintained at 5:1 throughout the treatment (Murphy et al., 1984). Therefore products with low LH activity have been developed.

Human Menopausal Gonadotrophin (hMG)

Human menopausal gonadotrophin (hMG) which is rich in gonadotrophins was discovered in 1964 in the urine of post-menopausal women (Donini, Puzzuoli and Montezeniold, 1964). It induces a similar response to that of FSH-P (McGowan, Braithewaite, Jochle and Mapletoft, 1985) though it is not widely used for veterinary applications.

1.3.5 Factors Affecting Superovulation

Superovulation is affected by factors related to the animal variability and to the hormone variability.

Animal Variability

The most important aspect of the animal variability is the state of follicular development at the time of superovulation. Moor and co-workers demonstrated that between days 8-10 of the cycle the bovine ovary contains the largest number of medium sized follicles which in turn are the class of follicles that respond most favourably to exogenous gonadotrophins (Moor, Kruip and Green, 1984). Other factors that contribute to the animal variability are the genetic make-up of the animal, the presence of a dominant follicle at the time of superovulation and finally, repeated superovulatory treatments in the same animal.

Hormone Variability

Hormone variability may be due to inaccuracy in estimation of the drug potency of commercially available preparations, differences in biological half-life and differences in the FSH:LH activity ratio.

There are real differences in potency of commercially available gonadotrophins. It is possible to estimate the potency of a hormone preparation using either bioassays and immunoassays. Immunoassays tend to be more reproducible and highly sensitive but a potency estimate based on immunoassay does not necessarily reflect biological activity. Bioassay in animals is less sensitive and costly.

In addition to real differences, potencies of different drugs are expressed in different units. The potency of PMSG is expressed in international units and there is no universally accepted standard for expression of potencies of pituitary FSH preparations.

As was discussed earlier, hormone preparations differ in their biological half-life. PMSG has a longer biological half-life than FSH. This is reflected in the need to give a single dose of PMSG but usually twice daily injections of FSH over 3-4 days.

1.3.6 Oestrus Detection, Synchronization and Artificial Insemination

Oestrus detection

The accurate detection of oestrus is of paramount importance in embryo transfer because of the need for close synchrony between the stage of development of the embryo and the uterine environment in the recipient (Broadbent and Hutchinson, 1988). The best method of oestrus detection is the visual examination of cattle loose in a yard for at least 30 minutes, three times a day. Aids to oestrus detection include chin ball markers, heat mount detectors, tail paint, vaginal electrical resistivity, and the use of teaser animals.

The Synchronization of Oestrus

The synchrony of donor and recipients must be close for embryo survival. Good results have been obtained with one day of difference between the stage of the cycle of donor and recipient (Sreenan, 1983). A large scale embryo transfer program found that pregnancy rates were not compromised when recipients were in oestrus as much as 36 hours before the donor (Hasler, McCauley, Lathrop and Foote, 1987).

There have been two approaches to the synchronization of oestrus; namely, artificially lengthening dioestrus using exogenous progestagens or shortening dioestrus with a luteolysin (Peters and Ball, 1987). The first approach uses either a subcutaneous implant (eg. Synchromate-B; Intervet) or progesterone releasing intravaginal coil (PRID; Ceva). The progesterone is absorbed by the animal mimicking the presence of a corpus luteum. With both types of treatment lysis of the natural corpus luteum is achieved using the luteolytic effect of oestradiol-17B either by injection or transvaginal absorption. On removal of the device, progesterone negative feedback on the hypothalamus is removed and the animal should come into oestrus in 2-4 days. Conception rates are generally lower in treated than in untreated animals.

The second approach is the use of prostaglandin as a luteolytic drug; artificial analogues are available. A corpus luteum is only responsive to PGF2a from about day 5 to day 15 of the cycle (Graves, Short, Randel, Bellows, Kattenbach and Dunn, 1974). To synchronise groups of animals at different stages of the oestrous cycle prostaglandins are injected twice 11 days apart.

It is also possible to combine these two methods using a regime such as progesterone or progestagen for 7-10 days and injection of PGF2a 24 hours prior to the removal of the progesterone source.

Artificial Insemination

Two inseminations carried out at 12 and 24 hours after first observed oestrus are recommended. However, a study did not find any significant difference in the percentage of oocytes fertilised and number of embryos with single, double or triple insemination regimes (Donaldson, 1985).

1.3.7 Embryo Recovery

Embryo recovery was initially carried out surgically. The various surgical procedures are described by Hafez (Hafez, 1987). Later, non-surgical methods were developed and are now widely adopted (Newcomb, Christie and Rowson, 1978). Non-surgical flushing was less damaging to the reproductive tract allowing repeated flushings of an individual donor, and is easily carried out on the farm.

Different types of catheters were developed for non-surgical flushing. Early workers used the two or three-way Foley catheter. Subsequently a two-way Rush catheter was preferred by German workers because it was longer (67cm) and stiffened for passage through the cervix (Schneider and Hahn, 1979). British workers described a three-way Franklin catheter, which required a speculum and introducer set. For best results this catheter is positioned within 5 cm of the tip of the horn (Newcomb et al., 1978). French researchers have promoted a three-way catheter with an extensible head and a length of 50 to 55 cm. Embryos are flushed by a continuous or interrupted flow closed circuit system or using the interrupted flow syringe technique. These techniques are clearly described in textbooks (Hafez, 1987; Arthur et al., 1989).

The embryo flushing technique used in this study is described in detail in Materials and Methods, Chapter 2.

1.3.8 Embryo Morphology and Evaluation

The diameter of the bovine embryo including the zona pellucida is estimated to be 150 to 190 um (Linder and Wright, 1983).

Generally, embryos recovered by flushing on day 7 vary in developmental stage from a morula to a hatched blastocyst. A morula has an estimated age of 5 days. The embryo has over 16 cells and resembles a mulberry. The cellular mass occupies most of the perivitelline space. When individual blastomeres have lost their sharp outline forming a compact mass of about 32 cells, the embryo is described as a compact morula. The embryo mass occupies 60 to 70 % of the perivitelline space and is approximately 6 days old. The early blastocyst has formed a fluid-filled cavity or blastocoele The embryo occupies 70 to 80 % of the perivitelline space and has about 100 cells. Visual differentiation between trophoblast and the inner cell mass may be possible at this stage. The estimated age is 7 days. In the blastocyst, pronounced differentiation of the outer trophoblast layer and the darker, more compact inner cell mass is evident. The blastocoele is highly prominent with the embryo occupying most of the perivitelline space. Again the estimated age is 7 days. As the embryo becomes an expanded blastocyst the overall diameter of the embryo increases, with concurrent thinning of the zona pellucida. Finally the embryo sheds the zona pellucida to become a hatched blastocyst. A hatched blastocyst may be spherical with a well defined blastocoele or collapsed. The estimated age is 9 to 10 days.

Visual evaluation of embryos under a microscope is based on the shape, distribution of cells, colour, and stage of development. Embryos are graded : -

Excellent: an ideal embryo, spherical, symmetrical with cells of uniform size, colour and texture.

Good: trivial imperfections such as a few extruded blastomeres, irregular shape, few vesicles.

Fair: definite but not severe problems, presence of extruded blastomeres, vesiculation, few degenerated cells.

Poor: severe problems, numerous extruded blastomeres, degenerated cells, cells of varying size, large numerous vesicles but a viable looking embryo mass.

1.4 Ultrasound

Ultrasound is defined as sound waves of frequency greater than those audible to the human ear. Diagnostic ultrasound usually employs sound-waves of frequency between 1 and 10 Mhz.

1.4.1 Introduction

From the 1970s real-time or dynamic imaging has developed, allowing study of the internal reproductive organs in large domestic animals via the transrectal route (Pierson, Kastelic and Ginther, 1988). In the 1980s several reports described the use of ultrasound to examine the normal bovine uterus in the non-pregnant and pregnant animal (Pierson and Ginther, 1984; Pierson and Ginther, 1984; Reeves, Rantanen and Hauser, 1984). Its application for the study of normal and pathological ovarian structures was described in 1986 (Edmondson, Fissore, Pashen and Bondurant, 1986). Recently ultrasonography has been used to monitor follicular dynamics during the oestrous cycle in cattle (Quirk et al, 1986). The most innovative application of ultrasound is the transvaginal ultrasound-guided follicular aspiration of bovine oocytes for *in vitro* fertilization developed by Pieterse and coworkers (Pieterse, Vos, Kruip, Wurth, Van Beneden, Willemse and Taverne, 1991).

Despite the widespread use of ultrasonography, its application in the embryo transfer industry remains low, taking into consideration its capability for ovarian evaluation and examination of the reproductive tract in both donor and recipients.

1.4.2 Production of the Image

Several reports have described the principles of ultrasound (Park, Nyland, Lattimer, Miller and Lebel, 1981; Pierson et al., 1988; Barr, 1990). The crystals of the ultrasound transducer have piezo electric properties. When electrically stimulated they become deformed and consequently release sound waves of characteristic frequency. When the transducer is placed in contact with the rectal wall, the sound waves travel through the tissues. Interfaces between tissues of different densities or 'acoustic impedance' reflect part or all of the beam back towards the transducer. The returning echoes are received by the same crystals and converted by means of

the piezo electric effect into electrical signals, which are analysed according to the strength and depth of reflection, and displayed on an oscilloscope screen. The characteristics of a tissue determine what proportion of the sound beam will be reflected. The reflected portion is represented on the ultrasound image by shades of grey, extending from black to white. Liquids do not reflect sound waves (nonechogenic or anechoic); therefore the image of a liquid-containing structure appears black on the screen. Other tissues are seen in various shades of grey depending upon their echogenecity or ability to reflect sound wave (Pierson et al., 1988).

1.4.2 Equipment

Resolution of the equipment depends on the frequency of the sound waves. High frequency equipment generating high number of vibrations of the acoustic source per second provides greater detail, whereas lower frequency provides greater tissue penetration. The ultrasound equipment generally used for cattle is B-mode, real-time. A B-mode ultrasound image is two dimensional and real time refers to the live or moving display (Pierson et al., 1988).

The Ultrasound Scanner

The image display on a scanner is one of three types: amplitude mode (A-mode), brightness mode (B-mode) or motion mode (M-mode).

Amplitude mode is the simplest form of image display which gives peaks along a horizontal line view. The height of each peak denotes the strength of the echo, whilst the horizontal axis illustrates the depth of the reflecting structure.

In the **Brightness mode** multiple lines or beams are utilised and represented on the screen as dots. The strength of the returning echo is expressed by the brightness of the dot. Consequently a two-dimensional image representing a slice through the body is built up and displayed on the screen. With real time scanning, the image produced is continuously updated; the image moves as the structure moves. This mode is commonly used in Veterinary Medicine.

Motion Mode is an adaptation of real-time scanning. A single ultrasound beam is used, and the returning echoes are displayed as a series of dots along a vertical line. The position of the dot along that line represents

the depth of the reflecting structure, and the brightness of the dot refer to the strength of the echo. This line is continuously updated as the screen scrolls horizontally.

The Transducer

Transducers with high frequency or high number of vibrations of the acoustic source per second provide greater detail, whereas lower frequency transducer provides greater tissue penetration (Pierson et al., 1988). There are two main types of transducer, namely linear and sector depending on the arrangement of the piezo electric crystals.

Linear Array Transducers are usually made of between 60 and 256 crystals arranged in line and produce a rectangular-shaped field of view (Barr, 1990). They have the advantage of facilitating a large field of view, even close to the scanning surface which in turn helps the recognition of structures and the anatomical relationship between them (Barr, 1990). This type of transducer is preferred for ultrasound examination of the ovaries in physiological and pathological conditions in cattle (Pierson and Ginther, 1984).

Sector Transducers have a small number of crystals which are mechanically or electronically driven to swing out in a fan shaped beam. The advantage of a sector transducer is that it allows more structures to be seen but on the other hand it yields poorer resolution than a linear array transducer. In the veterinary field its main application is for small animal diagnosis (Barr, 1990).

1.4.3 The Application of Ultrasound Scanning

Ultrasonography has been used widely in human medicine in a range of fields for a number of years. In the human, ultrasound is extensively used in obstetrical medicine. In veterinary medicine ultrasound has been rapidly adopted for small animal use (Bondestam, Karkkainen, Alitalo and Forss, 1984; England and Allen, 1989; Fluckiger, 1990), non domestic animals such as primates (Nyland, Hill, Hendrickx, Farver, McGahan, Henrickson, Anderson and Phillips, 1984), dolphins (Williamson, Gales and Lister, 1990) and ferrets (Peter, Bell, Manning and Bosu, 1990)). There are numerous reports dealing with farm animal applications, outlining the use of ultrasound to study the back fat deposition and pork carcass composition in the pig (Terry, Javell, Recio and Cross, 1989); pregnancy diagnosis and prediction of calving date in red deer (Wilson and Bingham, 1990); pregnancy diagnosis and determination of foetal number in sheep (Watt, Anderson and Campbell, 1984) and ovarian study in llamas (Adams, Sumar and Ginther, 1991).

Veterinary transrectal ultrasound was first reported in 1980 with visualization of the equine conceptus from day 14 of pregnancy (Palmer and Driancourt, 1980). Later studies gave a detailed description of ultrasonography of reproductive events in the mare (Ginther, 1986). Other work in the mare included further study of early pregnancy detection (Allen and Goddard, 1984).

The use of ultrasound for the study of physiological and pathological conditions of the uterus in cattle was outlined by Fissore and co-workers, who reported pregnancy diagnosis as early as 22 days of gestation; and a definite recognition of the conceptus by day 27 of gestation (Fissore, Edmondson, Pashen and Bondurant, 1986). A later report found the diagnosis of pregnancy possible as early as 13 days using a high frequency transducer (Boyd, Omran and Ayliffe, 1988). Similarly Pierson and Ginther identified embryonic vesicles as non-echogenic areas as early as 12 days (Pierson and Ginther, 1984). Pieterse and coworkers outlined the sensitivity of ultrasound scanning between days 21 to 25 of pregnancy as only 44.8% (Pieterse, Szenci, Willemse, Bajcsy, Dieleman and Taverne, 1990). The applications of ultrasound for early pregnancy diagnosis in recipient cows after embryo transfer has been reported (Pierson and Ginther, 1984; Kastelic, Curran, Pierson and Ginther, 1988; Jones, Marek, Wilson and Looney, 1990).

Folliculogenesis in cattle has been monitored using ultrasound by Sirois and Fortune (1988) who found that follicles as small as 2 mm could be

identified. Knopf and co-workers detailed follicular growth in two, three or four waves during the oestrous cycle in heifers (Knopf et al., 1989). Ultrasonography was used to assess ovarian structures; Pierson and Ginther suggested that ultrasonography was a highly accurate estimator of the diameter of the largest follicle per ovary (Pierson and Ginther, 1987). Additionally they found that the corpus luteum gave a different echogenic pattern than that of the surrounding tissue. Kastelic and colleagues indicated that ultrasonic assessment was a viable alternative to plasma progesterone levels for the assessment of luteal function of nulliparous heifers (Kastelic, Bergfelt and Ginther, 1990). In a later report the same authors implied that the central luteal cavities detected by ultrasound did not affect pregnancy rate or the length of the interovulatory interval (Kastelic, Pierson and Ginther, 1990). Pieterse and colleagues compared the accuracy of vaginal ultrasound and manual rectal palpation with the post mortem findings when used to detect follicles and corpora lutea. They found poor accuracy for both techniques when used to detect young corpora lutea. In contrast, mid-cycle corpora lutea were well detected by both techniques. Rectal palpation was slightly better for detecting old corpora lutea due to the similar density of old corpora lutea and the ovarian stroma (Pieterse, Taverne, Kruip and Willemse, 1990).

Normal ovarian structures such as follicles, corpus luteum, corpus albicans and pathological structures such as follicular and luteal cysts were detected using ultrasound (Edmondson et al., 1986; Carrol, Pierson, Hauser, Grummer and Combs, 1990). Follicular cysts were described as possessing non-echogenic areas of 25-55 mm diameter with thin walls; luteal cysts were described as non-echogenic areas surrounded by echogenic tissue of varying thickness (2-5mm).

Monitoring of the superovulatory response by ultrasonography was first reported in 1984 (Pierson and Ginther, 1984). They estimated the number of ovulations from the number of corpora lutea seen by ultrasound. The same authors in a later paper gave descriptions of ovarian structures in superovulated cows as well as in the normal oestrous cycle (Pierson and Ginther, 1988). Grasso and colleagues stated the importance of ultrasound examination prior to superovulation to control the presence of large follicles which presumably lowered the superovulatory response (Grasso, Guibault, Roy and Lussier, 1989). Two other reports have been published monitoring follicular dynamics in response to superovulation (Schallenberger, Knopf, Veh, Tenhumberg and Aumuller, 1988; Driancourt, Thatcher, Terqui and

Andrieu, 1991). Driancourt and colleagues concluded that ultrasonography was a valuable technique when used to monitor follicular dynamics. The latest application of ultrasonography to reproductive technology is transvaginal ultrasound-guided follicular aspiration (Pieterse et al., 1991) .

Chapter 2

2.1 Experimental Animals
2.2 Experimental Design
2.3 Methods
2.3.1 Oestrus Induction
2.3.2 Oestrus Detection
2.3.3 Superovulation
2.3.4 Artificial Insemination
2.3.5 Collection of Blood Samples
2.3.6 Hormone Radioimmunoassay
2.3.7 Ultrasonography
2.3.8 Embryo Collection
2.3.9 Histological Methods
2.4 Figures

Materials and Methods

2.1 Experimental Animals

Non-pregnant cycling females were chosen for the study. They were divided in three groups according to the availability. Group 1 consisted of two non-lactating Friesian cows and two heifers. Group 2 consisted of three, and group 3 consisted of four, non-lactating Friesian cows. Animals were housed in a byre and fed hay and concentrates.

2.2 Experimental Design

The animals were monitored through two oestrous cycles. The first was called the control cycle and the second called the superovulated cycle. In both cycles oestrus was induced by injection of prostaglandin. Superovulation was induced by injection of 3000 or 1500 i.u. of PMSG. Cows were inseminated during the superovulated cycle 12 and 24 hours after onset of standing oestrus. Ovarian changes were monitored by ultrasonography. Cows in each group were scanned by ultrasound daily, then twice daily around oestrus in both the control and the superovulated cycles and daily thereafter until embryo flushing. Animals in group 1 were monitored until return to oestrus after embryo collection. Animals in groups 2 and 3 were slaughtered after embryo collection. Progesterone levels were determined from blood samples taken at predetermined time points throughout the experiment. Experimental design is shown in Figure 2.1.

2.3 Methods

2.3.1 Oestrus Induction

Oestrus was induced in the control and in the superovulated cycle by the intramuscular injection of 2ml of the synthetic prostaglandin, cloprostenol (Estrumate, Coopers Animal Health Ltd,). The first injection was given in the mid luteal phase of the control cycle; the second injection was given on day 11-13 of the cycle two days after PMSG injection.

2.3.2 Superovulation

Superovulation was achieved by the intramuscular injection of Pregnant Mare Serum Gonadotrophin (PMSG) (Folligon, Intervet Ltd, Cambridge) in the mid luteal phase (days 9-11). Cows in groups 1 and 2 received 3000 i.u. and cows in group 3 received 1500 i.u..

2.3.3 Oestrus Detection

Oestrus detection was carried out by observation of the animals loose in a yard three times a day for twenty minutes at approximately 8.00 a.m., 4.00 p.m. and 11.00 p.m.. Oestrous behaviour was identified as standing to be mounted for more than 30 seconds. The duration of standing oestrus was also recorded.

2.3.4 Artificial Insemination

Cows were inseminated twice at 12h and 24h after the onset of oestrus during the superovulated cycle using frozen semen from two Friesian bulls. Straws of 0.25 ml were removed from liquid nitrogen and thawed in water at 37ºC for 1 minute prior to insemination. The time of insemination was recorded in addition to name of bull, breed and freezing batch. Periodic assessment of semen motility was carried out.

2.3.5 Collection of Blood Samples

In order to make frequent sampling possible the jugular vein of each animal was cannulated at the time of prostaglandin injection both in the control and in the superovulated cycle. Samples were taken every 6 hours for 24 hours, then at hourly intervals until 42 hours after prostaglandin injection, then back to 6 hourly samples until postovulation. Thereafter blood samples were taken once daily. This sampling frequency was designed to monitor the preovulatory LH surge. Blood samples were collected in heparinized blood collection tubes. After collection samples were centrifuged in order to separate the plasma which was transfered into 7 ml plastic tubes and stored frozen at -20 ^{O}C until hormone assay was carried out. In this study, a single daily sample was assayed for plasma progesterone.

2.3.6 Hormone Radioimmunoassay

Plasma progesterone concentrations were determined using a double antibody radioimmunoassay (RIA) developed by Dr I. A. Jeffcoate. All reagents were diluted using 0.05M phosphate buffered saline with 0.25% bovine serum albumin (BSA). Samples and standards were thawed at room temperature. The first antibody (provided by Dr B. Cook, The Royal Infirmary, Glasgow, UK.) was raised in sheep against 11a-hydroprogesterone hemisuccinate BSA and used at 1:20,000 dilution to give approximatedly 30 to 35% binding of labelled progesterone. The cross reaction of the antibody with 11a-hydroxyprogesterone, 11-deoxycorticosterone, 17a-hydroxyprogesterone and 20a-hydroxypregn-4-en-3-one were 61, 4, 1.5 and 1% respectively and < 1% with other steroids. 200 ul of sample or standard (Sigma, 0.3-2 ng/ml) was extracted in duplicate with 3 ml diethyl ether (Analar grade, May & Baker, Dagenham, Essex, UK) by vortex mixing for 5 minutes on a multi-tube vortexer. The ether phase was decanted into glass tubes, after prior freezing the aqueous phase in a methanol and dry ice bath, and subsequently evaporated under air. The iodinated progesterone was prepared by iodination of progesterone 11a-glucuronyl tyramine and purified by solvent extraction and TLC (Corrie, Hunter and MacPherson, 1975). Tracer (100 ul approximately 10,000 cpm) and primary antibody (200 ul, dilution 1:20,000) were added to the dried ether extract tubes which were incubated at 37 ^{o}C for 45 minutes. After that 400 ul of second antibody containing 1:20 donkey antisheep/goat serum plus 1:200 normal goat serum (SAPU, Law Hospital, Carluke, Strathclyde, UK) was added to the tubes and were incubated at 4^{o}C overnight.

The next day the tubes were centrifugated at 2000g for 10 minutes, then the supernatant was aspirated and the precipitate containing the antibody-bound fraction was counted in a Packard Auto-Gamma Minaxi 5000 series counter. The assay sensitivity was 0.2 $\pm$ 0.1 ng/ml. The intra-assay coefficient for variation for two pools containing high and low progesterone was 10% and 6%. The inter-assay coefficient of variation was 11 % and 7%.

2.3.7 Ultrasonography

Two B-mode real-time two dimensional ultrasound scanners were used in this study (Concept I and Concept II, Dynamic Imaging, Livingston, Scotland). The scanners possessed a freeze mode, a magnification or zoom mode and an image storage memory with recall. Additionally the scanners were provided with a press control keyboard with annotation facilities permitting images to be permanently identified on videotapes and photographs. A linear rectal transducer of 7.5 Mhz frequency with axial and lateral resolution of 0.5 and 1 mm was used in this study.

Ultrasound scans were routinely recorded on videotape using a videorecorder with play-back facilities. Taped ultrasound scans were reviewed on a high quality viewer with a slow motion and freeze frame facility to allow the reassessment of all information.

Ultrasonic measurement

Structures were measured by using a light pen to measure images on the scanner screen. Alternatively videoprints were made of important structures and the structures measured using a specially scaled ruler. Follicles were measured at the widest diameter horizontally then vertically and the mean value for two measurements used to express the size of the structure.

Interpretation of Images

Fluid does not reflect sound waves and appears black on the screen. Such an image would be given by follicular fluid and is termed non-echogenic (Figure 2.2.a). Dense tissue such as bone reflects sound waves. The image appears white on the screen and is termed hyper-echogenic. Soft tissues reflect sound waves to different degrees and produce a grey echogenic image. An example of this would be a corpus luteum (Figure 2.2.e and f).

Artifacts

Artifacts are defined as any dot showing in the ultrasound image which does not conform to a real echo in the patient. The most common artifacts are as follows:

Specular Reflection: generally seen when a portion of the beam strikes a smooth reflective surface wider than the beam and parallel to the transducer; the upper and lower surface of a fluid filled structure such as a cyst creates a highly echogenic reflection on the ultrasound image (Figure 2.3a).

Non-specular Reflection: is produced when the ultrasound pulses strike a rough surface that is narrower than the beam. The echoes are scattered in many directions. The transducer received many scattered sounds simultaneously which are display as a net of bright echoes. Figure 2.3b shows non-specular reflection caused by the sound waves hitting luteal cells in a corpus luteum.

Reverberation: Reverberation artifacts are generally encountered in transrectal ultrasound scanning owing to gas or air filled segments of the digestive tract. These artifacts are produced when the sound pulse is bounced back and forth between two highly reflective interfaces like the bowel and the transducer. They appear as bright echoes because of the delay of each returning as perceived by the transducer (Figure 2.3.c).

Enhanced Through Transmission: this artifact is often encountered underneath fluid filled structures like follicles or cysts. It is produced because the sound waves passing through the fluid are not attenuated like the waves passing through the adjacent tissue. A bright echo appears beneath the fluid filled structure.(Figure 2.3.d).

Refraction: This artifact is usually found in fluid filled structures like cysts. It is produced when a portion of the sound beam strikes on a curved boundary area of the structure at less than 90^{o}. Therefore a refraction or reflection is formed causing shadowing or lack of echo beyond the place of refraction.

Shadowing: shadowing is characterised by lack of echo beneath a very dense structure and caused by the absorption of the ultrasound waves. It may be seen when the sound waves are obstructed by bowel gas, lack of close contact between the transducer and the rectal wall, or in the presence of faecal material on the transducer. This artifact is shown in Figure.2.3.e.

Beam-Width Artifact: this is produced when portions of the sound sample hit two interfaces of different reflective characteristics (Figure 2.3.f).

Electronic Interferences: This can be reduced by having the machine properly tuned and adjusted. It appears as a double image with static lines.

In addition to artifacts encountered during rectal examination of the bovine reproductive tract, for the purpose of this study it was necessary to identify certain structures which could be confused with the structures on the ovaries which were actually under study. Figure 2.2.b and c. shows the

uterine horn lying close to the ovary under examination. The horn, particularly during oestrus when there is a fluid filled lumen, could be confused with the ovary. Figure 2.2 d. shows both the circular and longitudinal appearance of blood vessels. Blood vessels can be identified by turning the transducer such that the circular appearance becomes a longitudinal 'sausage ' shape. Figures 2.2.e. and f. highlight the need to make a thorough inspection of the ovary. A single scan gives a single cross sectional picture of the ovary which can obviously miss structures.

2.3.8 Embryo Collection

The cow was prepared for embryo flushing by sedation using 0.02 mg/kg of acetylpromazine maleate (ACP, 10 mg/ml) and contained in a crush elevated 30 cm at the front. To produce smooth muscle relaxation the cow was injected with either 10 ml of Clenbuterol (Planipart, Boehringer Ingelhein Ltd, Berkshire) or 20 ml Buscopan (Boehringer Ingelhein Ltd, Berkshire). The second treatment was used where the animal was scheduled for slaughter on the day after flushing. Finally an epidural anaesthesia was administered by the injection of 7 ml of 2.0% Lignocaine Hydrochloride (Lignol, Arnolds Veterinary Products, Romford).

After the sedative and uterine relaxant were administered the rectum was emptied of faeces prior to the epidural injection. The tail was tied out of the way and the area washed. An estimation of the number of corpora lutea was carried out by manual palpation. A lubricated speculum was then inserted into the vagina. A lubricated introducer (14g) was then passed via the speculum through the cervix and guided per rectum into the uterine horn as far as can easily be achieved without causing trauma. The central core was withdrawn and sterile PVC three lumen Franklin catheter (TFX Medical, High Wycombe) was passed through it by an assistant. The work was more easily carried out by previously marking the catheter at the correct position; the marks showed exactly when the cuff lay in front of the intercornual ligaments. The catheter was prefilled with medium in order to prevent air entering uterus and lubricated with silicon spray. When the catheter was correctly placed the cuff was inflated with 7 to 10 ml of saline (0.9% normal saline). The uterus was flushed with phosphate-buffered saline containing 1% foetal calf serum, penicillin (100 i.u./ml) and streptomycin (100 mg/ml). Six to eight syringes of medium (300 to 350 ml) were flushed through each horn. The medium flushed through the catheter was collected by an assistant

in a warmed glass bottle. When the flushing was finished the cuff was deflated and the procedure repeated with the other horn using the same or another catheter.

Once flushing was accomplished 5mg/kg of Ampicillin (Penbritin, 500 mg per vial, Beecham Animal Health, Middlessex) was infused into the horns using a short PVC catheter. This step was omitted then the animal was scheduled for slaughter on the following day.

After flushing, the medium was left to settle for 30 minutes in a incubator at 39ºC, 5% CO_2 in air. The top two-thirds were discarded and the remaining portion examined under a stereo microscope. Embryos were identified and graded as described in Chapter 1.

2.3.9 Histological Methods

The post-fixation histological preparation was carried out by the Surgery Department histologists. Fixation of specimens was carried out using Bouin's fluid as described by Culling (1974). Specimens were fixed for 24 hours. The Martius scarlet blue stain was used following the method of Lendrum (Lendrum, Fraser, Sliddess and Henderson, 1962).

Figure 2.1 Experimental Design

Animals were monitored through a control cycle and a superovulated cycle. In both cycles oestrus was induced by injection of prostaglandin (PG). Superovulation was induced by injection of 3000 (groups 1 and 2) or 1500 (group 3) i.u. of PMSG. Cows were inseminated (AI) during the superovulated cycle 12 and 24 hours after onset of standing oestrus (Oe). Ovarian changes were monitored by ultrasonography. Cows in each group were scanned by ultrasound daily, then twice daily around oestrus in both the control and the superovulated cycles and daily thereafter until embryo flushing. Animals in group 1 were monitored until return to oestrus after embryo collection. Animals in groups 2 and 3 were slaughtered after embryo collection.

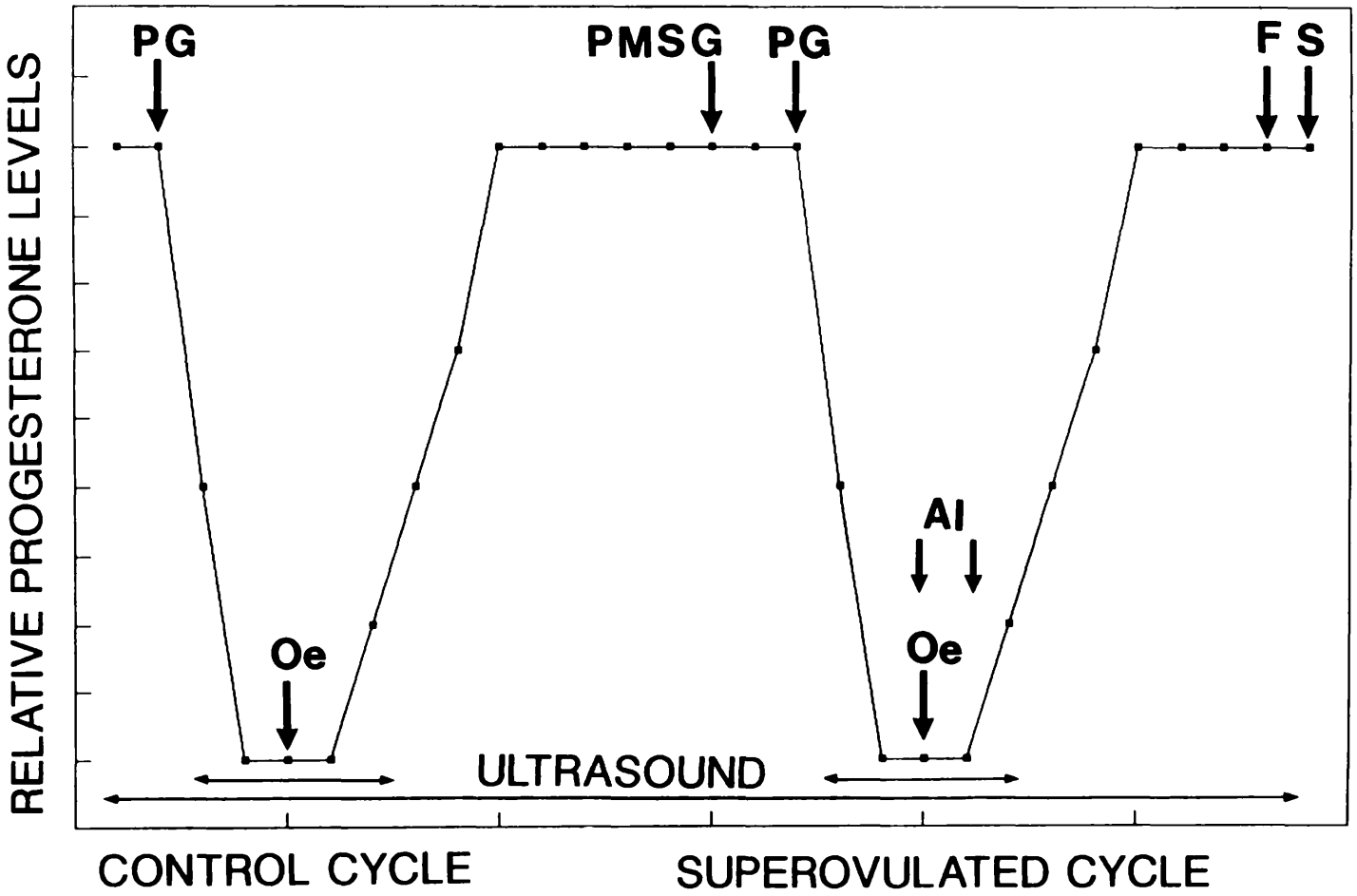

PG
PMSG
PG
F S
RELATIVE PROGESTERONE LEVELS
AI
Oe
Oe
ULTRASOUND
CONTROL CYCLE
SUPEROVULATED CYCLE

FIGURE 2.1

Figure 2.2

a) Intepretation of the ultrasound image. A non-echogenic area representing a typical follicle is marked A. A small poorly outlined follicle is marked B. The outline of the ovary is arrowed and the echogenic ovarian stroma is marked C.

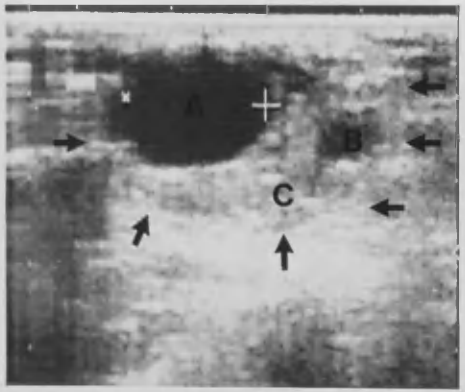
b) A cross section of the midcycle uterine horn (H) lying below the ovary is arrowed.

c) The uterine horn (H) lying next to an ovary containing follicles (F) during oestrus. Note the central fluid 'star' of the oestrous horn

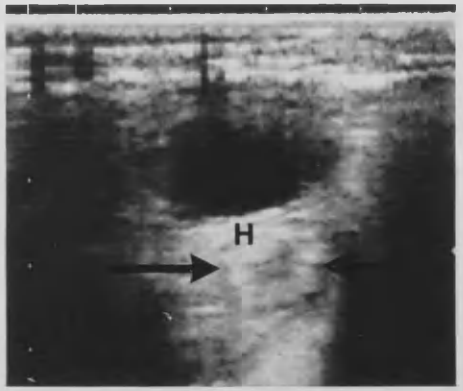
d) Blood vessels. Note circular and longitudinal aspects.

e) Twin corpora lutea on an ovary. Note echogenicity.

f) Same ovary examined at a different angle. Change of orientation presents a different picture with only one corpus luteum visible (A) plus a follicle.



a



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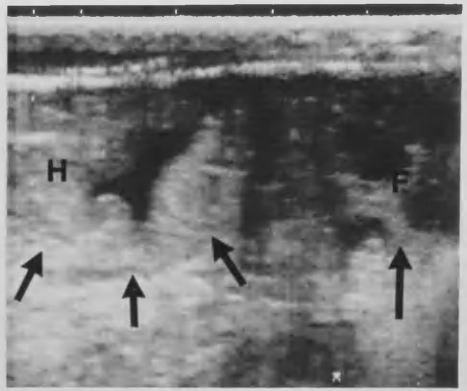
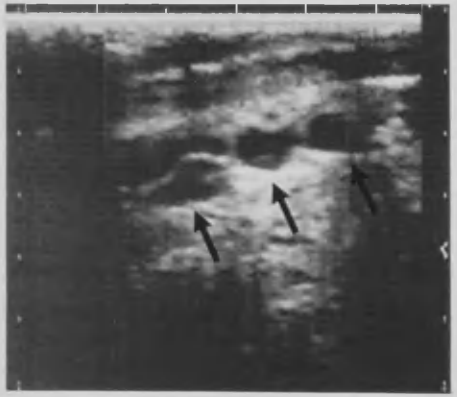


c



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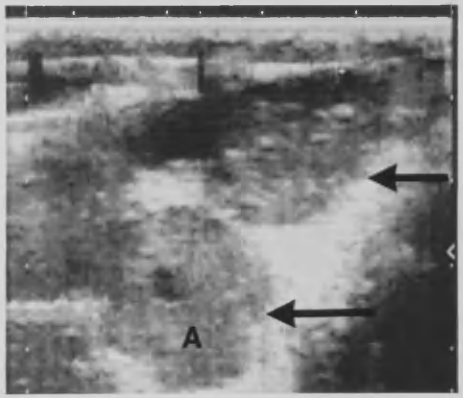
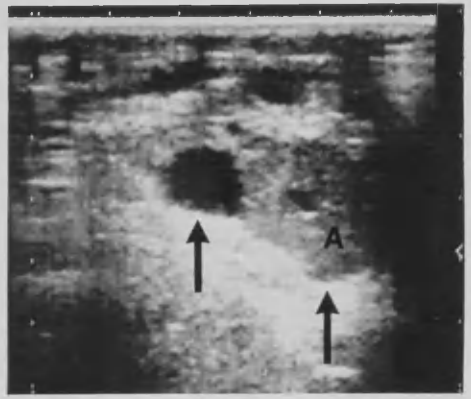


e



f

Figure 2.3

a) Specular reflection marked on a follicular cyst.

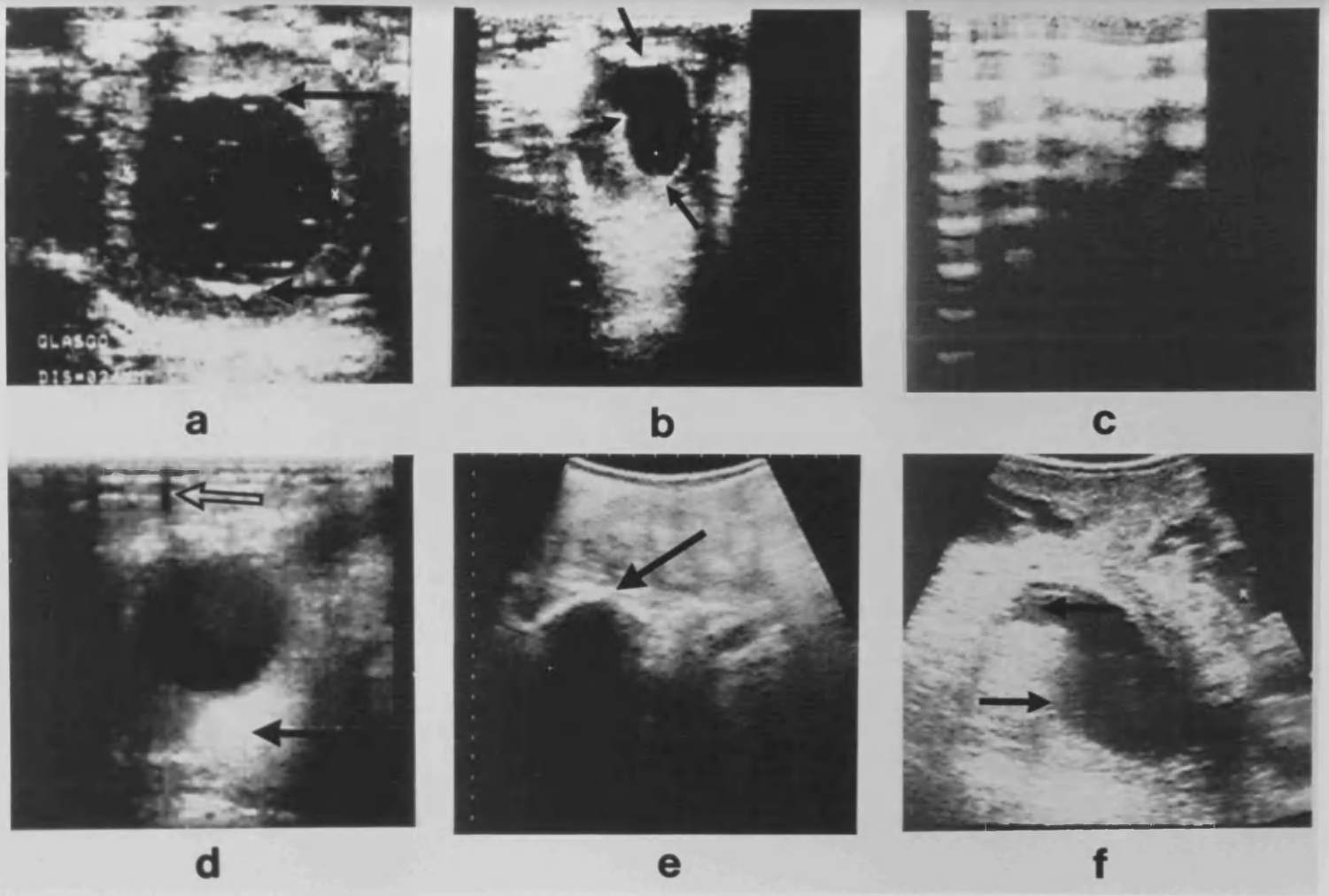
b) Non-specular reflection marked on a corpus luteum.

c) Reverberation

d) Acoustical enhancement. Area of enhancement below the follicle is arrowed. White arrow marks an area showing loss of image due to damage to the transducer

e) Shadow artifacts. The beam is absorbed by foetal bone.

f) Beam width artifact.


a
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Chapter 3

Section 1 Study of Superovulation using Ultrasound

3.1.1 Introduction
3.1.2 Results
Response Subsequent to Prostaglandin Injection
Group 1
Groups 2 and 3
Post Mortem Examination of the Ovaries
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Section 2 Endocrine Findings

3.2.1 Introduction
3.2.2 Results
3.2.3 Figures

Section 3 Histology Studies

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3.3.2 Histological Findings
3.3.3 Figures

Section 1

Study of Superovulation using Ultrasound

3.1.1 Introduction

Embryo transfer in cattle has become a standard husbandry technique. However, cost effective embryo recovery is dependent upon successful superovulation to increase the ovulation rate and thus embryo yield. Various studies have identified problems with repeatability and predictability of response to superovulation. It has been generally accepted that none of the stimulatory treatments available can change the individual variability of the donor.

This study is an in-depth examination of the usefulness of B-mode real time ultrasound scanning for the purpose of monitoring follicular development, ovulation, and formation of the corpus luteum in superovulated cows.

Ultrasound examination was used to study follicular growth and ovulation during both normal cycles and after superovulatory stimulation. The aims of this study were to monitor and quantify follicular growth, to identify ovulation, if possible to quantify ovulation and to identify the spread of timing of ovulation. In addition, formation of corpora lutea was monitored and used to predict possible embryo numbers at flushing.

To confirm the interpretation of ultrasound scans ovarian changes were correlated with measurement of plasma progesterone. In addition, in group 1, preovulatory plasma LH was monitored. In groups 2 and 3, animals were slaughtered after embryo collection and reproductive tracts recovered for examination. The ovaries were retrieved for further gross and histological examination.

Oestrus was induced in all cycles, with PGF2a, and superovulation achieved using 3000 or 1500 i.u. PMSG. Individual animal response to these pharmacological treatments varied. The responses are tabulated and discussed.

3.1.2 Experimental Results

Response Subsequent to Prostaglandin Injection.

The timing of events after prostaglandin injection was recorded for all eleven animals in the three groups. This is summarised in Table 1. The average time from prostaglandin injection to oestrus in the control cycle was 77 hours (range 60-96 hours). In the superovulated cycle this time was reduced by approximately half to 36 hours (range 18-48 hours). The average duration of oestrous behaviour in the control cycle was 16 hours with a range from < 6 hours to 54 hours. A single cow (73) showed oestrous behaviour for 54 hours. Two cows (40 and 44) showed no oestrous behaviour. In the superovulated cycle the average length of oestrous behaviour for ten cows was 23 hours with a range from 12-48 hours. A single cow (54) showed oestrous behaviour intermittantly for 157 hours. The average length of time from first recorded behavioural oestrus to ovulation (as determined by ultrasonography) in the control cycle was 40 hours with a range of 6-72 hours. During the superovulated cycle the average time from oestrus to ovulation was 25 hours (0-36 hours). Generally ovulation occurred after the end of oestrus in the control cycle. Of the two cows which failed to show oestrus during the control cycle, one (40) ovulated 96 hours after prostaglandin and the second (44) failed to ovulate. In contrast, ovulation frequently occurred during oestrus in the superovulated cycle. The average length of time from prostaglandin injection to ovulation was 115 hours (72-144) in the control cycle and 61 hours (36-84) in the superovulated cycle.

These results showed a general pattern of a halving of the length of time from prostaglandin to oestrus and ovulation in the superovulated cycle compared with the control cycle. The duration of oestrous behaviour was markedly increased in the superovulated cycle. Oestrus in the superovulated cycle was marked by a quicker onset and longer duration of more intense oestrus behaviour.

Ultrasound Monitoring of Ovarian Structures

Group 1

Group 1 were monitored through a control cycle and superovulated by injection of 3000 i.u. PMSG. Ultrasound scanning was carried out to identify

follicular growth and identify ovulation in the unstimulated and stimulated cycle. Ovulation was confirmed by LH assay and measurement of rising levels of plasma progesterone. Group 1 was monitored until return to next oestrus after the superovulated oestrus.

In all groups events observed by scanning during the control cycle were similar; representative videoprints are shown in Figure 3.1. Initially the principal feature was a mature corpus luteum on one ovary. After prostaglandin injection luteolysis and follicular growth were monitored. A single growing follicle was usually identified. Follicles reached an average preovulatory size of 1.4 cm. Ovulation was identified as the partial collapse of the monitored follicle and increased echogenicity of the corpus haemorrhagicum formed. Finally, the mature corpus luteum was identified 4-5 days after ovulation.

After treatment with 3000 i.u. PMSG follicular growth was rapid. Only small follicles were visible at the time of injection of PMSG (Figure 3.2.a). At the time of injection of prostaglandin 2 days later, multiple medium sized follicles were visible (Figure 3.2.b). By the day of oestrus multiple large follicles (>1 cm) were evident (Figure 3.2.c). It was difficult to identify and follow individual follicles. Ovulation was identified by decrease in follicular number. Corpora haemorrhagia were identified as poorly defined, echogenic areas (Figure 3.2.d). Ovulation was confirmed in retrospect by identification of an LH surge and increasing plasma progesterone concentrations. It was not possible to recognise a spread in the timing of ovulation.

For group 1, the number of follicles identified on each ovary, the estimated number of ovulations and the number of corpora lutea were recorded. This is shown in Table 2. The number of follicles recorded per cow ranged from 9-17. Only 5-7 ovulations were recognised per cow. This number did not correlate particularly well with the number of corpora lutea observed on day 6 nor at all well with the number of corpora lutea recorded on day 15 post oestrus.

Group 1 were then examined every 3-4 days until return to oestrus. Changes in the ovaries continued. On most ovaries a number of large follicles developed which had not been evident immediately post-ovulation (Figure 3.3). This is demonstrated clearly in Cow 647 from group 1, where multiple follicles are visible 9 days after ovulation, and Cow 274 from group 2, where no follicles were visible post-ovulation but multiple large (>1.5 cm) follicles were seen 5 days later (Figure 3.3). In group 1 by day 8 post-

ovulation, multiple corpora lutea were evident as expected; corporal lutea with and without lacunae were visible (Figure 3.3.d). By as early as 9 days after ovulation large cystic structures were identified on one or both ovaries of three cows. These were characterised by large size (> 3 cm) and remained present on the ovaries for more than 10 days.

Group 2

Having determined that it was possible to identify ovulation on superovulated ovaries a second study was set up to correlate ultrasound with post mortem findings. The poor correlation between follicles, ovulations and corpora lutea in group 1 presented a confusing picture. In addition we wanted to correlate ultrasound estimates of corpora lutea with plasma progesterone and finally, with embryo recovery. Three more cows were superovulated with a dose of 3000 i.u. PMSG (group 2) and four cows were superovulated with a reduced dose of 1500 i.u. PMSG (group 3).

Ovaries of cows in both groups were examined on the day of PMSG (day 9-12 of the cycle). The majority of follicles observed were small although most cows had 1 follicle > 1cm. In response to PMSG (either dose), follicular growth was dramatic; a large number of follicles could be counted on each ovary. Follicle measurements at PMSG injection, at oestrus and at the last scanning carried out before ovulation are tabulated in Table 3. The general trend of an increase in the number and the size of follicles was recognisable. Usually a number of follicles > 10 mm were seen and it was those which were expected to ovulate. Interestingly cow 73 which susequently produced 14 embryos had only 3 follicles > 10 mm at the last scan before ovulation. This does suggest that follicular growth immediately prior to ovulation must be very rapid. Representative pictures of follicular development and ovulation from group 2 are shown in Figure 3.4.

In group 3, superovulated using 1500 i.u. PMSG, follicular development in response to PMSG was similar to those given 3000 i.u. Cow 94 is shown in Figure 3.5. On average the same number of follicles in the same size range (either < or > 1 cm) were evident regardless of dosage (Table 3). However, ovulation was much more obvious on scanning group 3, being identified as a measurable reduction in the size of the ovary as well as a disappearance of follicles (Figure 3.5).

Post Mortem Examination of the Ovaries

On examination it was evident that all pairs of ovaries were very much larger than normal ovaries. The average right ovary equalled 7.6 x 5.3 cm; the average left ovary measured 6.0 x 3.9 cm.

A number of different structures were identified on the ovaries. A striking feature was the presence of a large number of follicles. For example

there were 19 follicles on dissection of the right ovary of cow 54, group 2, 3000 i.u. PMSG (Figure 3.6.a). A large number of these follicles were over 1 cm, many reaching 2.5 cm in size. Similar findings were observed on the ovaries of a number of other cows. The number of follicles identified post mortem exceeded the number identified preovulation by ultrasound.

The number of corpora lutea identified varied between cows corresponding, we thought, to ovulation rate. In some ovaries it was clear that corpora lutea of different ages were present (Figure 3.7.b). It was also possible to identify corpora lutea with an ovulatory papilla but of widely differing sizes (Figure 3.6.c). The ovulatory papilla varied in structure between corpora lutea and it was difficult to determine whether some luteal structures were corpora lutea or fully luteinised anovulatory follicles. Typical of this appearance are the structures round the middle of the ovary in Figure 3.7.b; corpora lutea with small papillae are arrowed but other structures also have crowns which could be papillae.

In addition to follicles and corpora lutea, luteinised follicles were recognised. The degree of luteinisation varied from discrete patches of luteal tissue present on the wall of a follicle (Figure 3.7.a) to follicles with partially luteinsed walls (Fig 3.7.c, d and e) to fully luteinised follicles which were only distinguished from corpora lutea by absence of an ovulatory papillum. In addition haemorrhagic follicles were identified on several ovaries. These structures did not possess an ovulatory papillum (figure 3.6.b). On sectioning these structures were composed of luteinised areas plus areas of blood clot (3.6.d).

In Table 4 the number of corpora lutea identified using ultrasound was compared with the number of corpora lutea or luteal structures which looked like corpora lutea and number of embryos recovered. The correlation was close only in cow 94. Correlation was poor in the other cows and particularly poor in cows 73, 274 and 44 where a large number of luteal structures were present. There was no obvious correlation between dosage of PMSG and numbers of luteal structures nor was there an undiputable correlation between dose rate and follicular number. However, two cows in group 2, 59 and 54, dosed with 3000 i.u. PMSG showed the greatest number of follicles at post mortem. One such ovary, containing 19 follicles is shown in Figure 3.6.a.

Waterbath Ultrasound Studies

Ultrasonographic images of ovarian structures visualised in a waterbath are shown in Figure 3.8. Multiple follicles with thick walls, caused by the compression, surrounding non-echogenic follicular fluid were similar to the appearance in the live animal (Figure 3.8.a). Likewise, a single follicle examined in the waterbath showed a thin echogenic wall and non-echogenic follicular fluid as is usually seen in the living animal (Figure 3.8.c). A corpus luteum appeared as an echogenic rounded area with highly echogenic ovulatory papilla which was not observed in the living animal (Figure 3.8.b). A luteinized follicle was partially echogenic surrounded by a highly echogenic wall, in the waterbath. Moving the transducer gave different pictures according to whether the beam passed through luteal tissue or follicular fluid (Fig 3.8.d,e and f). This type of structure was not observed in the living animal.

Table 1 Individual animal response subsequent to prostaglandin injection

	Control Cycle				Superovulated Cycle			
Cow No	PG to Oestrus Hours	Length of Oestrus Hours	Oest to OVN Hours	PG to OVN Hours	PG to Oestrus Hours	Length of Oestrus Hours	Oest to OVN Hours	PG to OVN Hours
12	60	8	72	132	30	36	30	60
202	66	16	30	96	42	16	18	60
647	90	16	30	120	36	48 b	36	72
895	90	16	42	132	36	24	36	72
59	96	12	36	132	48	<12	0	48
54	66	8	6	72	35	157 b	13	48
274	78	<6	30	108	30	24	18	48
73	78	54	66	144	36	24	36	72
94	69	15	51	120	36	12	36	72
40	a	0		96	48	12	36	84
44	a	0			18	18	18	36
Mean	77	16	40	115	36	23	25	61
Range	60-96	<6-54	6-72	72-144	18-48	<12-48	0-36	36-84

a = animal did not display oestrus

b = broken oestrus, omitted from calculation

Table 2 Follicular development and ovulation after injection with 3000 iu PMSG

	No of Follicles at oestrus		No of Ovulations		No of corpora lutea (Day 6)		No of corpora lutea (Day 15)	
Cow No	RO	LO	RO	LO	RO	LO	RO	LO
12	11	6	3	3	2	1	2	1
202	5	4	4	3	1	2	4	4
647	6	3	3	2	1	2	1	3
895	4	6	3	2	3	2	5	4

Table 3. Distribution of growing follicles after treatment with PMSG

	PMSG				Oestrus				Pre-Ovulation			
Cow No	<5	6-10 mm	>10	>15	<5	6-10 mm	>10	>15	<5	6-10 mm	>10	>15
54	1	3	1	0	0	11	2	0	0	5	4	0
59	3	3	1	0	2	3	5	0	1	3	4	0
274	1	3	1	0	0	9	0	0	0	10	0	0
73	8	1	0	0	2	7	4	0	1	9	3	0
94	5	0	1	0	0	0	2	2	2	1	4	1
40	1	4	0	0	1	4	4	0	1	3	4	1
44	3	3	1	0	0	2	4	2	0	4	5	4

Table 4 A comparison of the number of luteal structures recognized scanning the live animal compared with the number counted at post mortem

		Ultrasound		Post mortem		
	Cow No	RO	LO	RO	LO	Embyro No
1500 iu	94	4	2	4	2	2
	73	7	4	14	7	14
	40	4	2	4	4	1
	44	6	4	16	10	2
3000 iu	54	2	0	4	2	0
	59	4	1	5	1	0
	274	4	5	18	9	1

Figure 3.1 A representative set of pictures from the control cycle. Cow 54 from group 2.

a) Two days after PG injection a regressing corpus luteum is visible on the right ovary; a growing follicle of 1.6 cm in diameter is visible on the left ovary.

b) Left ovary 12 hours later, the preovulatory follicle is now 1.8 cm in diameter.

c) Left ovary 12 hours later, ovulation has occured with formation of the corpus haemorrhagicum.

d) Corpus luteum of 1.8 cm diameter, on the left ovary 5 days after ovulation.

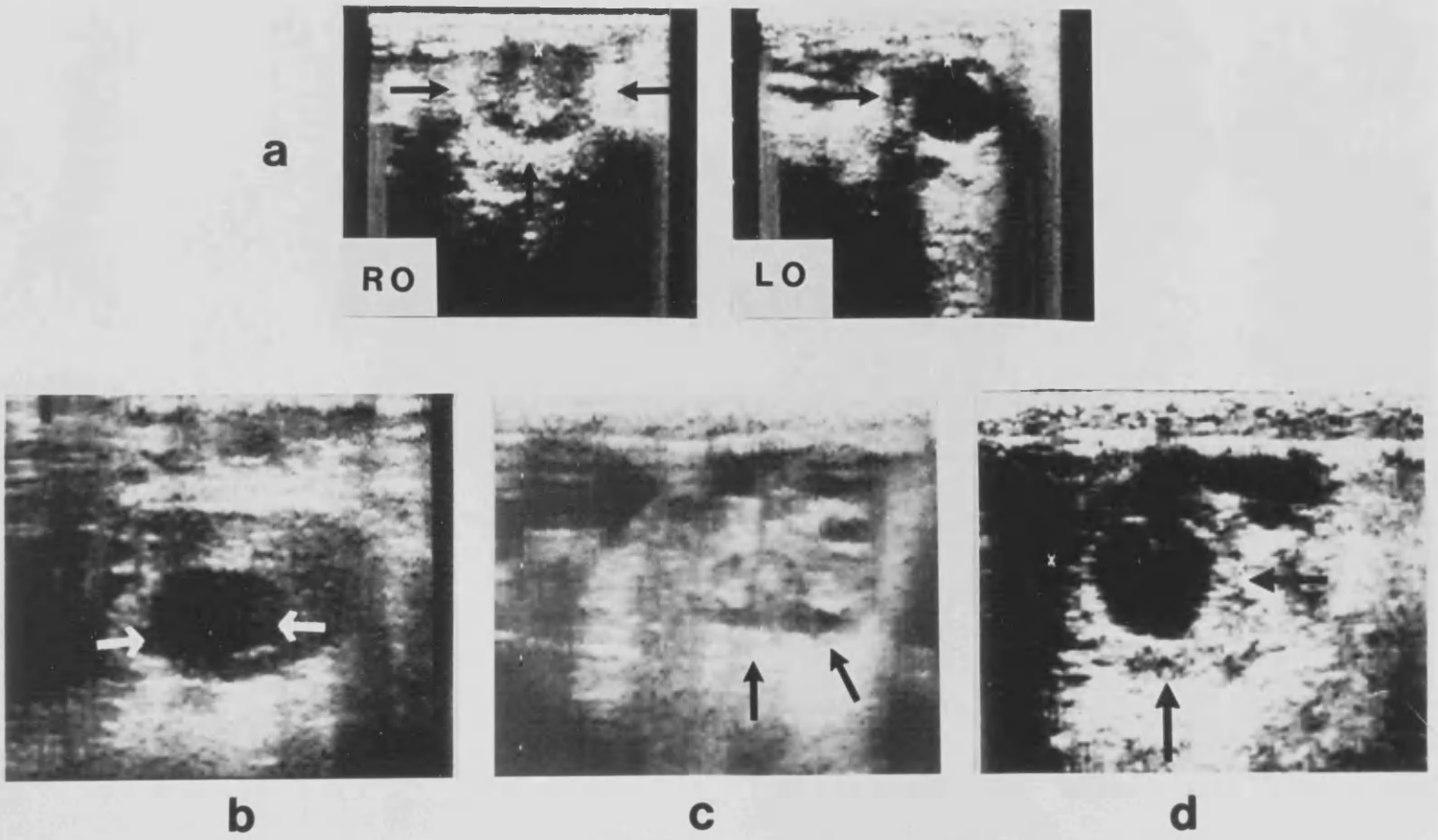

a
RO
LO
b
c
d

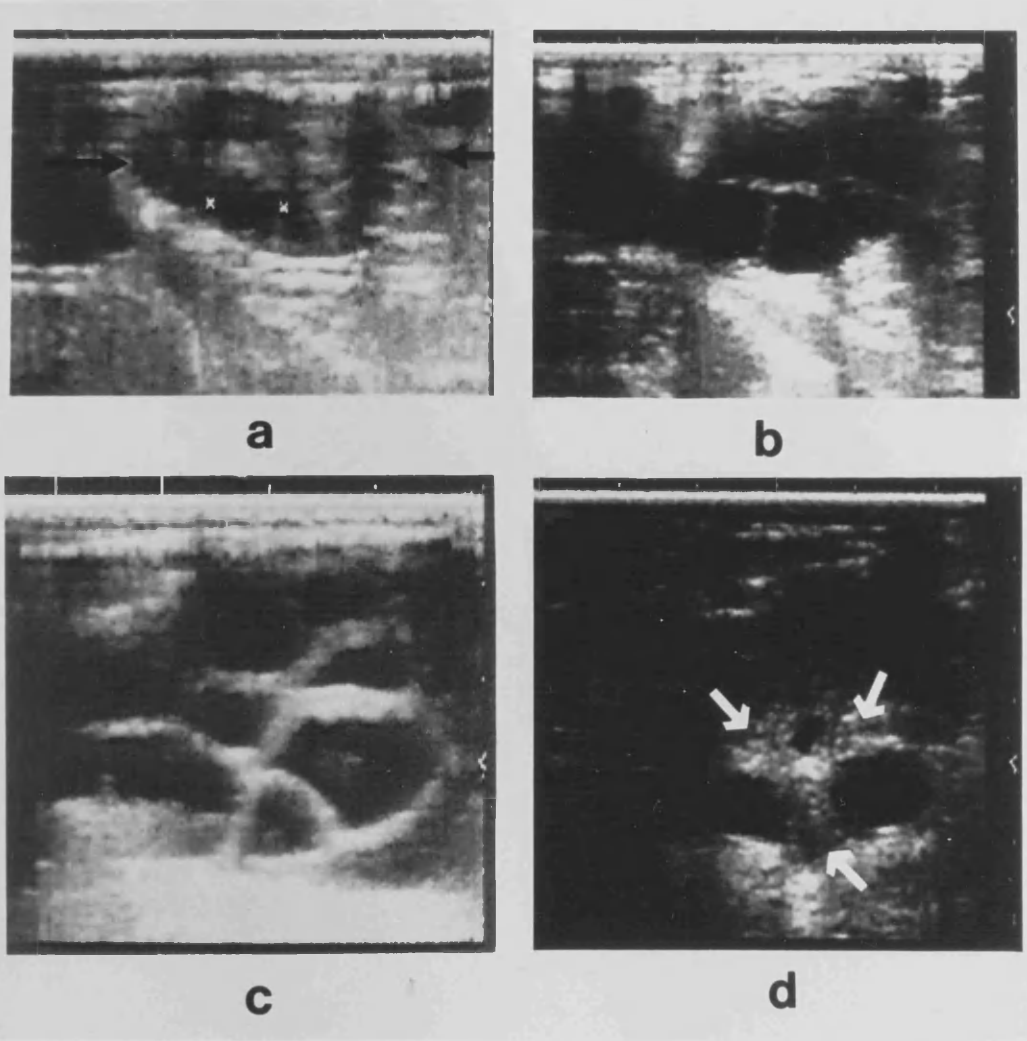
Figure 3.2 Representative pictures of ultrasound findings in group 1 after superovulation with 3000 i.u. PMSG.

a) Right ovary of cow 12 on day of PMSG injection. Note only small follicles (< 5mm) are visible.

b) Right ovary on day of PG injection 2 days later. Note follicular growth.

c) Follicular growth, 7 follicles visible at the last scan prior to ovulation.

d) Ovulation and formation of corpora haemorrhagica.


a
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d

Figure 3.3 Examination of the ovaries of group 1 during return to oestrus.
a) Right ovary of cow 647, 9 days after ovulation. Note the presence of multiple large follicles.
b) Right ovary of cow 647, 9 days after ovulation, again. Note the presence of a large cystic structure 3 cm x 2.4 cm.
c) Right ovary of cow 202 15 days after ovulation. Note a corpus luteum marked by black arrows 2 cm by 2.2 cm. Below the corpus luteum is a cystic structure 3 cm by 2.4 cm marked by white arrows.
d) Cow 895 8 days after ovulation. Three corporal lutea are visible in addition to a cystic structure of 2.75 diameter.
e) Cow 274, left ovary, from group 2. No follicles visible on the day of ovulation.
f) Same ovary 5 days later. Note corpus luteum with a lacuna and three new large follicles.

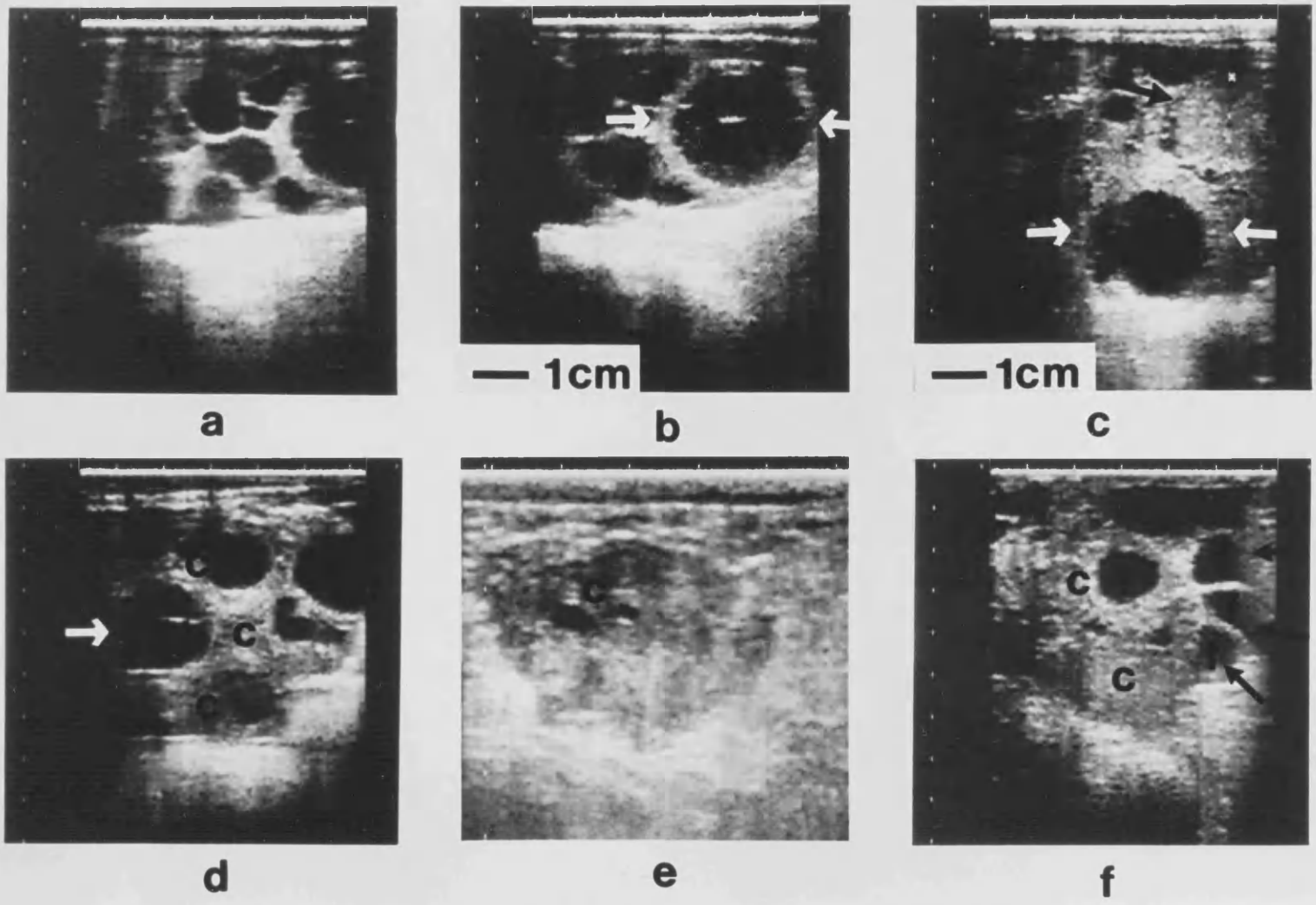

— 1cm
— 1cm
a
b
c
c
c
c
d
c
e
c
c
f

Figure 3.4 Group 2. Follicular development after superovulation with 3000 i.u., Cow 274.

a) Left ovary on day of prostaglandin injection.

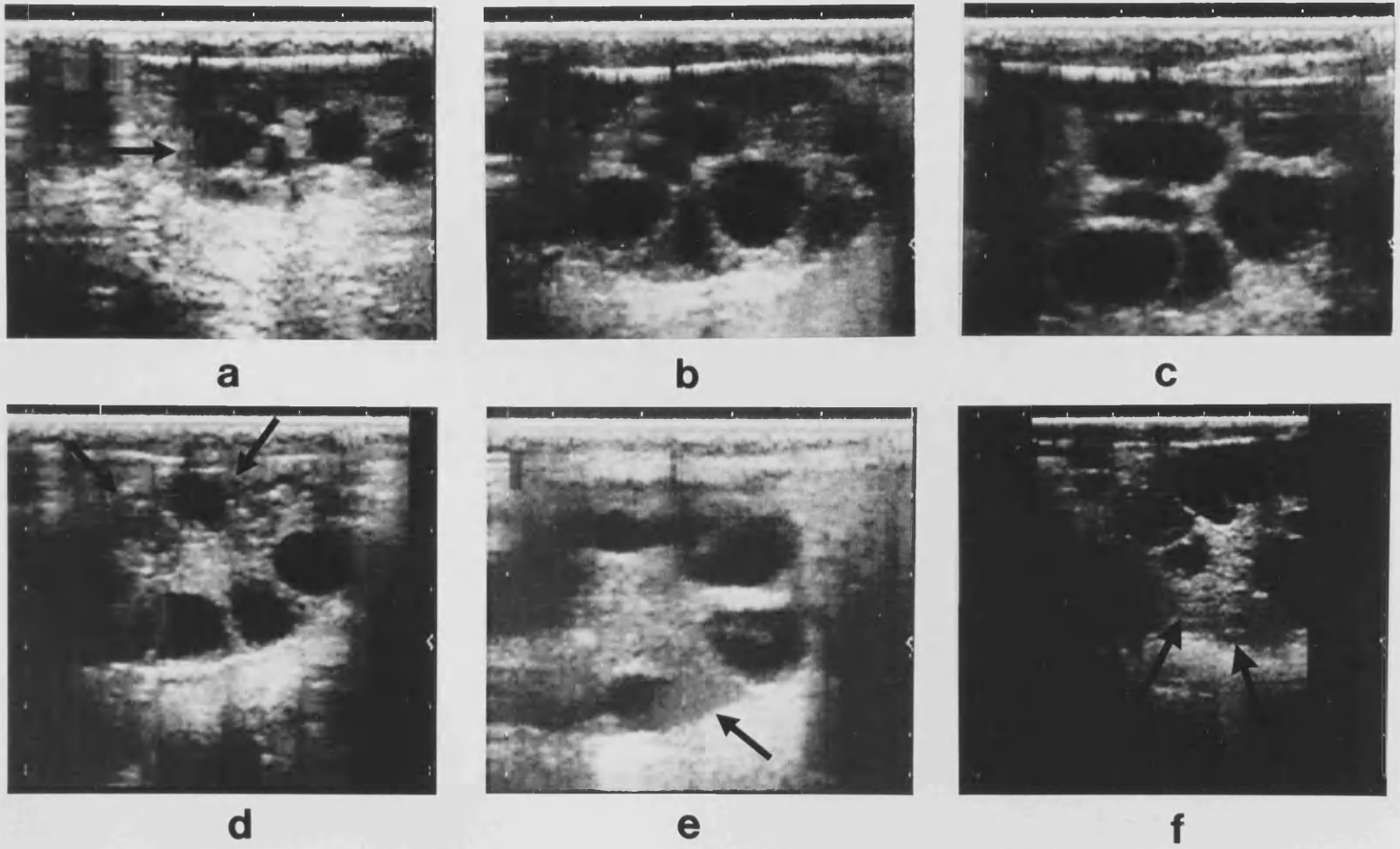
b) Left ovary 24 hours later, note increase in size of follicles.

c) Left ovary 24 hours later, preovulation.

d) Left ovary post-ovulation, corpora haemorrhagica is arrowed.

e) Left ovary 24 hours later, further ovulation may have occurred.

f) Left ovary five days later, two corpora lutea are arrowed. Note multiple large follicles.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e
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Figure 3.5 Follicular development and ovulation in response to 1500 i.u. PMSG, Cow 94.

a) Left ovary on the day of PMSG, follicle of 1 cm is present.

b) Right ovary on day of PMSG, corpus luteum with lacunae present.

c) Right ovary two days later at time of PG injection, four medium follicles are evident. Note the regressing corpus luteum (white arrows).

d) Right ovary at last scanning preovulation.

e) Right ovary, 3 follicles > 1 cm clearly visible

f) Right ovary 12 hours later, ovulation has occurred. Follicles are no longer evident.

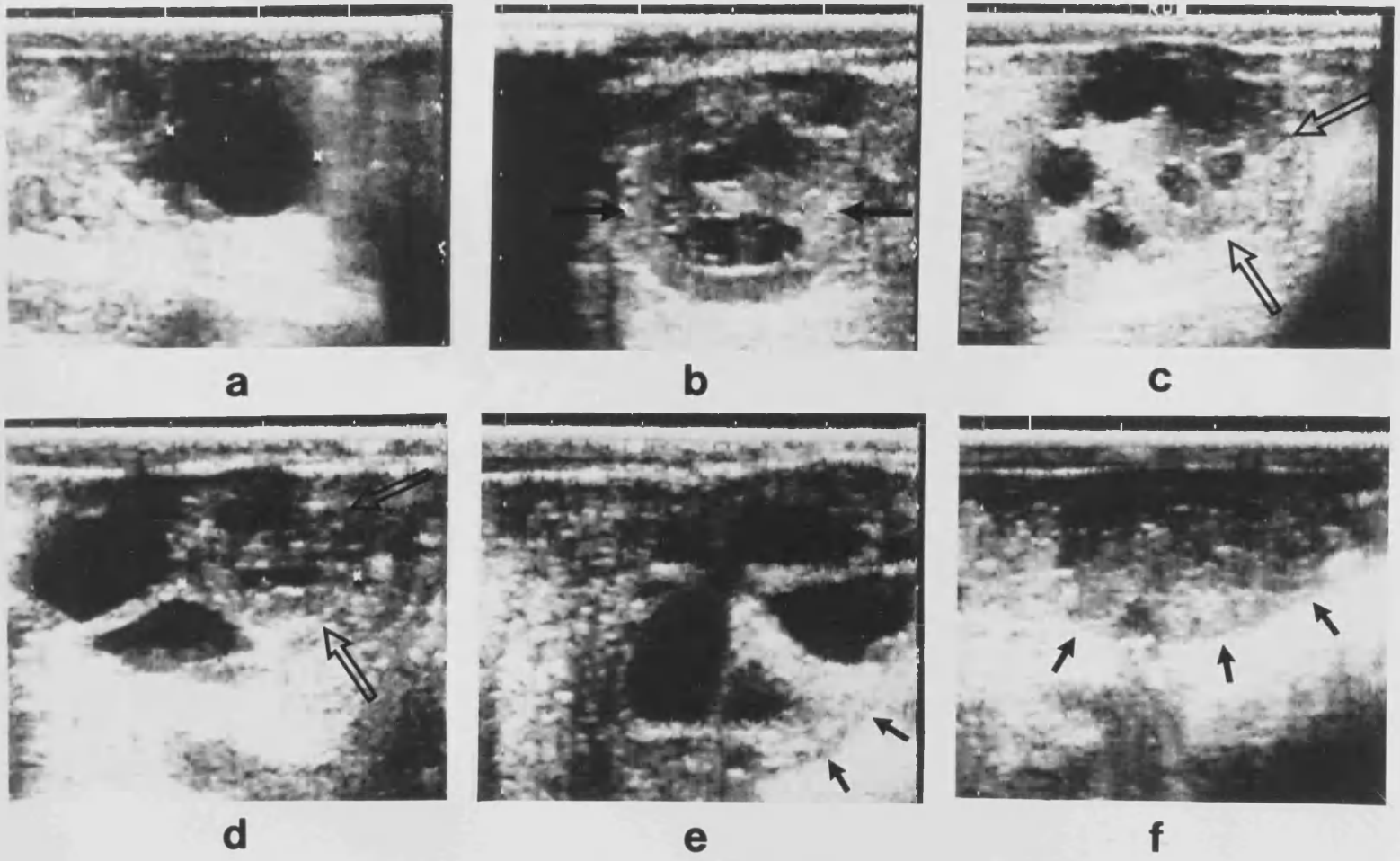
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Figure 3.6 Post Mortem examination of the ovaries.

a) Right ovary from cow 54, group 1, treated with 3000 i.u. PMSG. Note large size of ovary and large number of follicles.

b) Right ovary from cow 59, group 1, treated with 3000 i.u. PMSG. Three corpora lutea are visible. Note the anovulatory follicles on the lower aspect of the ovary, showing no ovulatory papilla. These are dissected in d).

c) Two corpora lutea dissected from cow 44, group 3, treated with 1500 i.u. PMSG. Left hand corpus luteum is apparently normal. Right hand corpus luteum is approximately 1 cm but has a distinct ovulatory papilla.

d) Right ovary of cow 54, group 2, 3000 iu. PMSG, dissected to show haemorrhagic follicles composed of a mixture of luteal tissue and blood clot. Structure seems to be composed of 3 follicles.

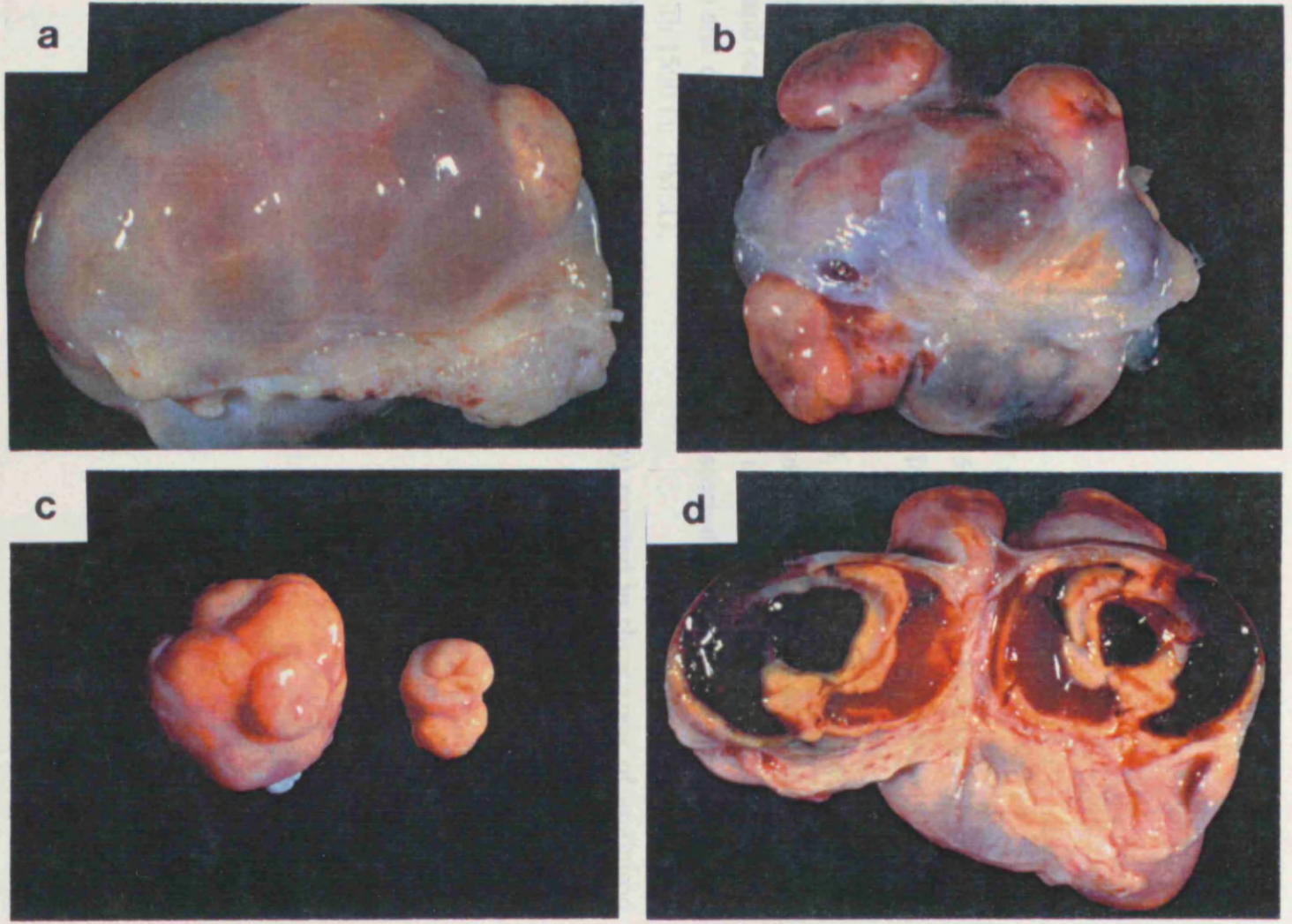

a
b
c
d

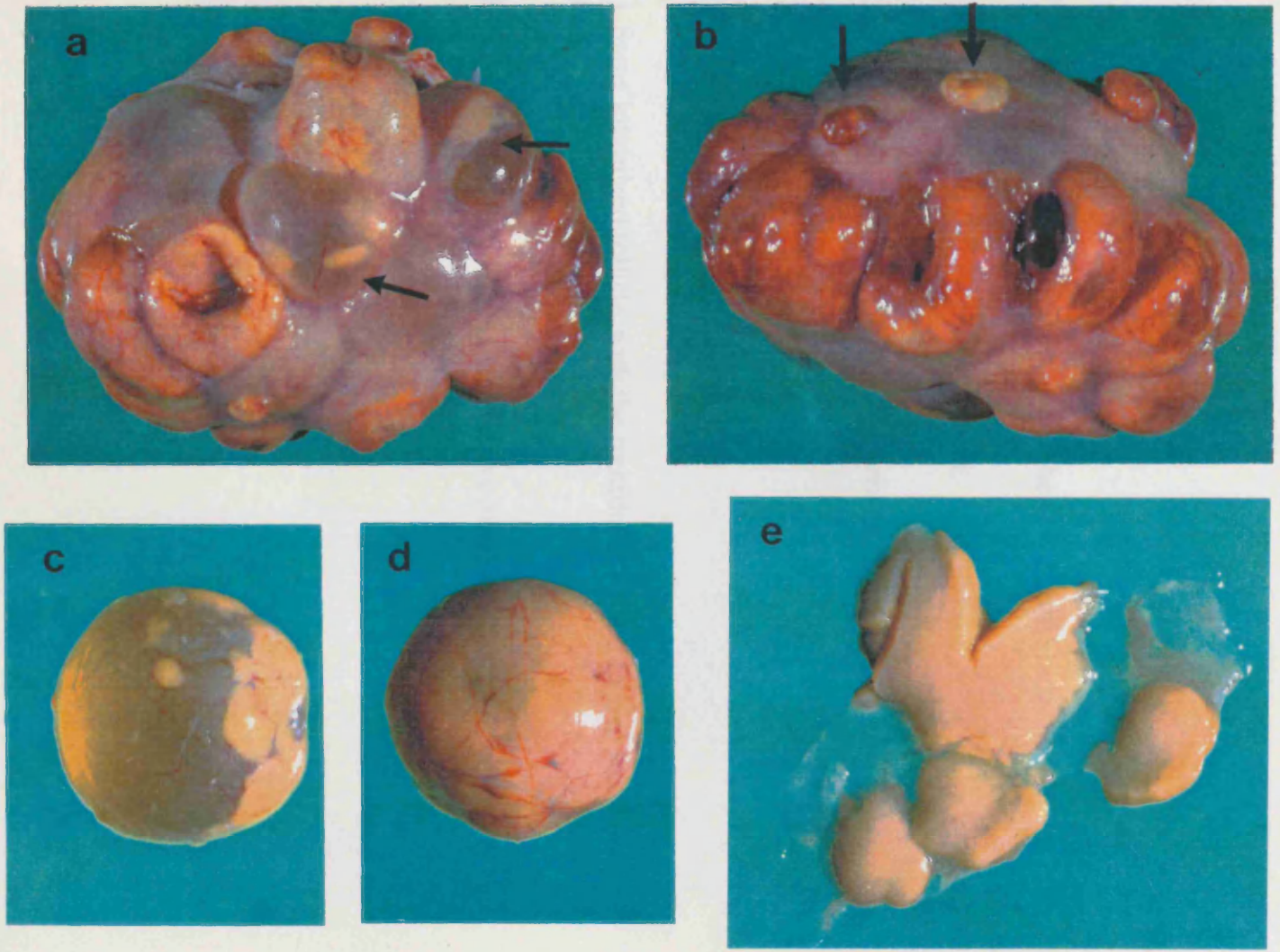
Figure 3.7 Post mortem examination of the ovaries

a) Right ovary from cow 44, group 3, 1500 i.u. PMSG. Follicles and corpora lutea are visible. Note the luteinised patches visible on the follicular walls (arrows).

b) Right ovary from Cow 44, group 3, treated with 1500 i.u. PMSG. Note the different colours of the two corpora lutea arrowed suggesting the left hand corpus luteum is less mature than that on the right.

c) and d) Partially luteinised follicle dissected from Cow 44, group 3, treated with 1500 i.u. PMSG.

e) Similar follicle (cow 44, right ovary) opened to show areas of luteinised tissue and areas of thin follicular wall.


a
b
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d
e

Figure 3.8 Representative pictures of the water bath study

a) Follicles from cow 44 post-ovulation.

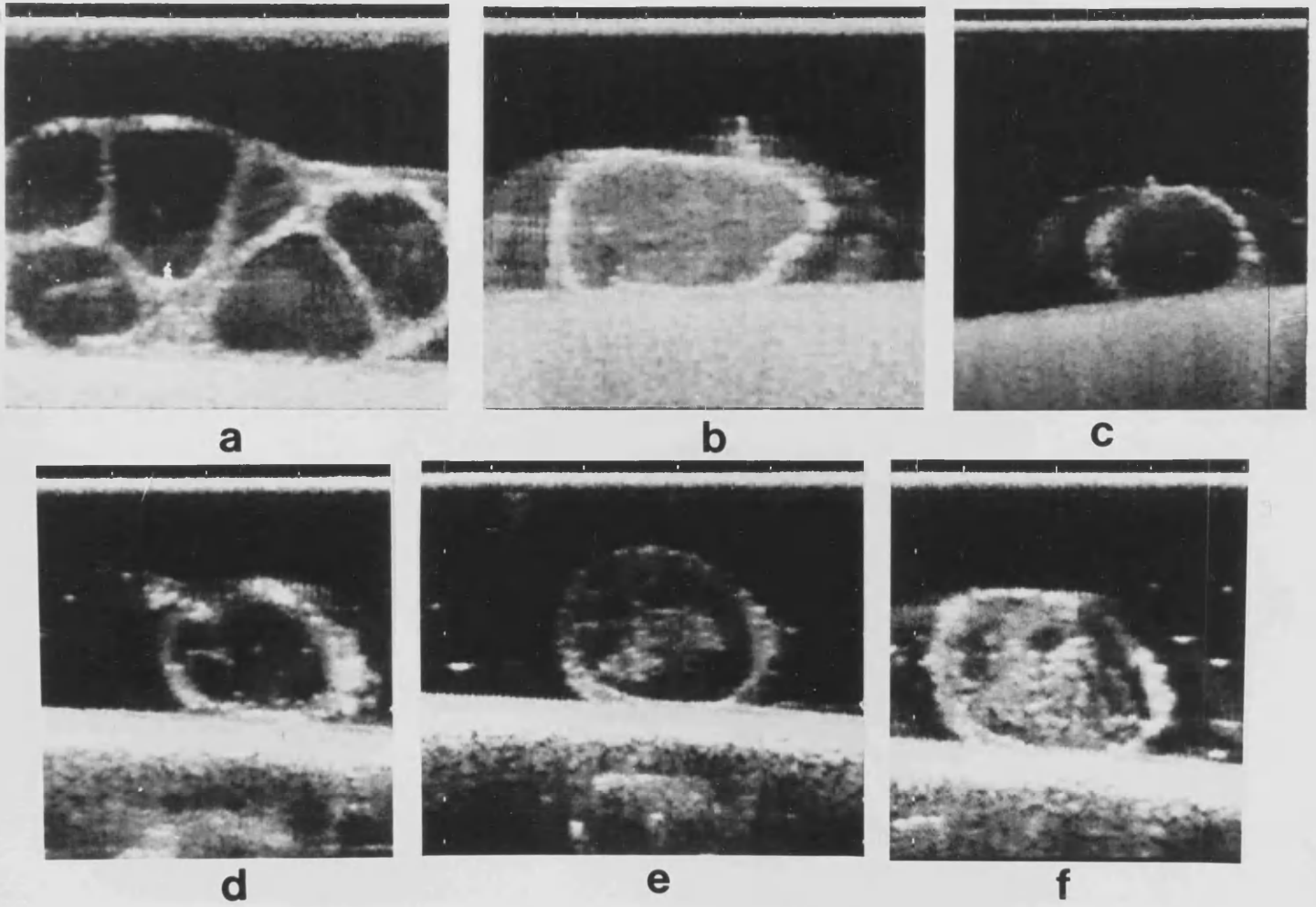
b) Corpus luteum from cow 44. Note ovulatory papilla.

c) Follicle from cow 44.

d) Luteinized follicle from cow 44.

e) Same luteinized follicle, with the transducer turned to give a different view.

f) Same luteinized follicle, turned to give another view.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Section 2

Endocrine Findings

3.2.1 Introduction

The purpose of measuring progesterone was to monitor changes in plasma progesterone levels through the control and superovulated cycles; to correlate progesterone levels with luteolysis after injection of prostaglandins, with oestrous behaviour, with ovulation as seen by ultrasound and to confirm formation of corpora lutea. In turn, this information was used to correlate plasma progesterone with numbers of luteal structures.

Pulished reports state that plasma progesterone varies through the normal oestrous cycle with the physiological state of the corpus luteum. During the periovulatory period, plasma progesterone is extremely low at 0.4 ng/ml. Levels gradually rise during the 4-5 days of metoestrus, as the corpus luteum attains full function, to 4-7 ng/ml. It then remains at this plateau level until day 17-18 of the cycle when luteolysis intervenes (Stabenfeldt, et al., 1969; Takeishi, Tsumagari, Nanba and Takagi, 1989).

Endocrine profiles have been constructed for superovulated cattle. There are are several publications showing progesterone, oestradiol-17B, LH and FSH profiles in the periovulatory period for cattle superovulated by different methods and dosage regimes (Booth, Newcomb, Strange and Rowson, 1975; Saumande, 1980; Jensen, Greve, Madej and Edqvist, 1982; Callesen, Greve and Hyttel, 1986; Goff, Greve, Bousquet and King, 1986; Callensen, Greve and Hyttel, 1987; Callensen, Greve and Hyttel, 1988; Bevers, Dieleman, van Tol, Blankenstein and van den Broek, 1989; Berardinelli and Adair, 1989; Martin, Swanson, Appell, Rowe and Stormshak, 1990; Wubishet, Kesler, Graves, Spahr and Favero, 1991).

3.2.2 Results

Hormonal assay for progesterone was carried out by myself and results of continuous monitoring through the control and superovulated cycle are presented for discussion. The LH hormonal assays were carried out by Dr I .A. Jeffcoate for group 1, during both the control and superovulated cycle.

Plasma Progesterone During the Control Cycle

The endocrine pattern during the control cycle was similar for all the cows with minor fluctuations in the actual levels. A representative cow is shown in Figure 3.9. Prior to prostaglandin injection the average plasma progesterone was 7 ng/ml (range 4.7-14.9 ng/ml). During the first 6 hours after prostaglandin, progesterone levels dropped rapidly to 2.9 ng/ml (range 1.6-7 ng/nml). By 24 hours after prostaglandin, levels averaged 1ng/ml (0.4 to 4 ng/ml).

During oestrus and ovulation, which occurred on average 105 hours and 4.4 days, respectively, after prostaglandin injection, values were low in all the animals, at or below 0.2 ng/ml. These levels remained low until 72-96 hours after ovulation when a rise to 1 ng/ml (0.9-1.9 ng/ml) was observed. Peak levels were reached about 6 days after ovulation at 6.4 ng/ml (3.1-16 ng/ml). In group 1 the LH peak was observed 60 and 96 hours after prostaglandin injection, respectively.

Animals 40 and 44 did not show oestrus, but the progesterone levels followed the general pattern, falling to 0.2 ng/ml by 72 and 78 hours after prostaglandin injection.

Plasma Progesterone During the Superovulated Cycle

A similar pattern of changes in plasma progesterone concentration was seen in the cows in groups 1-3 and individual profiles are shown in Figures 3.10-3.12. Animals were superovulated at midcycle (9-12 days post-oestrus). At this point plasma progesterone concentration averaged 8 ng/ml (3-17 ng/ml). Two days after PMSG treatment, progesterone levels were higher in seven of the eleven cows, averaging 11.9ng/ml. In the other four animals, levels dropped prior to prostaglandin injection. Prostaglandin caused a drop in the circulating progesterone levels to 1.1 ng/ml in all the

cows within 24 hours of injection. Levels remained low during oestrus and for 24 hours after ovulation with a mean value of 0.6 ng/ml. The LH surge occurred 37 hours after prostaglandin injection, ranging between 36 and 40 hours, in group 1.

Levels started to increase 72 hours after ovulation or 8 days after PMSG, with mean values of 1.7 ng/ml (0.4-4.4 ng/ml). With two cows progesterone levels started to rise 24 hours earlier. All cows reached peak progesterone production 10 ($\pm$ 1) days after prostaglandin injection.

In the superovulated cycle most cows presented a very high level of progesterone which was above the maximum standard of 20ng/ml for the assay. Therefore, plasma samples with high levels in groups 2 and 3 were reassayed. The samples were diluted 1:4 and the reading obtained was then multiplied by the dilution factor.

The peak progesterone levels and endocrine profiles of representative animals in group 1 are shown in Figure 3.10. They were very high for cows 647 (43.5 ng/ml), 202 (70 ng/ml) and 895 (88 ng/ml). In contrast, cow 12 peaked at only 10.9 ng/ml. In group 2, peak levels were high in all cows; cow 59 (64 ng/ml), cow 54 (40 ng/ml) and 274 (80 ng/ml). This is shown in Figure 3.11. In group 3, three cows showed high levels ranging from 64 ng/ml (cow 40) to 80 ng/ml (cows 73 and 44). One individual animal (cow 94) showed lower levels of 15 ng/ml (Figure 3.12).

Finally, a comparison between the luteal structures at post mortem and the peak progesterone levels was tabulated for groups 2 and 3 , where ovaries were recovered and examined post mortem (Table 5). There is no clear correlation between the number of luteal structures and the corresponding progesterone level. For example cow 73 had 21 structures, progesterone of 80 ng/ml and produced 14 embryos. In contrast, cow 274 had 27 structure, progesterone at 80 ng/ml and produced 1 embryo. Cow 94 showed 6 ovarian structures, progesterone of 15 ng/ml and produced 2 embryos. Cow 59 also showed 6 ovarian structures, produced progesterone of 64 ng/nl and yielded no embryos. These results would suggest that correlation between progesterone levels and luteal structures and particularly embryo yield is very poor. Further investigation of the ovarian structures was then carried out using histology.

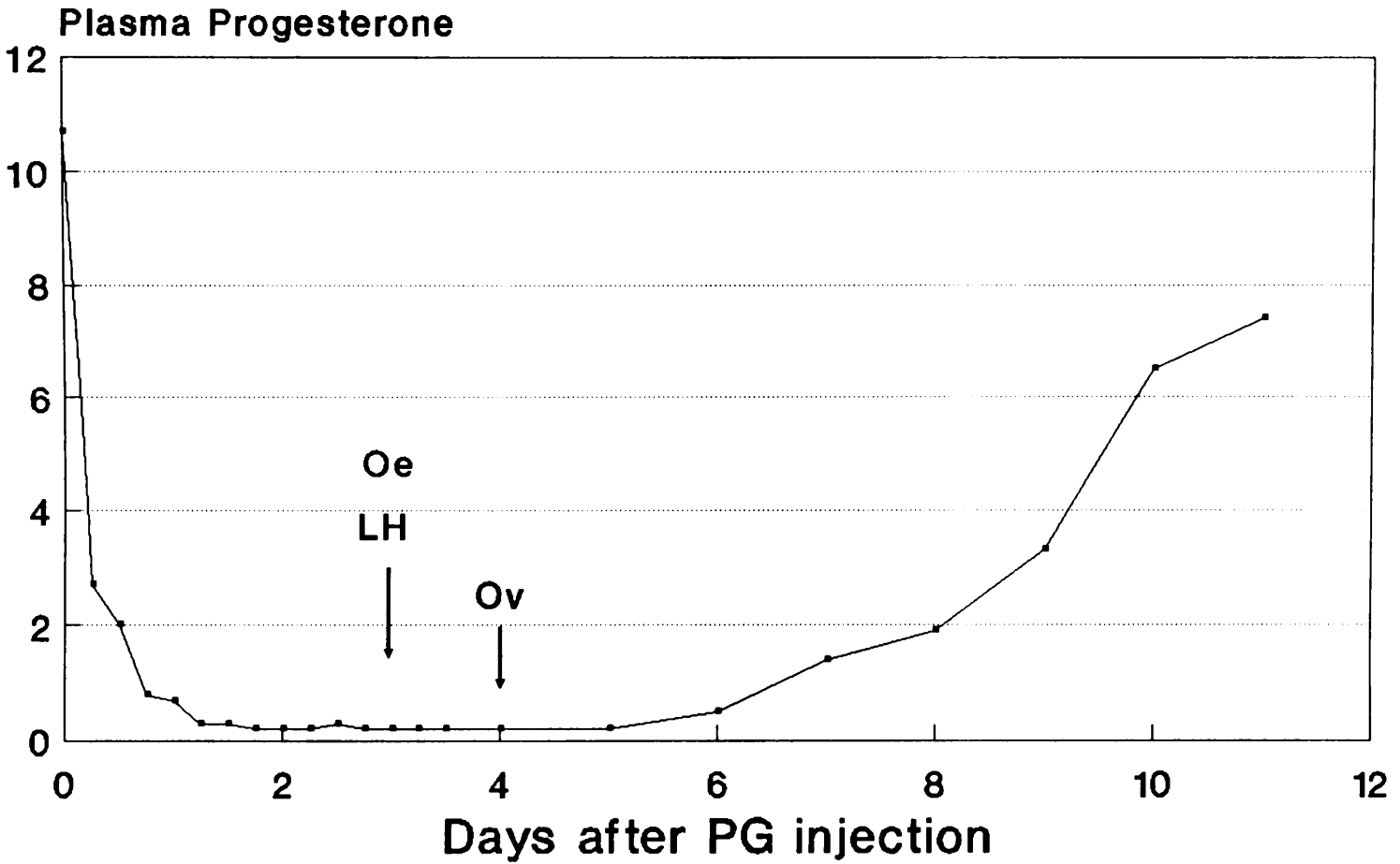
Table 5 Comparison betweeen luteal structures at post mortem and peak progesterone levels

	Cow No	Luteal structures at post motem	Peak Progesterone (ng/ml)
Group 3			
1500 iu	94	6	15
	73	21	80
	40	8	64
	44	26	80
Group 2			
3000 iu	54	6	40
	59	6	64
	274	27	80

Figure 3.9

Plasma progesterone concentration of cow 202 during the control cycle. Arrows indicate the day of prostaglandin injection (**PG**), day of luteinizing hormone peak(**LH**) and day of ovulation (**Ov**) determined by ultrasound. **Oe** indicate day(s) of oestrous behaviour.

Control Cycle Cow #202



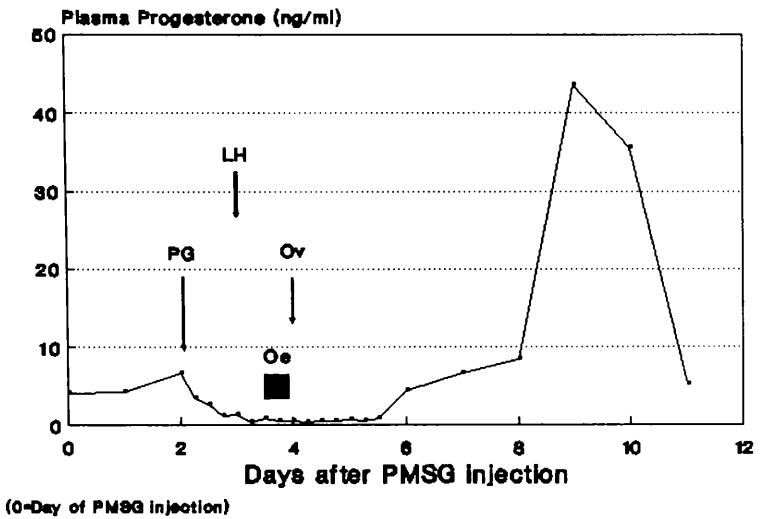
(0=Day of PG injection)

Figure 3.10

Plasma progesterone concentrations during the superovulated cycle of two cows from group 1, treated with 3000 i.u. PMSG. Arrows indicate the day of prostaglandin injection (**PG**), day of luteinizing hormone peak(**LH**) and day of ovulation (**Ov**) determined by ultrasound. **Square black box** indicate day(s) of oestrous behaviour (**Oe**).

3000 IU (1)

Cow #647



Cow #12

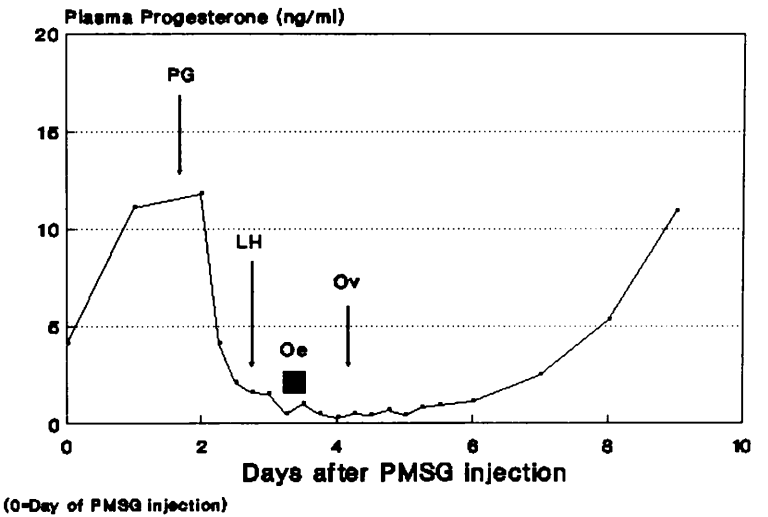
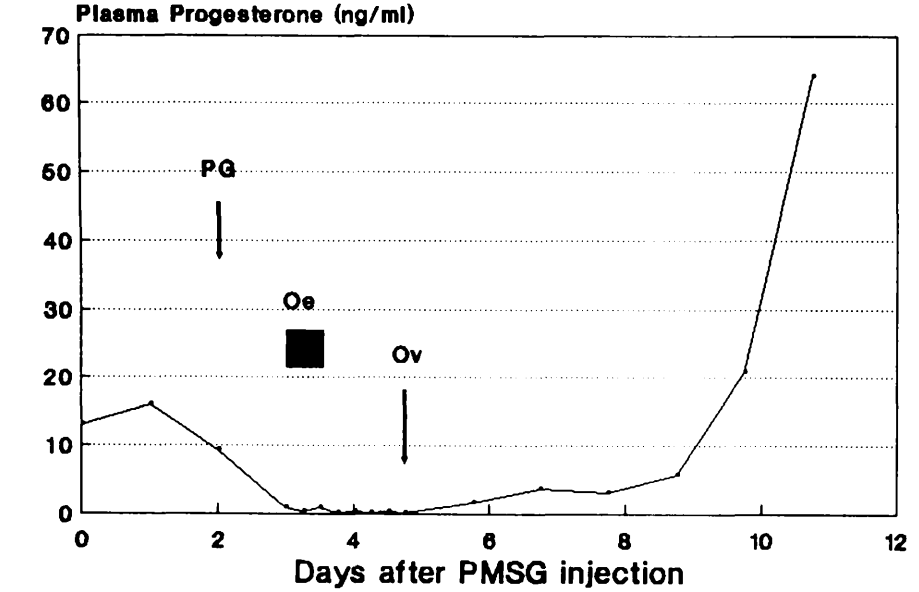


Figure 3.11

Plasma progesterone concentrations during the superovulated cycle of group 2, treated with 3000 i.u. PMSG. Arrows indicate the day of prostaglandin injection (**PG**) and day of ovulation (**Ov**) determined by ultrasound. **Square black box** indicate day(s) of oestrous behaviour (**Oe**).

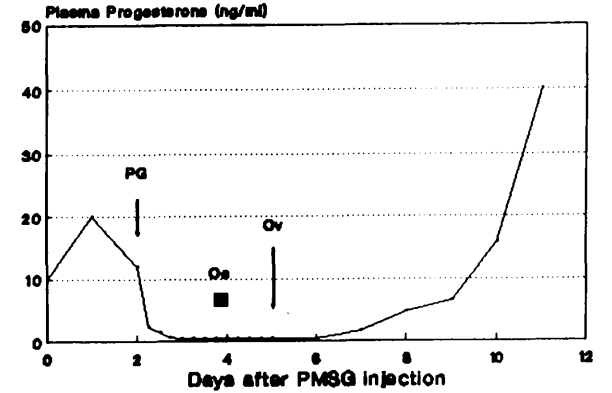
3000 IU PMSG (2)

Cow #59



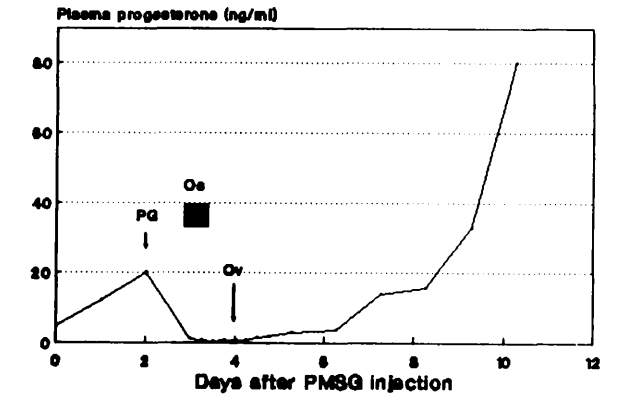
(0-Day of PMSG injection)

Cow #54



(0-Day of PMSG injection)

Cow #274



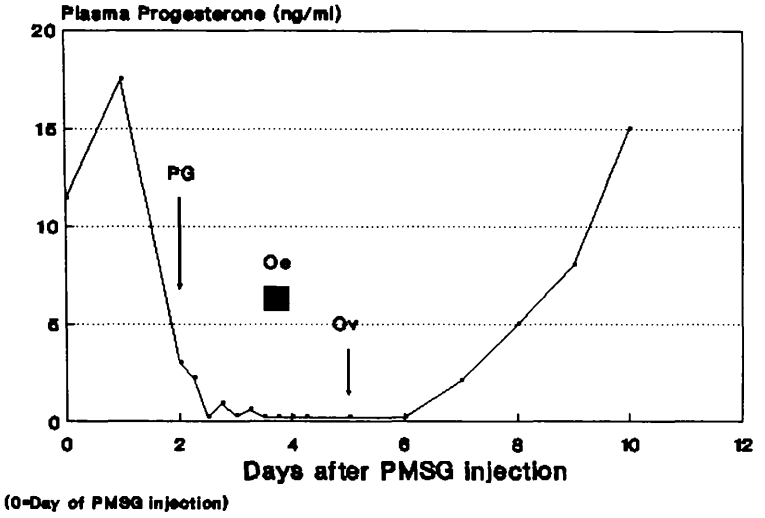
(0-Day of PMSG injection)

Figure 3.12

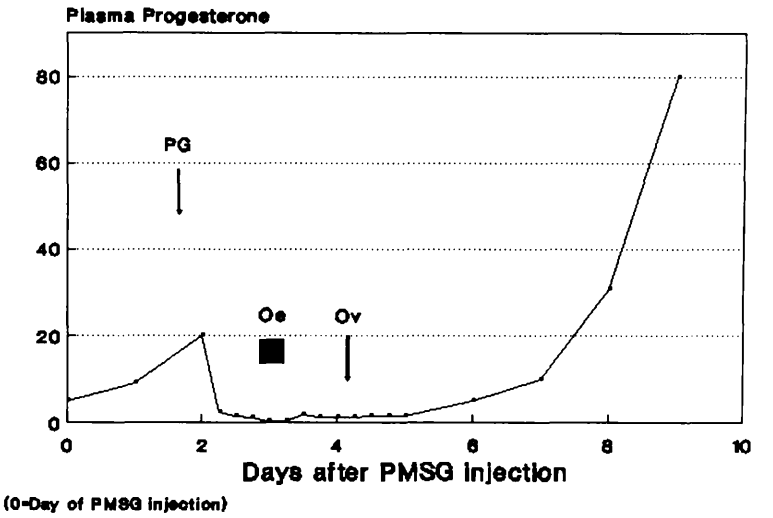
Plasma progesterone concentrations during the superovulated cycle of two cows from group 3 treated with 1500 i.u. PMSG. Arrows indicate the day of prostaglandin injection (**PG**), and day of ovulation (**Ov**) determined by ultrasound. **Square black box** indicate day(s) of oestrus behaviour (**Oe**).

1500 IU (3)

Cow #94



Cow #73



Section 3

Histological Studies

3.3.1. Introduction

In order to understand the histology of a superovulated ovary it is helpful to briefly review the histology of a normal ovary. The ovary consists of an outer zone called the cortex and an inner zone called the medulla. The ovary is covered in a superficial epithelium underneath which lies a dense connective tissue layer called the tunica albuginea. The ovarian cortex consists of stroma containing follicles at various stages of development. The ovarian medulla consists of loose connective tissue, blood vessels, nerves and lymphatics.

The majority of follicles in the stroma are primordial or unilaminar follicles, consisting of an ovum surrounded by a single layer of follicular epithelium. Primordial follicles are inactive; follicles classified as primary, secondary and mature are growing follicles. Primary follicles contain an ovum surrounded by a zona pellucida. The follicular cells become columnar in shape forming a stratified columnar epithelium. A sheath of stroma cells forms into the theca folliculi round the follicle. Secondary or vesicular follicles are characterised by fluid secretion from the multiple layers of granulosa cells, which eventually form a fluid-filled cavity called the antrum. As the follicle increases in size the theca folliculi is organised into a capsule consisting of the theca interna, an inner vascular layer, and the theca externa, an outer fibrous layer which merges with the ovarian stroma (Bloom and Fawcett, 1969). The total time required for follicular growth in large domestic mammals is not known but thought to be in the region of six months (Hafez, 1987). Antrum formation and final growth are FSH and LH dependent.

As the follicle reaches maturity there is an increased secretion of follicular fluid. The ovum is surrounded by a layer of granulosa cells called the cumulus oophorus which detaches from the granulosa layer. The follicle ruptures at an area called the stigma and the ovum is discharged in the follicular fluid. The follicular cavity fills with blood and lymph from broken vessels forming the corpus haemorrhagicum, characterised by a central blood clot. As the corpus luteum forms, the granulosa cells of the follicle differentiate into large pale-staining cells with vesicular nuclei called

granulosa lutein cells or large luteal cells. Cells of the theca interna form theca lutein cells or small luteal cells. It is thought that small luteal cells differentiate into large luteal cells later in the cycle. Lutein cells are large, polygonal, pale staining cells. Histological preparation dissolves lipid droplets from lutein cells leaving characteristic vacuoles. The component cells of the theca externa do not undergo transformation. As the corpus haemorrhagicum becomes the corpus luteum the central blood clot is converted into a fibrous core.

The desired result of a superovulatory treatment is to increase the number of follicles developing and ovulating with the subsequent development of a large number of functioning corpora lutea and the harvesting of a corresponding number of embryos. It is evident from the previous section that follicular growth was readily induced in response to PMSG. However, ovulation rate of follicles which attained a preovulatory size was low. In addition, embryo recovery was lower than estimated on the basis of the number of corpora lutea. Further, plasma progesterone profiles were found to correlate poorly with luteal structures.

To investigate these findings ovaries were recovered at slaughter and histological examination of specific ovarian structures was carried out. As a control, ovaries at various stages in the cycle and to all appearances 'normal' were also recovered and sectioned. These findings are presented first. Findings in the superovulated ovaries are then compared with the control ovaries. A second form of control was really neccessary in this study but severely limited by finances. This is the control which distinguishes the effect of PMSG from the effect of the endogenous LH surge. One cow was superovulated with 3000 i.u. PMSG and slaughtered 7 days later. This cow was not treated with prostaglandin, did not come in season and the ovaries showed no recent corpus luteum. Thus this cow was subjected to PMSG, with its gonadotrophin effects, but not subjected to an LH surge.

3.3.2 Histological Findings

Control Specimens

Control or 'normal' specimens were recovered from cattle slaughtered at the abbatoir. Exact cyclical stage of these control specimens is not known but the specimens serve to illustrate the classical features of ovarian histology.

Figure 3.13 shows multiple primordial follicles visible in the ovarian cortex. These follicles have an oocyte with no zona pellucida, surrounded by a single layer of flattened epithelial-type granulosa cells. A primary follicle is shown in Figure 3.14. The oocyte is surrounded by a zona pellucida (not visible). The follicle is surrounded by multiple layers of cuboidal granulosa cells. The granulosa cell layer is in turn surrounded by the theca folliculi formed from ovarian stroma.

The secondary follicle develops a fluid filled cavity called the antrum. Follicular fluid is secreted by the granulosa cells and contains steroids. The oocyte becomes surrounded by the cumulus oophorus formed from the granulosa layer round the oocyte (Figure 3.15). Theca interna and externa are distinct (Figure 3.16). This class of follicle may go on to ovulate but the majority of follicles become atretic. It is this class of follicle which is said to be 'rescued' by superovulatory treatments. Only antral follicle will be visible on ultrasound since it is the anechoic appearance of follicular fluid within the follicle which actually shows on scanning. Moreover, only antral follicles which have reached a certain size can be discerned. In this study it was possible to identify follicles of 5 mm using ultrasound.

The corpus haemorrhagicum is characterised by a central blood clot. Cells from the granulosa layer and the theca interna differentiate into large pale-staining cells with vesicular nuclei. Histological preparation dissolves lipid droplets from lutein cells leaving characteristic vacuoles (Figure 3.17). The component cells of the theca externa do not undergo transformation. The cells of the corpus luteum are larger, shown by the increase in cytoplasm relative to nucleus. The stippled cytoplasm of the steroid producing cell is characteristic (Figure 3.18). It becomes possible to differentiate between large and small luteal cells.

Superovulated structures

Follicular structures were examined microscopically under low (x10) and high magnification (x40, x100) and divided into 3 classes according to the initial macroscopic and microscopic appearance. In addition, corpora haemorrhagica and corpora lutea were identified.

The first class of follicles had the gross appearance of normal follicles and had the histological appearance similar to a vesicular follicle (Figure 3.19). However, on closer inspection the granulosa layer had an appearance best described as disaggregated. The stratified columnar appearance which should have been present was not clear. In addition, cells in the adjoining thecal layer had nuclei similar to luteal cells. In Figure 3.20 the granulosa layer is very obviously not columnar. In the adjoining theca interna, cells with the classical luteal appearance are visible.

The second class of follicles again looked grossly normal, but was obviously abnormal on microscopic examination. This type of follicle had no visible granulosa layer (Figure 3.21). The thecal layer most closely resembled the connective tissue of theca externa. Cells lining the antrum were long and fibrous and lacked cytoplasm. These cells stained blue, indicative of connective tissue and did not have the appearance of the steroid secreting cell.

The third class of follicles was those with macroscopically visible patches of luteal tissue such as shown in Figure 3.7. Histological examination of this type of follicle showed several interesting features. There was loss of the granulosa layer. The basement membrane lined the antrum. Fusiform cells were visible in the layer lining the antrum. Some areas of wall have only connective tissue and maybe a fusiform layer (Figure 3.22) as described in the previous class while other areas of follicular wall showed theca interna developed into wide pockets of luteal type tissue (Figure 3.23). The basement membrane is assumed to line the antrum because no other layer of connective tissue is present between the antrum and the luteal outpocketings (Figure3.24).

Corpora hemorragica were identified. Their appearance was similar to that described for the control structures (Figure 3.25). Corpora lutea with papilla were examined too (3.26). These structures showed large cells with a foamy appearence of the cytoplasm. In some cases small cells were present with dark nuclei and smaller volume of cytoplasm. This appearance, also, was very similar to that described for the control structure.

Superovulated Control Animal

The ovaries of this animal were sectioned. Representative results are shown in Figures 3.27 and 3.28. The findings were very interesting. It was very clear that large numbers of follicles were induced to become vesicular. A large proportion of these follicles had a unilaminar granulosa lining the follicular antrum. Other follicles had a 2 or 3 cell granulosa layer. This single layer granulosa would be more aptly described as cuboidal than columnar. This is shown clearly in Figure 3.28. In addition to an abnormal granulosa layer, the thecal layers are absent from a proportion, though not all, follicles. It can be seen in Figure 3.27 that thecal tissue is visible around some follicles. However many other follicles are surrounded by tissue which looks like ovarian stroma (Figure 3.28). This raises interesting questions about using PMSG to force vesicle formation in follicles at a rate of growth with which granulosa growth and theca formation cannot keep apace.

Figure 3.13 Primordial Follicles

Five primordial or unilaminar follicles pictured in the cortex of an unstimulated ovary (x100). Note the single layer of epithelial like granulosa layer.

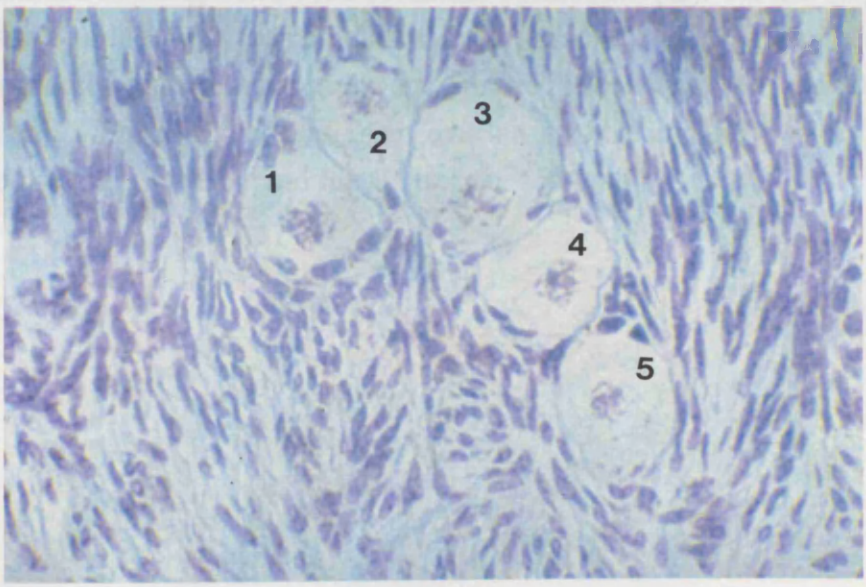

1
2
3
4
5

Figure 3.14 Primary Follicle

Primary follicle (x10). Note oocyte (O) surrounded by multilaminar granulosa forming a stratified columnar epithelium (G). At this stage the egg is surrounded by a zona pellucida (not visible). The granulosa layer is bounded by a distinct basement membrane (BM). The layer called the theca folliculi (TF) differentiates from ovarian stroma and further differentiates into the theca externa and theca interna.

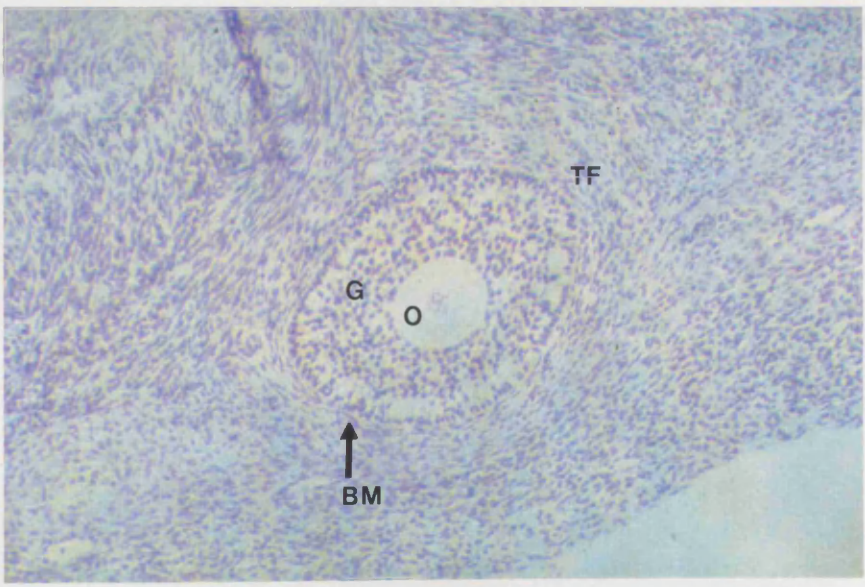

TF
G
O
BM

Figure 3.15 Vesicular follicle (x 10).

Note the follicular antrum has formed at this stage. Follicular fluid is obvious (FF). The antrum is bounded by multiple layers of granulosa (G), in turn bounded by the theca interna (TI) and theca externa (TE). The cumulus oophorus (CO) has formed though the oocyte is not visible in this section.

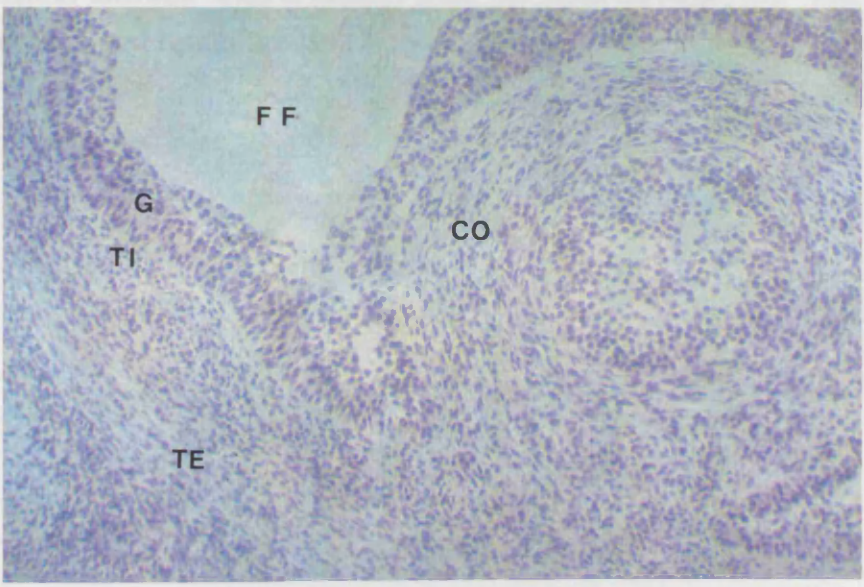

F F
G
TI
CO
TE

Figure 3.16 Same section (x 100).
Note the multilayered columnar nature of the granulosa layer (G) lining the follicular antrum. This is distinct from the theca interna (TI). The theca externa is and remains a connective tissue layer (TE).

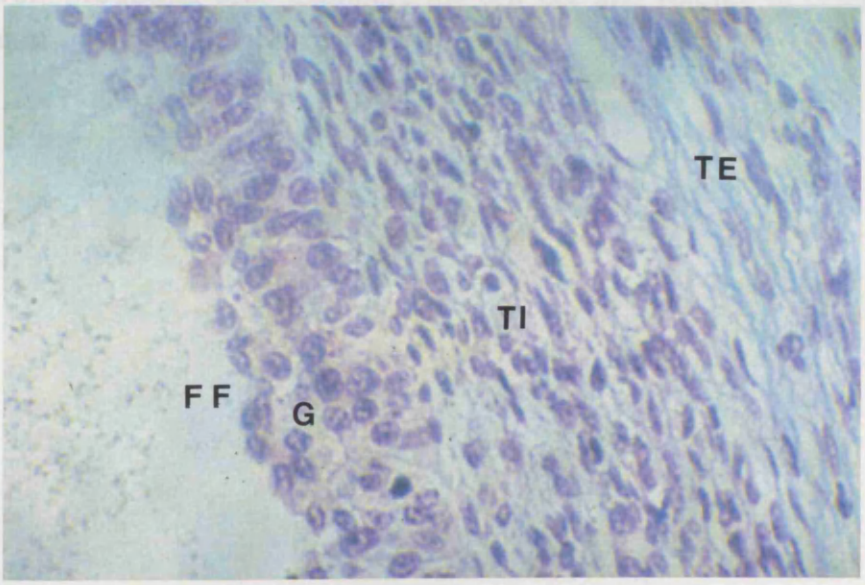

TE
TI
F F
G

Figure 3.17 Corpus Haemorrhagicum (x100).
Note the cell type has become the large, pale, vacuolated cell typical of the steroid producing cell. Compare the cell size and volume of cytoplasm with that of the the mature corpus luteum.

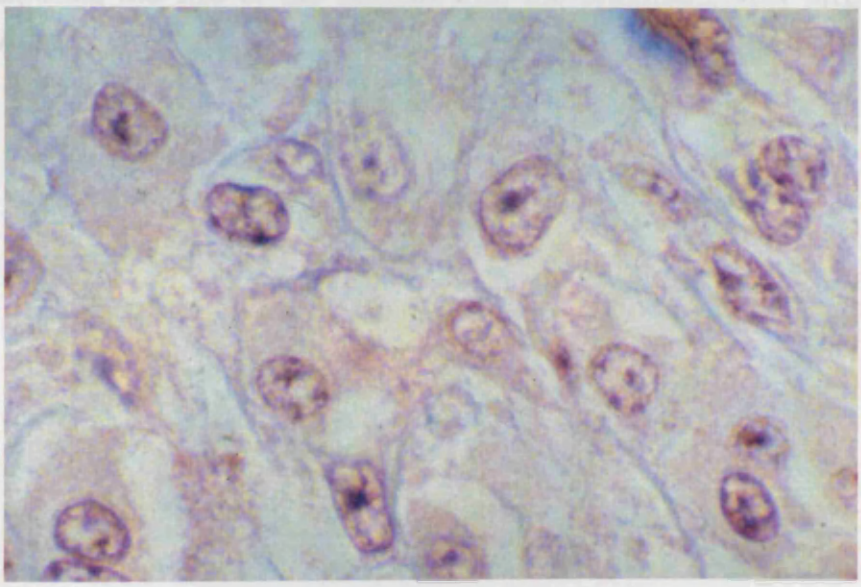


Figure 3.18 Corpus Luteum (x 100).
Note the pale staining, vacuolated or stippled appearance. Assessed by comparing the size of the nucleus with the cytoplasm, very large cells are obvious.

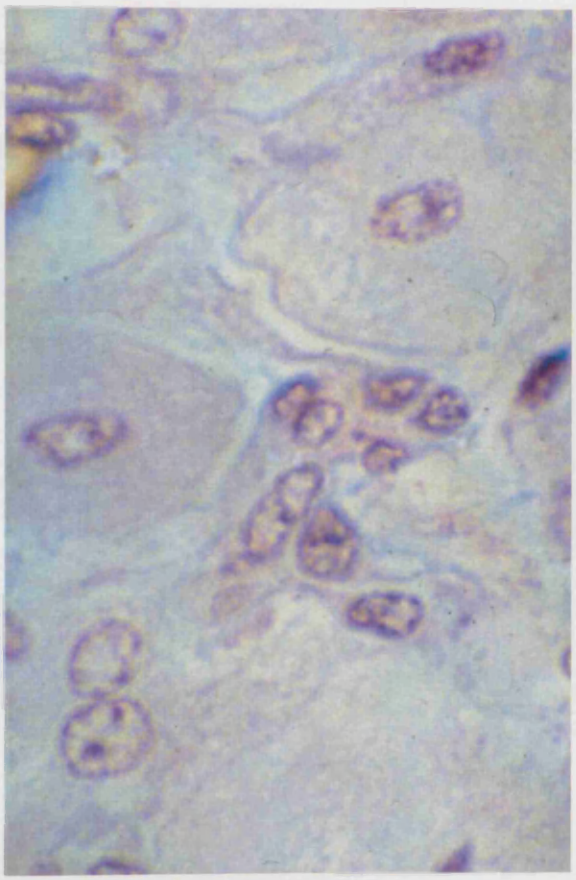


Figure 3.19 Section from a follicle with a normal gross appearance (x100). Cow 59, 3000 i.u. PMSG. Note disaggregation of the granulosa layer (G) and luteal type cells in the theca interna (TI). Follicular fluid fills the antrum (FF).

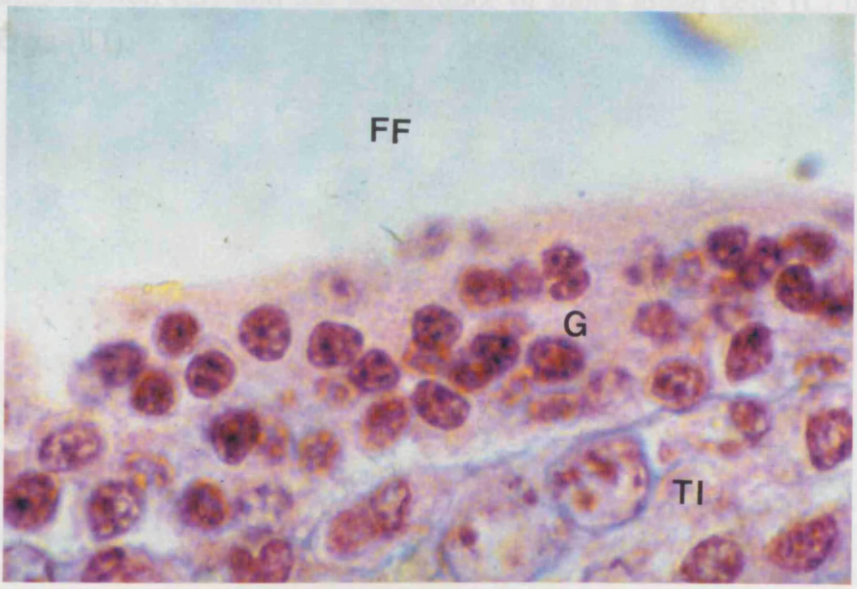

FF
G
TI

Figure 3.20 Section from a second follicle with a normal gross appearance (x 100). Cow 54, 3000 i.u. PMSG. Note the greater degree of granulosa cell disaggregation (G) and the appearance of more luteal cells (L) in the theca interna (TI).

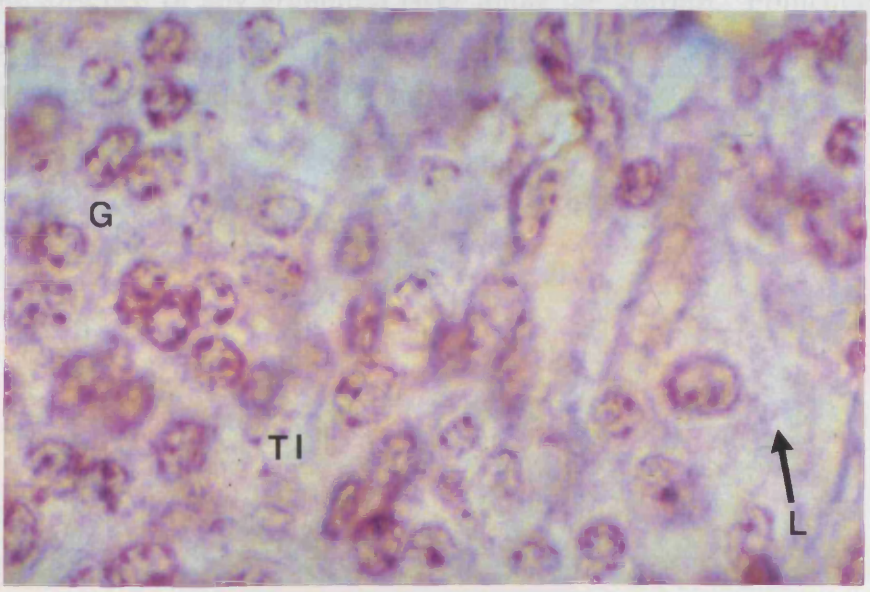

G
TI
L

Figure 3.21 Section from a follicle with a normal gross appearance but clearly abnormal histological appearance (x 100). Cow 40, 1500 i.u. PMSG. Note absence of any granulosa layer lining the antrum. Follicular fluid fills the antrum (FF). The thecal cells (T) have a fusiform appearance (T). Basement membrane lines the antrum.

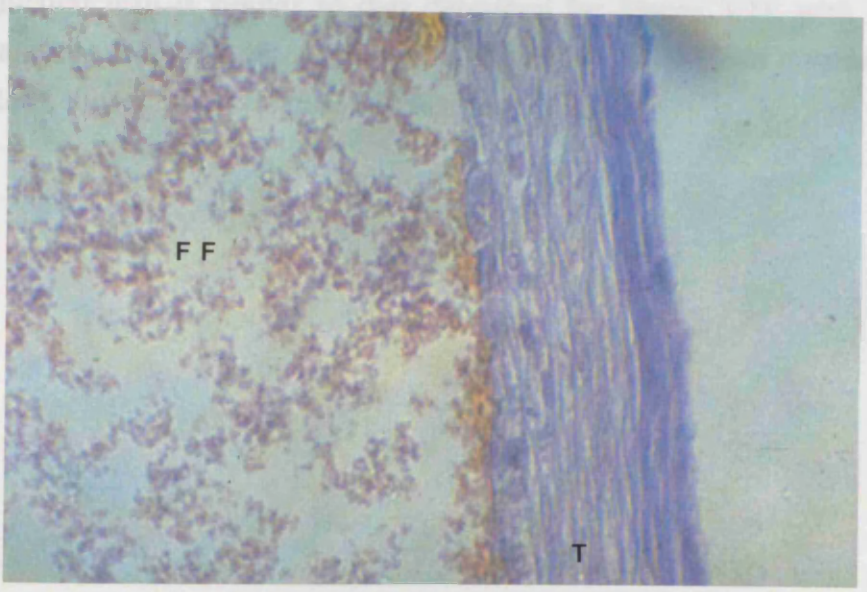

F F
T

Figure 3.22 Section from a follicle with grossly visible luteal patches (x 100). Cow 40, 1500 i.u. PMSG. The appearance of the non-luteinised portions of follicular wall is similar to that described for Figure 3.21, with loss of the granulosa layer, basement membrane lining the fluid filled antrum (FF), and a thin, thecal layer composed of fusiform cells (T).

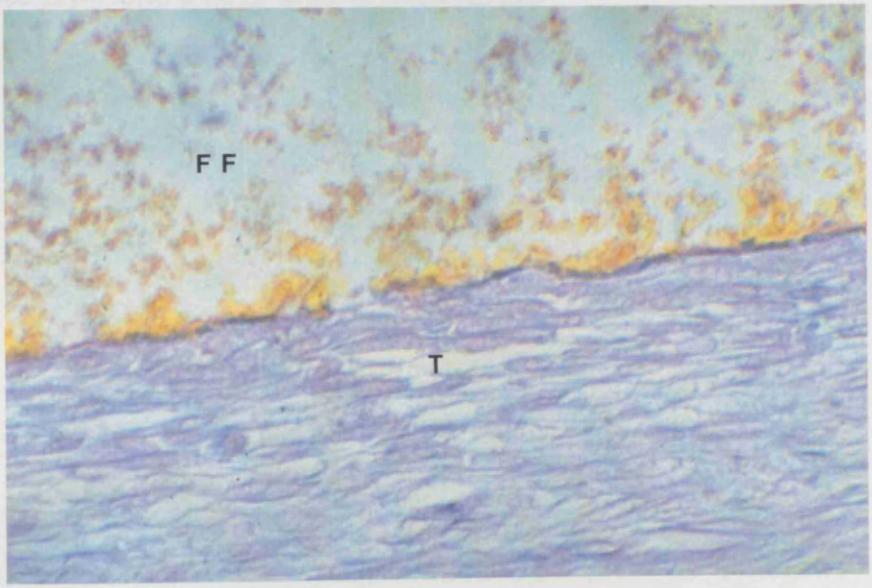

F F
T

Figure 3.23 Section from the junction of a thin walled and luteinised area of the same follicle (x 10). Cow 40, 1500 i.u. PMSG. Note the expansion of the theca layer into a large area of lutea tissue.

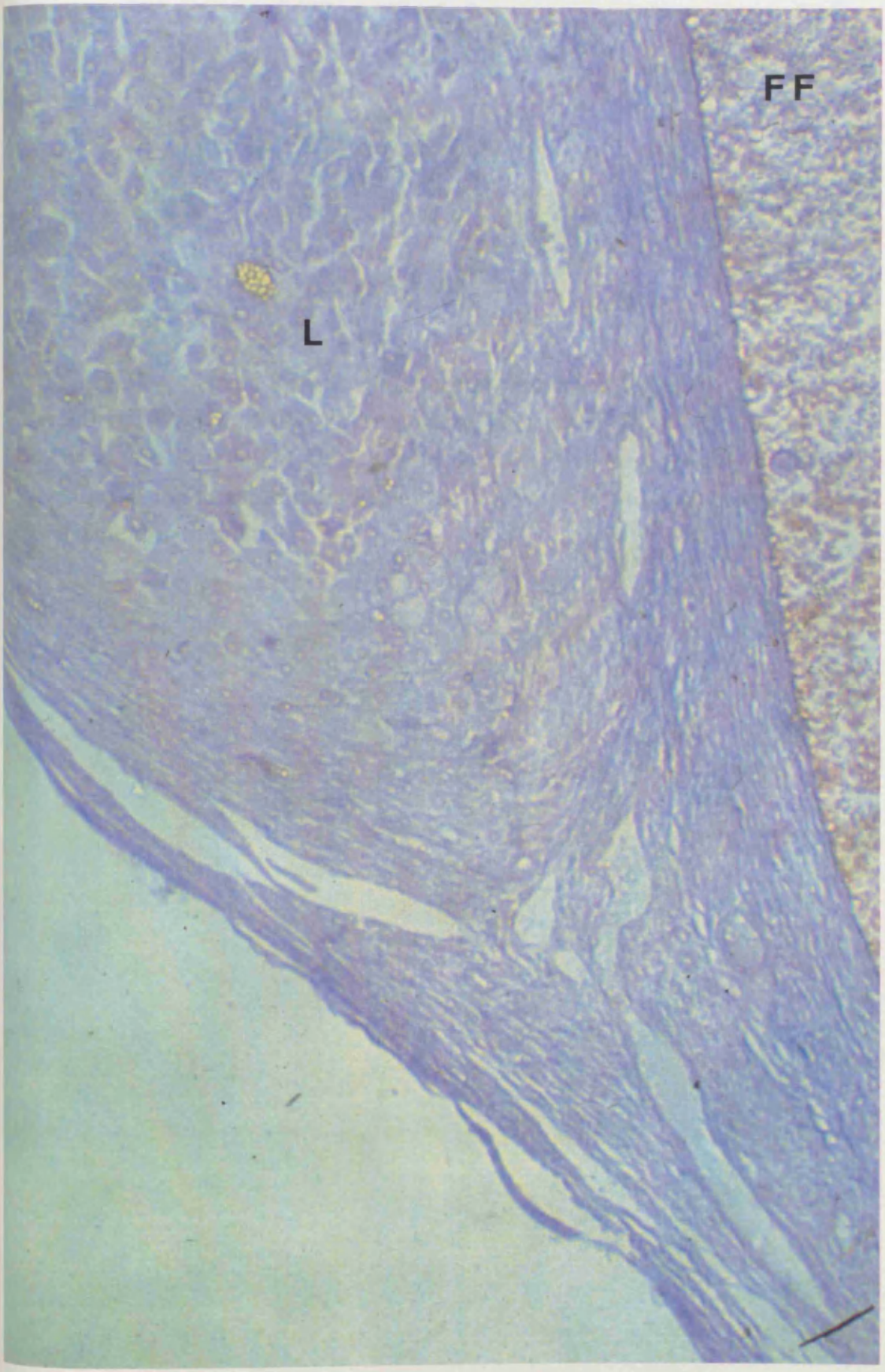

FF
L

Figure 3.24 Section from another partially luteinised follicle (x 40). Cow 44, 1500 i.u. PMSG. Note the expanse of lutea tissue (L) in the theca layer. There is no granulosa layer. Note that there is no bounding membrane visible in the luteal area inferring that the lining of the antrum must be the basement membrane.

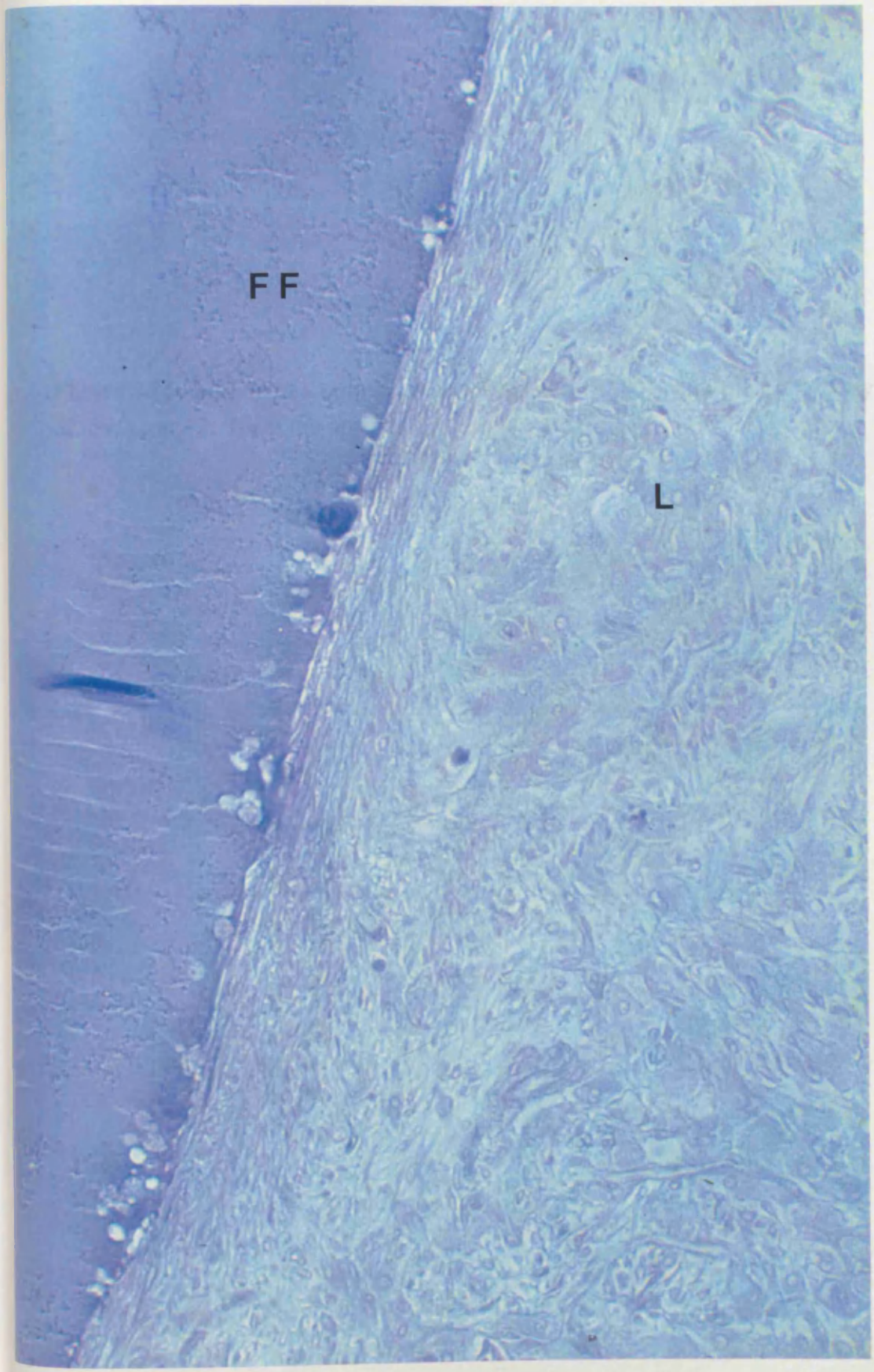

F F
L

Figure 3.25 Section of a corpus haemorrhagicum (x 100) from the right ovary of 59, group 2. Note the apparently normal structure.

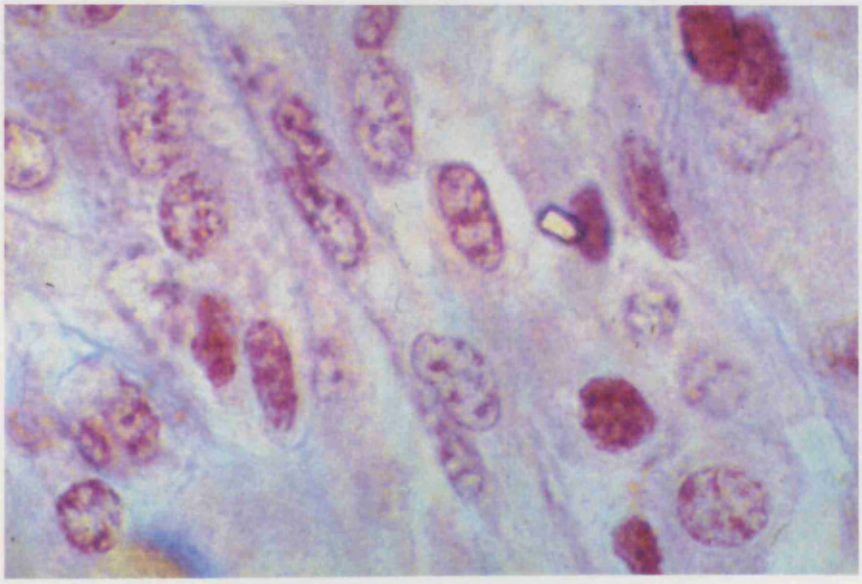


Figure 3.26 Section of a corpus luteum from the right ovary of cow 59, group 2 (x 100). Note the apparently normal structure.

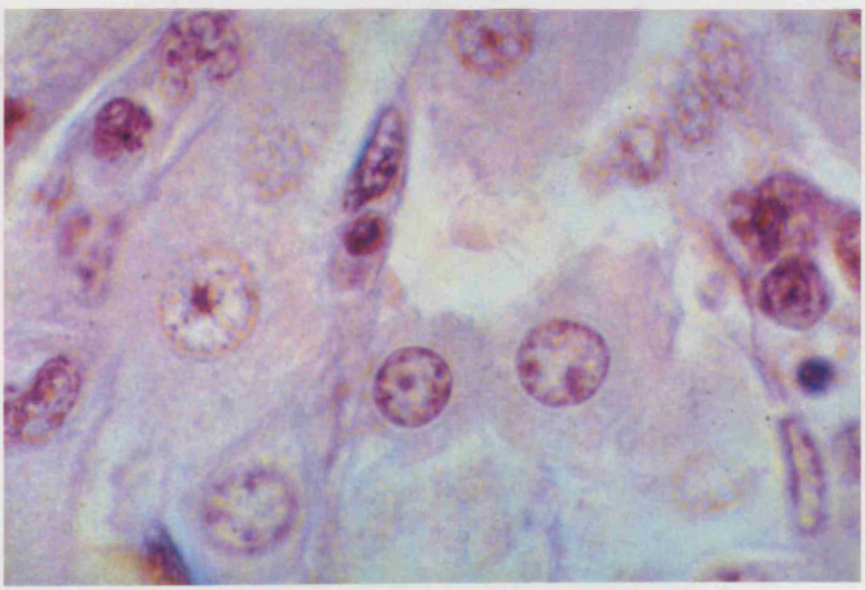


Figure 3.27 Control cow given 3000 i.u. PMSG but no prostaglandin (x 10). Note that multiple vesicular follicles have developed. Most are lined with one or few granulosa layers. Some luteal type tissue is evident (L) but a clear theca interna and externa is difficult to discern around most of these follicles.

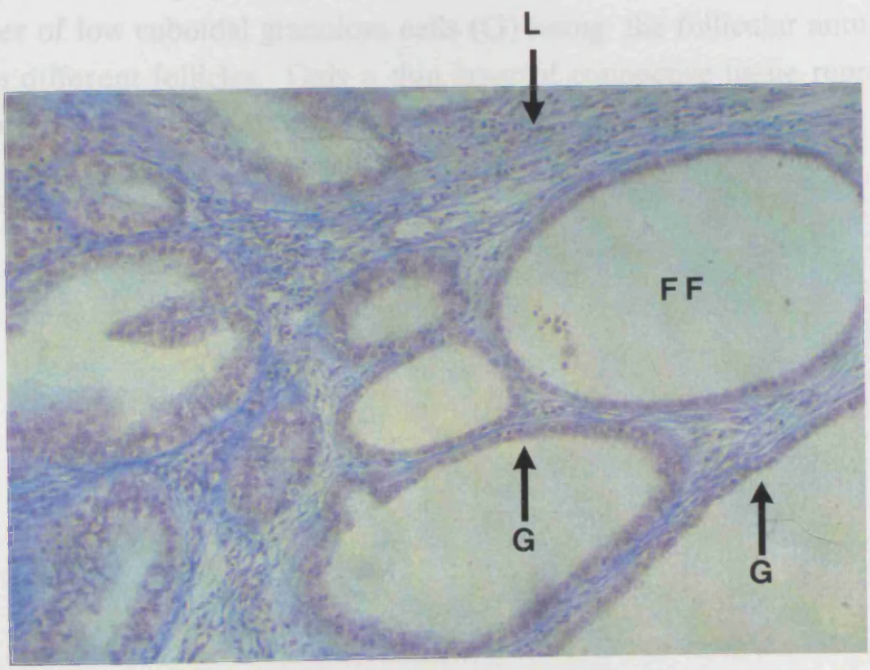
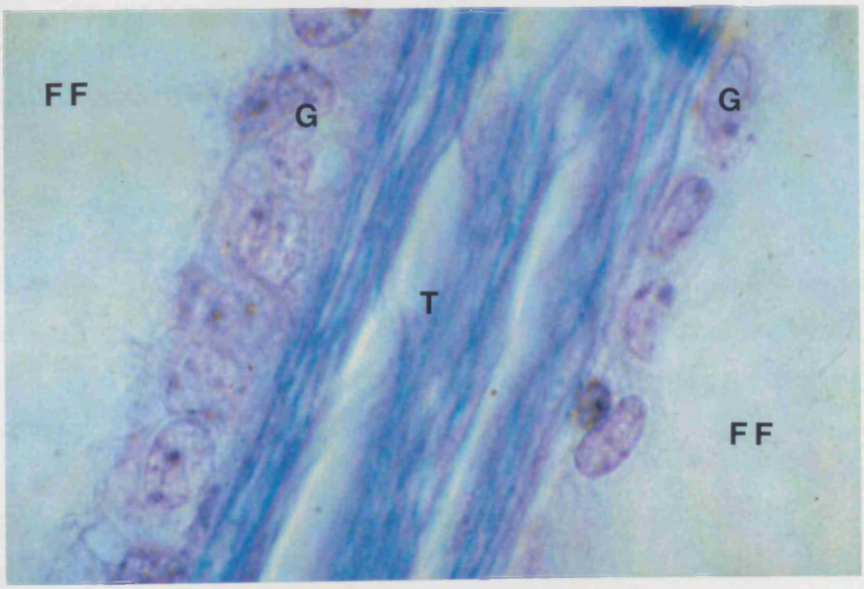

L
FF
G
G

Figure 3.28 High power picture of the same section (x 100). Note the single layer of low cuboidal granulosa cells (G) lining the follicular antum (FF) of two different follicles. Only a thin layer of connective tissue represents the theca between these follicles.


FF
G
G
T
FF

Chapter 4

Discussion

Discussion

Technical Problems

Certain problems were encountered when performing embryo collection, ultrasound examination and progesterone assay. It is worthwhile discussing these problems and the solutions.

The catheter and equipment was always tested and sterilized before use. All catheters were modified by widening the opening at the tip with a scalpel because maintaining a flow of liquid seemed to require a great deal of pressure on the medium syringes. Moreover, the state of the rubber cuff of the catheter was always checked by inflation with saline solution. This was done because often the cuff burst during flushing.

Embryo transfer required practical dexterity which I gained by practising with reproductive tracts, and later working with animals belonging to the Department. The first problem was to pass the introducer through the cervix. This exercise required the ability to move the cervix while keeping the catheter static. This was a cumbersome procedure with reproductive tracts but proved to be easier in the live animal, perhaps because of relaxation of the cervix after epidural anaesthesia. However, heifers with a narrow cervical canal still proved difficult. My previous experience with artificial insemination helped me to learn this technique.

Several other problems were encountered. One was positioning of the catheter in the uterine horn due to both the length of the horn in Friesian cows and the soft texture of a Franklin catheter. Having read the literature, the aim was to place the catheter 5 cm from the tip of the horn. It was difficult to feel the catheter end or the inflatable cuff. The solution was to mark the catheter at premeasured points. Coiling of the soft catheter was another minor problem. The catheter was introduced into the uterine horn and guided by the operator up to the marks. If resistance was felt, the assistant pulled the catheter back into the introducer. The operator then stretched the horn in front of the introducer while the assistant reintroduced the catheter. If no resistance was encountered, the normal procedure continued. Having correctly introduced the catheter it was necessary to optimise the volume of liquid needed to inflate the catheter cuff to prevent medium leakage but avoiding damage of the uterine walls by overinflation. The answer was to fill the cuff with 5 to 7 ml of fluid then check for

movement. If the catheter moved, the assistant added more fluid in 1 ml steps until no movement was perceived.

The techniques of ultrasonography were learned by attending ultrasound examinations carried out by staff members and postgraduate colleagues. Then the learning procedure became trial and error until my ability to identify the anatomical structures developed. Care of the equipment, especially the transducer, was of great concern considering the fragility of the transducer crystals. During the study many problems were pin-pointed. The animals reacted to rectal scanning with strong peristaltic movements which tired the operator. Frequent scanning irritated the rectum in two heifers in group 1, an effect not encountered when examining cows.

To allow frequent blood sampling the cows were canulated by Dr I. A. Jeffcoate. I blood sampled the cows with the help of collaborators. During blood sampling, problems arose mainly in connection with the cannula, making it difficult to obtain a sample. Sometimes the cannula was obstructed or twisted, or damaged by the movements of the cow. Whenever a problem arose, it was usually possible to mend the catheter or, as a last resort, bleed from the tail vein.

Progesterone assay was another technique which had to be learned. Especial care was taken during this assay because it involved handling radioactive material. Initially I practised only with the standard curve. Then I ran some plasma samples from the animals. The first results were not satisfactory, so the assay was repeated. A common problem was the accurate pipetting of the sample and reagents.

Animal Response after Prostaglandin Injection

The average time from prostaglandin injection until oestrus was approximately halved in the superovulated cycle when compared to the control cycle. Likewise, the time from prostaglandin injection until ovulation was approximately halved in the superovulated cycle. The duration of oestrous behaviour was one third longer in the superovulated cycle. Some exceptions were noted in both the control and superovulated cycle. Two cows were not observed in oestrus in the control cycle despite conscientious observation and knowledge of oestrus dates from the previous cycle which allowed prostaglandin treatment to be carried out at mid-cycle. Ovulation was observed in one cow (40) by ultrasound scanning. Retrospective progesterone analysis showed that the second cow (44) too, had ovulated.

Ultrasound scanning failed to pin-point this ovulation because oestrus behaviour was the signal to switch from daily to 12 hourly scanning. Scanning at daily intervals was obviously too infrequent because the ovulatory follicle was not recognised and ovulation was not identified.

One cow (73) showed oestrous behaviour for 54 hours in the control cycle, compared with the average of 16 hours. The shortest oestrous period was cow 274 which was observed in oestrus at only one oestrus detection session and thus showed oestrus for < 6 hours. One has to consider that either cow 40 or 44 may have displayed oestrus for an even shorter period than 6 hours, thus going unobserved. These findings do highlight the real problems encountered in predicting physiological events from oestrous behaviour.

In the superovulated cycle, oestrus started sooner after prostaglandin and lasted longer. One consequence of this was that ovulation occurred during oestrus. In cow (59) oestrous behaviour was first recognised immediately before ovulation was pin-pointed by ultrasound. While there is not sufficient data from only 11 animals to draw conclusions it seems those treated with 1500 i.u. PMSG were in oestrus for a shorter time than those treated with 3000 i.u. This remains true even after omitting cow 54 from the average figures. Cow 54 was treated with 3000 i.u and showed oestrus over 157 hours, intermittently. Other workers have reported plasma oestrogens in superovulated heifers 4 times higher at oestrus and 8 times higher 5-6 days postovulation (Booth et al., 1975). These findings may explain the increased duration and intensity of oestrus behaviour observed at the superovulated oestrus.

Response to Superovulation

Subsequent to treatment with 3000 or 1500 i.u. PMSG massive follicular growth occurred. Large numbers of follicles developed on each ovary. There was no obvious dose related response.

Evaluation of Ultrasound for Monitoring Follicular Growth and Ovulation

Large numbers of follicles developed on each ovary in response to PMSG. Some specific problems of monitoring follicular growth and ovulation in the superovulated cycle were identified. Lack of an orientation point made it impossible to follow the development of individual follicles. The old corpus luteum served as an orientation point but was obscured as it regressed and follicles grew. Repeat examinations were carried out in full awareness of this problem but even careful examination did not result in the same picture every time. Large ovaries changed position possibly due to the weight of tissue. Follicles in a superovulated ovary were extremely well outlined and surrounded by a thick wall. In view of subsequent evidence of follicular luteinisation, it is appealing to speculate that the thick walled appearance represented luteinisation. However, it is suggested that this appearance is simply due to pressure from the growing follicles (Pierson and Ginther, 1988). This pressure causes follicles to adopt irregular shapes and can make it difficult to determine the follicular diameter accurately.

Despite these problems, recent reports suggest ultrasound is a useful tool for monitoring growth of follicular populations after FSH or PMSG stimulation (Grasso et al., 1989; Driancourt et al., 1991). This study found it possible to monitor changes in populations of different sizes, from 5 mm upwards (Table 3).

In the control cycle a single follicle was monitored growing to a preovulatory size of 1.4 cm, (range 1.1 to 1.9 cm). This is in agreement with findings of Sirois and Fortune (1988). It was possible to identify ovulation of this follicle, and formation of the corpus haemorrhagicum is illustrated. There are several reports of ultrasound used to identify ovulation in the unstimulated ovary. Used in human medicine, ultrasound scanning identified ovulation in 80% of cycles studied (Queenan, O'Brien, Bains, Simpson, Collins and Campbell, 1980) and has been used successfully to diagnosis anovulation in infertile women (Liukkonen, Koskimies, Tenhunen and Ylostalo, 1984). The process of ovulation in the mare has been clearly

described (Ginther, 1986). Edmondson and co-workers reported preovulatory follicular growth to 1.9 cm in the bovine, followed by appearance of 'filling' images and loss of acoustic enhancement of the deeper structures as the corpus haemorrhagicum formed. Pierson and Ginther (1988) more simply defined ovulation as disappearance of a large follicle which was previously present. Omran (1989) presented a detailed description of ovulation, identifying changes in the shape of the follicle before ovulation and identifying rapid and slow evacuation of the follicle.

Identification of ovulation and corpora haemorrhagica in the superovulated cycle was also possible. However, this was not always easy and quantitation proved inaccurate. Where relatively few follicles developed in response to stimulation (such as in cow 94) it was much easier to detect ovulation. Ultrasound did not prove to be suitable for identifying a spread in the timing of ovulation after the LH peak though it was clear from post mortem that corpora haemorrhagica and corpora lutea coexisted in the same ovaries, suggesting that a spread in ovulation had occurred. Figure 3.4 shows an ovary on two successive days with what looks like further ovulation on the second day. However, one has to be aware that the appearance of corpora haemorrhagica change markedly immediately after ovulation (Omran, 1989) making it difficult to interpret this finding. It was certainly easier to identify ovulation in cows treated with 1500 i.u PMSG because the whole ovary decreased in size in addition to disappearance of follicles. We did not recorded the actual process of ovulation, due to the minimum interval between examinations being 12 hours. Where ovulation has been recorded, ovaries were monitored at 3-4 hourly intervals (Omran, 1989).

Postovulatory Changes Observed by Ultrasound

The structures of primary interest postovulation were the corpora lutea. Many studies of the corpus luteum in the bovine have been carried out using ultrasound (Pierson and Ginther, 1984; Edmondson et al., 1986; Pierson and Ginther 1988; Kastelic et al., 1990; Omran, 1989; Pieterse et al., 1990; Patel, 1990). An accurate method of correlating corpora lutea after superovulation with embryo recovery would be an aid to embryo transfer. However, in this study the number of luteal structures identified on the ovary prior to embryo flushing often correlated poorly with the number counted at post mortem. Pieterse and co-workers suggested ultrasound was inaccurate for the detection of young and old corpora lutea though accurate for the

detection of mid-cycle corpora lutea (Pieterse et al., 1990). We suggest that ultrasound is also inaccurate when there are > 4 mid-cycle corpora lutea on an ovary.

Follicular growth did not remain static post-ovulation. Follicular growth continued, resulting in large numbers of follicles at slaughter. This can be seen in Figure 3.6. The presence of large follicles after ovulation in PMSG stimulated cycles has been reported previously and incriminated as a cause of abnormal oocyte maturation, and poor embryo viability (Booth et al., 1975; Saumande, 1980; Monniaux et al., 1983). In Group 1, monitored until first oestrus post-flushing, luteal cysts were identified according to descriptions in the literature (Edmondson et al., 1986; Fissore et al., 1986; Carrol et al., 1990). Structures with this appearance were also identified at post mortem. This type of structure could not be confused with a corpus luteum at post mortem because the cyst was large, round and fluid-filled.

Embryo flushing was carried out in most of the living animals and repeated post mortem for all animals. Embryo recovery did not correlate well with number of corpora lutea observed at post-mortem. One likely explanation for this is the recurring problem of differentiating corpora lutea from anovulatory follicles. The ovulation rate may have been lower than the number of luteal structures suggested. In addition, the size of a superovulated ovary may interfere mechanically with ovum transport or cause ovulation into the peritoneum. This has been reported previously (Hunter, 1980). It has been reported that high peri-ovulatory oestrogen levels accelerate transport of embryos through the oviduct such that embryos are further down the horn than expected at flushing. Moreover, expulsion of the embryos into the vagina may occur (Booth et al., 1975).

Progesterone Levels

Control Cycle

Plasma progesterone levels during the control cycle were in general agreement with previous reports. Levels at the time of prostaglandin injection indicated the presence of a normal functional corpus luteum; this was confirmed by ultrasound examination. Previous studies have shown peak or mid-luteal levels of 6-10 ng/ml, which is in agreement with our findings. Levels dropped sharply 24 hours after prostaglandin injection to 1 ng/ml. During oestrus and ovulation, progesterone levels were low, at 0.2 ng/ml. Levels remained low for 96 h then rose to 1 ng/ml. Peak levels reached 6 to 18 ng/ml, in the presence of a mature corpus luteum (Stabenfeldt, et al., 1969; Berardinelli and Adair, 1989). The mean time from prostaglandin injection to the LH peak in this study was 81 hours, making the peak slightly later in this study than that previously reported (Jensen et al., 1982). In conclusion, the control cycle endocrine profiles were within normal physiological levels in all cows, as had been expected.

Superovulated Cycle

The mean progesterone level of 8 ng/ml at PMSG injection was within the physiological bounds expected at the mid-luteal phase prior to the superovulatory treatment and was similar to that in the control cycle. One cow (44) showed a high level of 17 ng/ml. As discussed above, this cow did not demonstrate oestrus during the control cycle, but it is not known whether these facts are connected

Subsequent to PMSG, progesterone rose in 7 of the 11 cows. Similar findings have been shown by others (Saumande, 1980; Jensen et al., 1982) and may be quite normal after PMSG treatment. Most cows showed a rise in levels prior to prostaglandin injection independent of dose of PMSG or subsequent number of corpora lutea. This rise might be caused by early luteinization of follicles present at the time of PMSG injection (Monniaux et al., 1983). Another possible explanation is that the LH content of PMSG enhanced progesterone production by the existing corpus luteum, as reported in *in vitro* studies (Martin et al., 1990).

Prostaglandin caused luteolysis with a subsequent drop in progesterone within 24 hours to 1.1 ng/ml in accordance with Jensen et al., (1982). The mean progesterone level during oestrus was 0.6 ng/ml which is

somewhat higher than that of the control cycle (0.2 ng/ml) but it is not known whether this difference is of importance. In this study progesterone levels started to increase 72 hours after ovulation (8 days after PMSG). Similar results have been reported (Boland, Foulkes, MacDonnell and Sauer, 1985). Progesterone levels in cows 73 and 895 started to rise 24 hours earlier (1.5 ng/ml, mean value) possibly suggesting that premature luteal structures were present on the ovaries of these cows. The LH surge at 37 hours after prostaglandin was slightly earlier than reported by others for a superovulated cycle, and much earlier than in a control cycle (Jensen et al., 1982; Bevers and Dieleman, 1987; Callesen et al., 1988).

Plasma samples from animals with postovulatory progesterone levels of more than 20ng/ml were reassayed, by diluting the samples 1:4. The values obtained were then multiplied by the dilution factor to allow a more precise determination of levels. The resulting values were as high as 80 ng/ml at flushing. High progesterone levels have been reported previously in both plasma and milk following superovulation (Booth et al., 1975; Greve 1980; Saumande, 1980; Boland et al., 1985). In this study, high progesterone levels did not necessarily coincide with an abnormal response. For example, cow 73 had a plasma progesterone concentration of 80 ng/ml and yielded 14 embryos and at post mortem examination presented 21 corpora lutea.

An Explanation for the High Progesterone Levels

Post mortem and histological examination of the ovaries was carried out to try and elucidate the reasons behind findings of high progesterone production but low embryo recovery. Follicles, luteinised follicles, corpora haemorrhagica, corpora lutea and luteal structures which were possibly luteinised follicles were identified at post mortem. Histology showed that the follicles examined were not normal but showed degeneration of the granulosa and theca interna. Atresia has been described as invasion of the granulosa layer by vascularised connective tissue, with subsequent shedding of the granulosa layer. In advanced atresia the basement membrane is transformed into a thick layer of hyaline substance. The large cells of the theca interna form into cords separated by smaller fusiform cells. These theca cells are identical to theca lutein cells but no information was found about the progesterone secreting ability of these cells (Bloom and Fawcett, 1969).

Partially luteinised follicles showed patches of luteal tissue, no visible granulosa layer and an obvious follicular antrum lined by basement

membrane. After PMSG stimulation in sheep a range of partially and completely luteinised follicles were identified, similar in description to luteinised follicles in this study. Cran reported that many follicles lost the granulosa layer, as evidenced by degenerate cells in the antrum and lack of cells internal to the basement membrane (Cran, 1983). Fusiform cells thought to be of thecal origin lined the antrum. In this study, cells with this same appearance lined the antrum of some luteinised follicles. Cran (1983), used 3B-hydroxysteroid dehydrogenase activity to identify progesterone production by the luteinised follicles and enzyme activity was found to be widespread. By extrapolation, this finding offers a likely explanation for the high progesterone production but low ovulation rate and embryo yield. Luteinised follicles and corpora lutea are probably producing progesterone.

Some areas of the theca derived cells differentiate into luteal type cells and some areas adopt this fusiform cell appearance. This probably reflects the distribution of LH receptors in follicles at different stages of development prior to PMSG.

One category of structures seen at post mortem defied confident identification. Those structures were solid luteal tissue with large flat papillae-like crowns and no antrum. Such structures are visible in Figure 3.7b, circling the center of the ovary. The papillae-like crowns could be flattened luteal tissue due to crowding and pressure within the superovulated ovary rather than ovulatory papillae. This problem has been recognised previously (Monniaux et al.,1983; Cran, 1983). Monniaux and co-workers recognized luteal structures which had the appearance of pseudo-corpora lutea and reported that it was often impossible to distinguish between luteinised follicles and corpora lutea. The problem of identification could only be overcome by post mortem 48 hours after ovulation or serial histological sectioning to identify a degenerate oocyte. These workers ranked identification of ovulation by counting corpora lutea with papillae at 48 hrs after LH peak as 85% accurate, but ranked counting all luteal structures as only 42% accurate. They even suggested that the number of embryos recovered may be a better estimator of ovulatory response than counting luteal structures, such was the confusing influence of anovulatory follicles.

The corpora haemorrhagica and corpora lutea identified in the present study, after PMSG treatment, had a normal histological appearance. However corpora haemorrhagica and corpora lutea are not usually present on the ovary at the same time, unless a spread in the time of multiple ovulation has occurred.

Problems Encountered Using PMSG for Superovulation

PMSG is cheap, easy to administer and stimulates follicular growth. However, it has an active LH component which is said to cause premature ovulation of large follicles and luteinisation of atretic follicles (Monniaux et al., 1983; Callesen et al., 1987). Other peri-ovulatory endocrine events are abnormal. It has been reported that the endogenous LH surge is inhibited in 16% of cattle after PMSG (Bevers and Dieleman, 1987). The half-life of PMSG is around 5 days (Schams et al., 1978). One effect of this is stimulation of a second wave of follicular growth soon after ovulation. High oestrogens produced at this time are said to be detrimental to oocyte maturation and gamete transport (Booth et al., 1975). However, at present the alternative is using FSH preparations which are expensive, must be administered in up to 10 doses, and are still likely to have a variable LH contaminant.

Conclusions

1. Ultrasound examination was useful to monitor follicular response to superovulation.
2. Ovulation could be identified but not accurately quantified in the superovulated cycle.
3. In general, crowding of the ovaries made it difficult to follow individual structures, whether follicular or luteal.
4. The number of corpora lutea identified by ultrasound correlated poorly with post mortem findings or embryo recovery.
5. Post mortem revealed a complex situation which was basically ovulation failure after PMSG stimulation. Structures which looked like follicles showed early degenerative changes. Many follicles showed gross luteinisation. In retrospect, some structure identified as corpora lutea may have been anovulatory, luteinised follicles.
6. Post-ovulatory plasma progesterone levels correlated poorly with embryo recovery. This was considered to be due to progesterone production by anovulatory follicles.
7. The same factors which rendered progesterone levels, laparotomy, laparoscopy or rectal palpation poor predictors of embryo yield, namely inability to differentiate luteinised anovulatory follicles from corpora lutea, applied to the use of ultrasound. This problem is a function of using PMSG as a superovulatory agent, probably an effect of the LH component.
8. In conclusion, with the results drawn together, this study presents a summary of the various problems known to plague effective embryo transfer ie. failure to detect oestrus, variable follicular development, variable oestrus duration, failure of ovulation, ovulation extended over an abnormal period, failure to recover the predicted number of embryos and recovery of poor quality embryos. As such it is a valuable lesson to this author.

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