

# **An Alternative Approach to Premature Luteal Regression**

By

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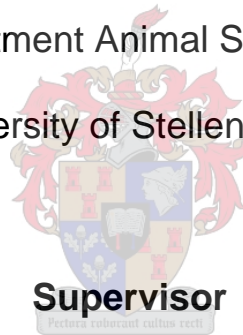
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## Dedicated to the influences in my life

My Saviour the Lord Jesus Christ,

My wife and inspiration Marelize,

Our supporting parents and

Our family and friends who make life

“a box of chocolates ...”



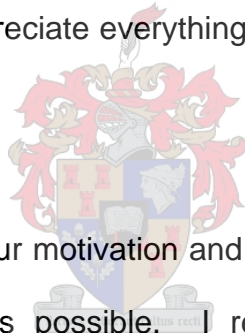
*"Cogito ergo sum"*

**René Descartes**

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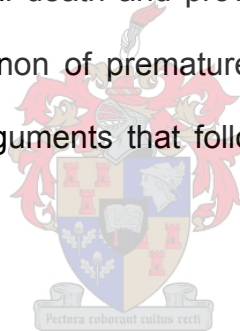
To Marelize, thank you for your motivation and for allowing me the time away from you to have made this possible. I really love you so much and appreciate your patience with me.

To my friend's Doc, Joss for all the encouragement I will never forget it.

# ABSTRACT

Premature luteal regression occurs on average in 30% of superovulated sheep ewes. This phenomenon occurs early in the cycle before the embryo's can be collected and is a major contributor to failure in embryo transfer programs. This research was done to understand the physiological mechanisms involved.

Chapter two provides a general background of the physiology of natural luteolysis and the maternal recognition of pregnancy. The chapter introduces some new concepts on the topic of cell death and provides a recent literature review on research done on the phenomenon of premature luteal regression. This chapter forms the base of ideas and arguments that follows in the two studies containing new original work in this field.



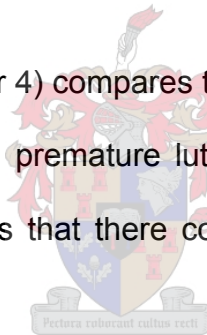
The research contained in this thesis comprises of two *in vivo* studies. The first study (Chapter 3) compare premature luteal regression to Prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ) induced regression with emphasis on the changes in levels of the steroid hormones progesterone ( $P_4$ ) and estradiol -  $17\beta$  ( $E_2-17\beta$ ) and changes in structure and ultra structure. The following conclusions were made:

1. Premature luteal regression is not merely inadequate luteal support, but indeed early luteal regression, since seasonal influences could merely be nutritional influences, and a definitive increase in  $P_4$  were recorded in animals exhibiting the phenomena.

2. Nutritional influences could play a role, but the type and quality of nutrients and mechanism involved, is still unclear.
3.  $\text{PGF}_{2\alpha}$ -induced regression differs from premature luteal regression in that:
  - a) The progression of functional and structural regression in  $\text{PGF}_{2\alpha}$  - induced regression is slower than in premature luteal regression.
  - b) Regressed corpora lutea do not occur with normal functioning corpora lutea.
4. There is a distinct second  $\text{E}_2$ - $17\beta$  peak preceding the decline in  $\text{P}_4$  in animals that exhibits signs of premature luteal regression.

A threshold initiating premature luteal regression was not established.

The second study (Chapter 4) compares the changes in the ovine  $\beta$  estradiol -  $17\beta$  receptor ( $\text{oER}\beta$ ) between premature luteal regression and  $\text{PGF}_{2\alpha}$  induced regression. The study concludes that there could be a potential role for  $\text{oER}\beta$  in premature luteal regression.



The findings of these two studies raise some questions about the conventional perception that early release of  $\text{PGF}_{2\alpha}$  is the cause of premature luteal regression. The thesis concludes in a hypothesis (Chapter 4) explaining the phenomenon.

# OPSOMMING

Premature luteale regressie kom gemiddeld in 30% van gesuperovuleerde skaap-ooie voor. Die verskynsel kom vroeg in die siklus voor, voor die embryos gekollekteer kan word, en is een van die belangrikste oorsaake van mislukkinge in 'n embryo-oorplaasingsprogram. Die huidige navorsing poog om die fisiologiese meganismes betrokke by premature luteale regressie te verstaan.

Hoofstuk twee verskaf 'n algemene agtergrond van die fisiologiese aspekte betrokke by natuurlike luteale regressie en maternale herkenning van swangerskap. Die hoofstuk stel nuwe konsepte voor oor sel afsterwing en verskaf 'n opgedateerde literatuuroorsig met betrekking tot die navorsing wat in die veld oor die verskynsel gedoen is. Die hoofstuk vorm die basis vir die idees en argumente, wat volg in die twee studies en wat oorspronklike nuwe navorsing bevat oor die onderwerp.

Die navorsing in die tesis bestaan uit twee *in vivo* studies. Die eerste studie (Hoofstuk 3) vergelyk premature luteale regressie en prostaglandien  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ) geïnduseerde regressie met 'n klem op die vlakke van die steröïedhormone progesteron ( $P_4$ ) en estradiol -  $17\beta$  ( $E_2-17\beta$ ) en veranderinge in die mikroskopiese struktuur en ultra struktuur van die corpus luteum. Die studie bevind:

1. Premature luteale regressie is nie slegs onvoldoende luteale funksie nie, maar vroë luteale regressie aangesien seisoenale invloede eitlik voedings

invloede kan wees en  $P_4$  gestyg het in diere waar die verskynsel voorgekom het.

2. Voeding kan 'n rol speel maar die tiepe en gehalte van die voedingstowwe en die meganismes betrokke is nie duidelik nie.
3.  $PGF_{2\alpha}$  - ge-induseerde regressie verskil van premature regressie in dat:
  - a) Die verloop van funksionele en strukturele regressie is stadiger in  $PGF_{2\alpha}$  - ge-induseerde regressie in vergelyking met premature luteale regressie.
  - b) Corpora lutea wat regressie ondergaan het kom nie voor saam met corpora lutea wat normal voorkom nie.
4. Daar die 'n duidelike tweede piek van  $E_2-17\beta$  gaan die afname in  $P_4$  vooraf in diere waar premature regressie voorkom.
5. Daar is nie geslaag om 'n drempel vas te stel waar premature regressie ge-inisieer word nie.



Die tweede studie vergelyk die veranderinge in estradiol- $17\beta$  reseptore ( $oER\beta$ ) in die skaap tydens premature luteale regressie en  $PGF_{2\alpha}$  geinduseerde regressie. Die studie bevind dat daar 'n moontlike rol is vir  $ER\beta$  in premature luteale regressie.

Die bevindinge van die twee studies bevestig die konvensionele opvatting dat vroeë vrystelling van  $PGF_{2\alpha}$  verantwoordelik is vir premature luteale regressie. Die tesis sluit af met 'n nuwe hipotese om die verskynsel te verduidelik.

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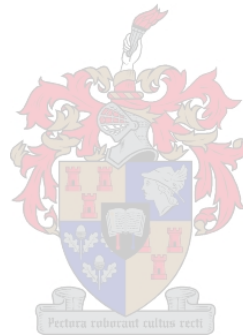
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# List of Abbreviations

c-AMP	Adenosine 3'-5' cyclic monophosphate
RFLP's	Chromosome restriction fragment length polymorphisms
R <sup>2</sup>	Coefficient of determination
R <sup>2</sup> <sub>adjusted</sub>	Coefficient of determination adjusted for small sample size
CV	Coefficient of variation
CL	Corpora lutea
Caspases	Cystein aspartate-specific proteases
C <sub>21</sub> P-450 <sub>SCC</sub> C <sub>21</sub>	Cytochrome P-450 side chain cleavage
C <sub>27</sub> P-450 <sub>SCC</sub>	C <sub>27</sub> Cytochrome P-450 side chain cleavage
P-450 <sub>AR</sub>	Cytochrome P-450 aromatase
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonucleic acid endonuclease I
DFF	DNA fragmentation factor
PGFM	13,14-dihydro-15-keto-PGF <sub>2</sub>
ET	Embryo transfer
E <sub>2</sub> -17β	Estradiol - 17β
ECP	Estradiol Cypionate
E <sub>2</sub> -17βR	Estradiol - 17β receptors
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
EDB	Estradiol benzoate
eCG	Equine chorionic gonadotropin
FDA	Food and drug administration
FSH	Follicle stimulating hormone
FSH-P	Follicle stimulating hormone (pituitary extract)
rFSH	Follicle stimulating hormone (recombinant)
GC/MS	Gas chromatography / mass spectrometry
GnRH	Gonadotropin releasing hormone
H-I	Hemagglutination-inhibition assay
HbsAg	Hepatitis B surface antigen
HDL	High-density lipoproteins
hCG	Human chorionic gonadotropin
HCV	Human cytomegalo virus
HIV	Human immune deficiency virus
hMG	Human menopausal gonadotropin
3β -HSD	Δ <sup>5</sup> -3β -hydroxysteroid dehydrogenase: Δ <sup>5-4</sup> - isomerase
17β-HSD	17β-hydroxysteroid dehydrogenase
20α-HSD	20α-hydroxysteroid dehydrogenase
20αOHP	20α-hydroxyprogesterone
ICE	Interleukin-1β converting enzyme
IGF	Insulin like growth factor
IGFR	Insulin like growth factor receptor
IGFBPs	Insulin like growth factor binding proteins
IVF	<i>In vitro</i> fertilization
i.v.	Intravenously
LDL	Low-density lipoproteins



LDLR	Low-density lipoprotein receptor
LH	Luteinizing hormone
LHR	Luteinizing hormone receptors
MT	Melatonin
MTR	Melatonin receptors
mRNA	Messenger ribonucleic acid
MUP	4-Methylumbelliferyl Phosphate
MU	Methylumbelliferone
MEIA	Microparticle enzyme immunoassay
MV	Microvilli
NCCLS	National committee for clinical laboratory standards
NAD <sup>+</sup>	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogenase
NE	Nuclear envelope
NT	Nuclear transfer
oERβ	Ovine β Estradiol - 17 β receptor
o-TP-1	Ovine trophoblast protein 1
Ox	Oxytocin
OxR	Oxytocin receptors
P-lase	Phospholipases
P-lase A <sub>1</sub>	Phospholipase A <sub>1</sub>
P-lase A <sub>2</sub>	Phospholipase A <sub>2</sub>
P-lase C	Phospholipase C
P-lase D	Phospholipase D
PK-A	Phosphokinase A
PK-C	Phosphokinase C
PARP	Poly (ADP-ribose) polymerase
P <sub>4</sub>	Progesterone
PR	Progesterone pessaries
PGE <sub>2</sub>	Prostaglandin E <sub>2</sub>
PGF <sub>2</sub>	Prostaglandin F <sub>2</sub>
PGF <sub>2α</sub>	Prostaglandin F <sub>2α</sub>
PGF <sub>2α</sub> R	Prostaglandin F <sub>2α</sub> receptor
PGG <sub>2</sub>	Prostaglandin G <sub>2</sub>
PGH <sub>2</sub>	Prostaglandin H <sub>2</sub>
PES	Prostaglandin endoperoxide synthase
PES <sub>cyc</sub>	Prostaglandin endoperoxide synthase cyclooxygenase
PES <sub>per</sub>	Prostaglandin endoperoxide synthase peroxidase
PES <sub>red</sub>	Prostaglandin endoperoxide reductase
PES <sub>iso</sub>	Prostaglandin endoperoxide isomerase
PK-C	Protein kinase C
RV	Reaction vessel
RIA	Radioimmunoassay
SST	Serum separating tube
SER	Smooth endoplasmic reticulum
SEM	Standard error of mean
SD	Standard deviation
SREBP	Sterol regulatory element binding protein
<sup>3</sup> H-PGF <sub>2α</sub>	Tritium labelled prostaglandin F <sub>2α</sub>

# Chapter 1

## 1.1 Introduction

“In the midst of life we are in death” – ID Bowen [1]; from day to day, season to season we are reminded of this strange paradox. Without death there can be no life, as we know it. In an ecological context, life and death form essential parts of a natural cycle.

The estrous cycle of farm animals and menstrual cycle of humans is a beautiful illustration of these philosophical ideas by the inventors of the cellular process known as apoptosis. For a female to conceive a follicle must develop to a mature dominant follicle. During this development phase granulosa cells reproduce and are born by means of a cellular process of cell division, or rather multiplication, called mitosis. After ovulation the nurturing granulosa and theca cells differentiate into progesterone producing granulosa lutein cells and theca lutein cells that specialize in the production of progesterone. The remains of the follicle after ovulation, are used to form the vital structure known as the corpus luteum. If the female fails to conceive, the cells in the corpus luteum must die or regress to make another cycle possible. Failure or imbalance of this natural cycle of birth, change and death of these interesting cells will make the creation of a new living being impossible.

Recent interest in the fate of the corpus luteum was generated by the abnormal phenomenon of premature luteal regression seen in superovulated females. Premature luteal regression can be defined as regression of the corpus luteum earlier in the estrous cycle before the onset of natural luteal regression were regression takes place between day 13 and 14 of the cycle in the sheep. Superovulation can be defined as an increase in the amount of ovulating follicles in females by increasing circulating gonadotropins. This can be accomplished by supplementing exogenous gonadotropins or stimulating release of endogenous gonadotropins.

Superovulation is one of the ways for improving reproductive efficiency. We live in an era in which new ways to rapidly propagate or design domestic animals are being developed. These new techniques take advantage of procedures in cellular and molecular biology, including the identification of genes or chromosome restriction fragment length polymorphisms (RFLP's) that are associated with important production traits, and the ability to transfer genes into the genome of domestic animals. These techniques assist in the direct and rapid selection of a particular trait. The molecular approach compliments techniques in reproductive physiology, like *in vitro* fertilization (IVF), multiplication of embryos by cloning and splitting, nuclear transfer (NT), the production of transgenic animals and embryo transfer (ET).

Premature luteal regression occurs on average in 30% of superovulated sheep ewes. The phenomenon results in the loss of embryos in these animals. This impacts in two ways : a) it slows progress in these

programs and b) the phenomenon adds to the cost of these programs. Prevention of the phenomenon or the reduction of the incidence of premature luteal regression will be an important contribution in assuring future survival of these programs and accelerating development in this exciting field. Prevention or reduction in incidence can only be achieved if the physiological mechanism of the phenomenon is known.

The aim of this thesis is:

- 1 To try and unravel the mechanism behind premature luteal regression.
2. To formulate a new hypothesis that explain the mechanism behind the phenomena.

## 1.2 References

1. **Bowen ID, Bowen,S.M., Jones,A.H.** Mitosis and apoptosis matters of life and death. London: Chapman & Hall; 1998

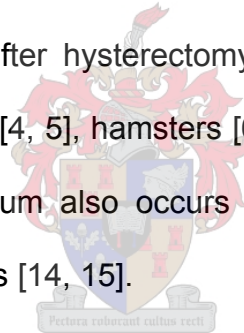
## Chapter 2

### General Background and Literature Review

#### SECTION A : NATURAL LUTEOLYSIS

##### 2.1 Uterine involvement in luteal regression

The first evidence of uterine involvement in the luteolytic process was presented in 1923, when Loeb [1] reported that removal of the uterus (hysterectomy) lengthened the lifespan of the corpus luteum in guinea pigs. Extension of the lifespan of the corpus luteum after hysterectomy was later also demonstrated in pseudo-pregnant rat [2, 3], mice [4, 5], hamsters [6-8] and rabbits [9-11]. Temporary maintenance of the corpus luteum also occurs after hysterectomy in cattle [12], sheep [12], swine [13] and horses [14, 15].



In other species, presence of the uterus is not required for luteolysis. Most notably, in several species of primates, including the rhesus monkey [16], cynomolgus monkey [17], and humans [18, 19], removal of the uterus has no effect on normal cyclic activity. Also, hysterectomy has no effect on the life span of the corpus luteum in the opossum [20], dog [21, 22], or ferret [23]. In contrast to pseudopregnant animals, the normal estrous cycle of the rat [24], mouse [25] and hamster [26, 27] is not prolonged by hysterectomy.

Although there is variation among species in the period that corpora lutea are maintained in hysterectomized animals, corpora lutea generally retain both structural and functional integrity for at least the duration of a normal pregnancy in guinea pigs [28-30], sheep [31-33], cattle [34, 35], pigs [13, 36-38] and rats [39]. In rabbits or horses, luteal function is maintained at levels lower than those established during the luteal phase after hysterectomy [14, 40-42].

In many species, effects of the uterus on luteal function are exerted only on one side of the median plane. Unilateral hysterectomy (removal of one uterine horn) in sheep [43, 44], cattle [45], guinea pigs [46-48], pseudopregnant hamsters [49], and pseudopregnant rats [50] results in luteal regression in the ovary adjacent to the intact horn, but prevents regression in the opposite ovary. The rabbit and horse are exceptions in that corpora lutea will undergo regression despite unilateral removal of the adjacent horn [15, 51]. It is thus apparent that the luteolytic factor emanating from the uterine horn in most species (with the exception of the rabbit and the horse), exerts its effect only on the adjacent ovary. Therefore, it is unlikely that the luteolytic factor of uterine origin is transported from the uterus to the ovary through the systemic circulation in these species. This, however, is not true for the rabbit and the horse.

Corpora lutea are maintained when the vascular, nervous and lymphatic connections between the ovary and the uterus are severed in ewes [44], cows [52], guinea pigs [53-55], and pseudopregnant rats [47, 50, 56]. Autotransplantation of either the uterus or ovary, while leaving the other organ *in situ*, results in extended luteal function in ewes [57, 58] and guinea pigs [59-61]. However, when the uterus

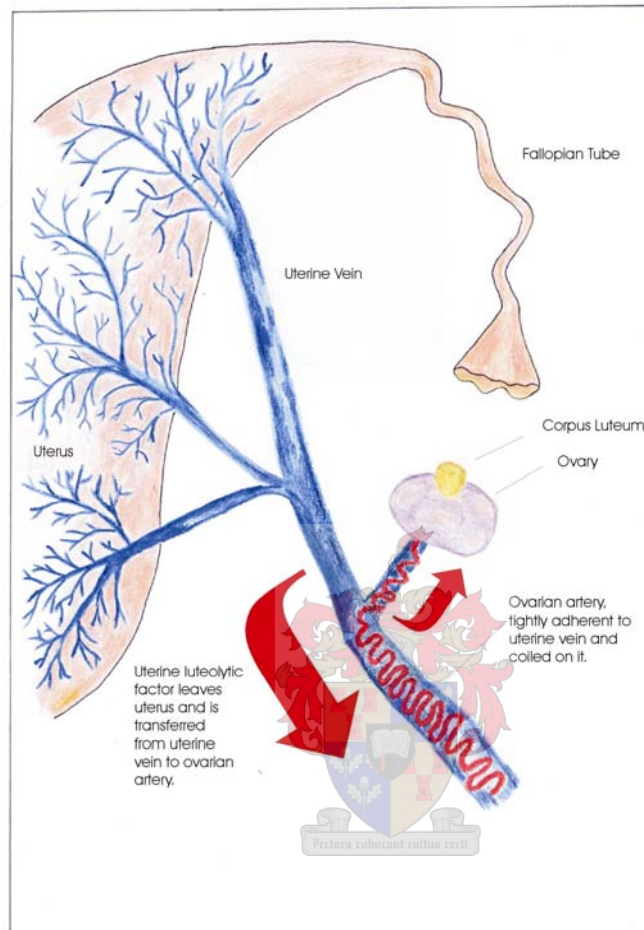
and ovaries are transplanted to the neck of the animal as a unit, normal ovarian behaviour continues in ewes [62].

The uterine luteolytic factor appears to pass directly from uterine vein to the ovarian artery in the ewe. This conclusion is based primarily on the results of the elegant studies of Ginter and colleagues [63]. If the uterine vein draining the intact horn of the unilaterally hysterectomized ewes is anastomosed to the uterine vein from the hysterectomized side, only corpora lutea on the hysterectomized side will regress [64, 65]. Reversal of the normal pattern of luteal regression in unilaterally hysterectomized ewes also occurs if the ovarian artery supplying the ovary on the intact side is anastomosed to the artery on the hysterectomized side [64, 65]. A similar effect of uterine venous and arterial anastomoses has been described for cattle [66].



Although there do not appear to be vascular connections between the uterine vein and ovarian artery, there are regions of extensive contact between these vessels throughout the broad ligament and up to their respective junctions with the Vena cava and aorta [67, 68] (Figure 1). The ovarian artery is extremely tortuous, and collateral channels and venules separate from the uterine vein, wrapping around the artery and thus increasing the area of contact between the two vessels [69]. The vessels share a common tunica adventitia and have thinner walls in the regions where they make contact [70]. Similar gross morphologic venoarterial relationships have been observed in guinea pigs, rats, hamsters [71], pigs [67] and cows [72].

Noteworthy is that there is very little contact between the uterine vein and ovarian artery in the rabbit and horse, two species in which the uterine luteolytic effect is not exerted locally [67, 71, 73].



**Figure 1** Diagram of the utero-ovarian vasculature in the sheep and the route the uterine luteolytic factor travels from the uterus to the ovary [68].

## 2.2 Identification of $\text{PGF}_{2\alpha}$ as the uterine luteolytic factor

Although the luteolytic effect of the uterus was recognized more than 60 years ago, the biochemical signal(s) involved were difficult to elucidate. A major breakthrough occurred in 1969 when the luteolytic actions of  $\text{PGF}_{2\alpha}$  were demonstrated in pseudopregnant rats [74]. This initial observation was rapidly extended to other species, and  $\text{PGF}_{2\alpha}$  has been shown to exert a luteolytic effect in



cattle [75-78], sheep [79-81], guinea pigs [82], goats [83], horses [84-86], pseudopregnant mice [87], hamsters [88], rabbits [89, 90], and to a limited extent in swine [91, 92].

On the other hand in rhesus monkeys [93, 94], humans [95, 96] and dogs [97], species in which the uterus has no clear luteolytic effect, there is a transitory suppression in the secretion of progesterone ( $P_4$ ) after short term infusions (up to 12 hours) with  $PGF_{2\alpha}$  during the luteal phase of the cycle. In fact, premature luteal regression can be induced in rhesus monkeys [98] and dogs [99] by more prolonged treatment regimes with high levels of  $PGF_{2\alpha}$ . Extremely high doses of  $PGF_{2\alpha}$  are ineffective in causing luteal regression in the cat [100]. If endogenous  $PGF_{2\alpha}$  is involved in the normal luteolytic process in these species, it is probably not of uterine origin.

Although the treatment of animals with  $PGF_{2\alpha}$  causes premature luteolysis, this does not conclusively prove that it is the uterine factor involved in the process. However, considerable evidence has accumulated to suggest that  $PGF_{2\alpha}$  is the factor of uterine origin responsible for luteolysis in domestic species and rodents.

Treatment of intact animals with inhibitors of prostaglandin synthesis, such as indomethacin and aspirin, will block spontaneous luteolysis in cattle, sheep and guinea pigs. Length of pseudopregnancy can be prolonged in rabbits, rats and mice by treatment with indomethacin. Thus, the synthesis of prostaglandins (presumably  $PGF_{2\alpha}$ ) appears to be required for luteolysis in these species [101, 102].

Perhaps the best evidence of  $\text{PGF}_{2\alpha}$  as the uterine luteolytic factor, is the fact that spontaneous luteal regression can be prevented by active or passive immunization against  $\text{PGF}_{2\alpha}$  of sheep [103, 104], cattle [105] and guinea pigs [28].

$\text{PGF}_{2\alpha}$  has been isolated from endometrial tissue of several species, and its concentration is maximal during the period of luteal regression in cattle [106], sheep [107], guinea pigs [108], horses [109], swine [110], pseudopregnant rats [111] and pseudopregnant rabbits [112].

Based on the available data, it seems reasonable to conclude that  $\text{PGF}_{2\alpha}$  is the factor, of uterine origin, responsible for luteolysis in large domestic species and rodents.

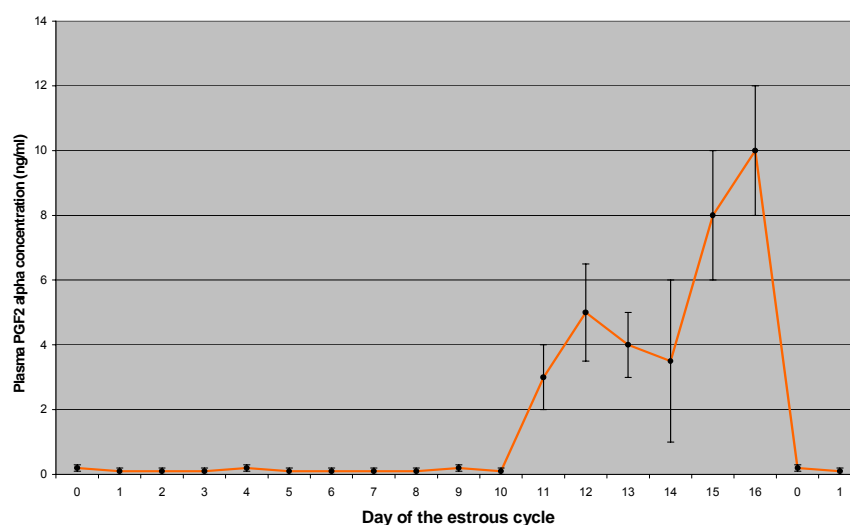
### 2.3 Pattern of $\text{PGF}_{2\alpha}$ secretion

With the discovery in 1970 that  $\text{PGF}_{2\alpha}$  is luteolytic in sheep [113], interest immediately arose to the pattern of  $\text{PGF}_{2\alpha}$  during the cycle. However, it was soon discovered that assessment of uterine  $\text{PGF}_{2\alpha}$  secretion *in vivo* is complicated by two factors. First, it must be measured in blood collected from the utero-ovarian artery, or uterine vein. The peripheral  $\text{PGF}_{2\alpha}$  concentrations is not a reliable index of uterine secretion rate, because a variety of tissues contribute to this pool. Prostaglandins are also almost completely metabolized in one passage through the lungs [114]. Secondly, because  $\text{PGF}_{2\alpha}$  is secreted episodically [115-119], blood samples must be collected relatively frequently to determine the actual secretory pattern. Investigators have used a number of approaches to overcome these technical problems, including blood collection via laparotomy [115], a cannula placed in the utero-ovarian vein

[117, 120-124], or autotransplantation of the ovary and uterus to the neck of the animal [115, 116, 125].

In early experiments,  $\text{PGF}_{2\alpha}$  was measured in a flock of sheep once daily to determine the secretory pattern. It was found that from day 2 to day 10 of the estrous cycle, there is little or no  $\text{PGF}_{2\alpha}$  release from the uterus [115, 120, 124, 125].  $\text{PGF}_{2\alpha}$  first starts to rise between day 11 and 13 of the cycle, and it increases over the next 2 to 3 days [112, 117-122]. The mean  $\text{PGF}_{2\alpha}$  concentration [126-128], in a group of animals when measured once daily show an increase late in the luteal phase, it remains relatively stable during the early follicular phase and then increases again to peak just before estrus [126] (Figure 2).

Very early it became clear that there was a high degree of variation in the mean concentration of  $\text{PGF}_{2\alpha}$  measured in the uterine vein in a flock of sheep at the time of luteolysis. This can clearly be seen in Figure 2, as a high standard error of the means (SEM's) from day 11 to 16 of the estrous cycle in the ewes was observed.



**Figure 2** The daily mean plasma  $\text{PGF}_{2\alpha}$  concentration in the uterine vein during the estrous cycle of the ewe (n=148) [126].

Horton & Poyser (1972) using a radio-immuno assay (RIA) method, reported that the concentration of  $\text{PGF}_{2\alpha}$  in the sheep ranges from 4 - 40 ng/ml in the uterine vein around the time of luteolysis [127]. This was confirmed by McCracken & coworkers (1976) using gaschromatography and mass spectrophotometry [128]. This would amount to a maximum secretion rate of 25  $\mu\text{g}$  of  $\text{PGF}_{2\alpha}$  per hour into the uterine vein. It was estimated that the counter current transfer between the uterine vein and the ovarian artery was about 10%. This gives an effective dose of approximately 2.5  $\mu\text{g}$  per hour that reaches the ovarian artery. Using the autotransplanted uterus and ovary as a model, it was shown that a constant infusion of 2.0 to 2.5  $\mu\text{g}$   $\text{PGF}_{2\alpha}$  per hour, for 6 to 18 hours into the arterial supply of the ovary, can cause complete luteolysis in sheep [128].

The concentration of  $\text{PGF}_{2\alpha}$  in the ovarian artery was based on the assumption that the counter current transfer between the uterine vein and the ovarian artery was 10%. Using tritium labelled prostaglandin  $\text{F}_{2\alpha}$  ( $^3\text{H-PGF}_{2\alpha}$ ), McCracken *et al* (1976) found that the transfer between the uterine vein and the ovarian artery was not 10% as assumed but only 2% [128].

This was confirmed by the laboratory of Scaramuzzi [129]. Thus, the concentration of  $\text{PGF}_{2\alpha}$  in the ovarian artery should range between 80 and 400 pg/ml. This was confirmed by actual measurements of  $\text{PGF}_{2\alpha}$  in the ovarian arterial supply [128, 129], when a more sensitive RIA was developed. These new facts implicated an effective dose of 0.5  $\mu\text{g}$  of  $\text{PGF}_{2\alpha}$  per hour in the ovarian arterial supply. Using the autotransplanted uterus and ovary as a model, it was shown that

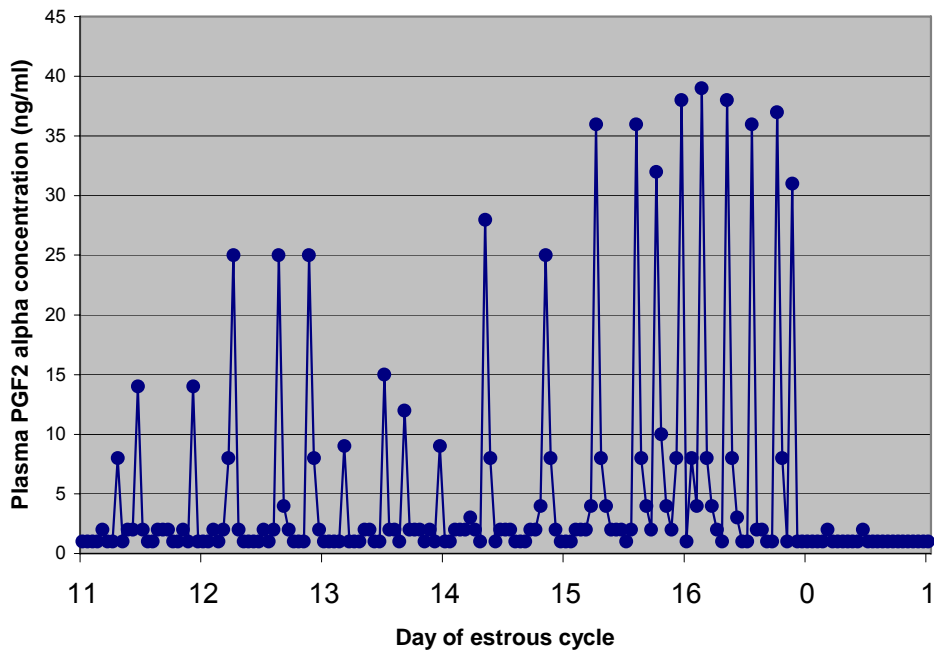
a constant infusion of 0.1 to 0.5  $\mu\text{g}$   $\text{PGF}_{2\alpha}$  per hour, for 6 to 18 hours into the arterial supply of the ovary, does not bring about complete luteolysis in sheep [130, 131].

In 1983 McCracken *et al* unravelled the problem. In a heroic effort hourly specimens were taken in both the uterine vein and ovarian artery. The typical pattern of secretion in sheep is shown in Figure 3 and Figure 4 [132].

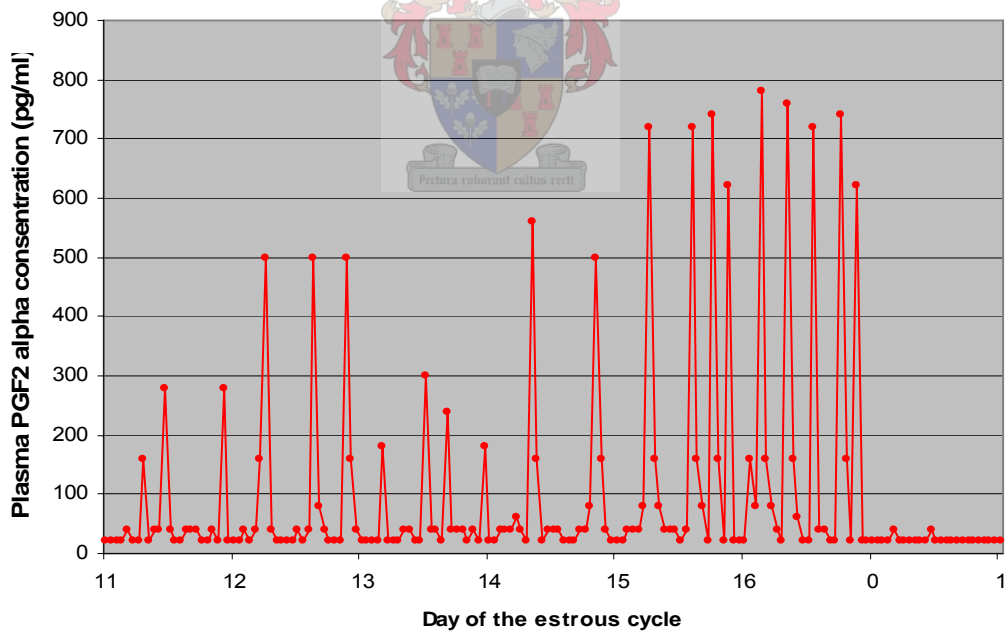
As can be seen in Figure 3, the concentration of  $\text{PGF}_{2\alpha}$  in the uterine vein during luteolysis varies dramatically. A pulse of  $\text{PGF}_{2\alpha}$  last about 1 hour, ranges from 8 ng/ml to 38 ng/ml and return to baseline of 1 - 2 ng /ml. This is consistent with the fact that  $\text{PGF}_{2\alpha}$  is almost completely metabolized with one passage through the lungs [114].

The pattern observed also explains the high degree of variation that was observed in  $\text{PGF}_{2\alpha}$  concentrations between days 11 to 16 of the estrous cycle, when blood samples were obtained only once a day (see Figure 2). Calculating the average and SEM of the 24 observations per day from the data in Figure 3, a pattern similar to that in Figure 2 is found (Figure 5).

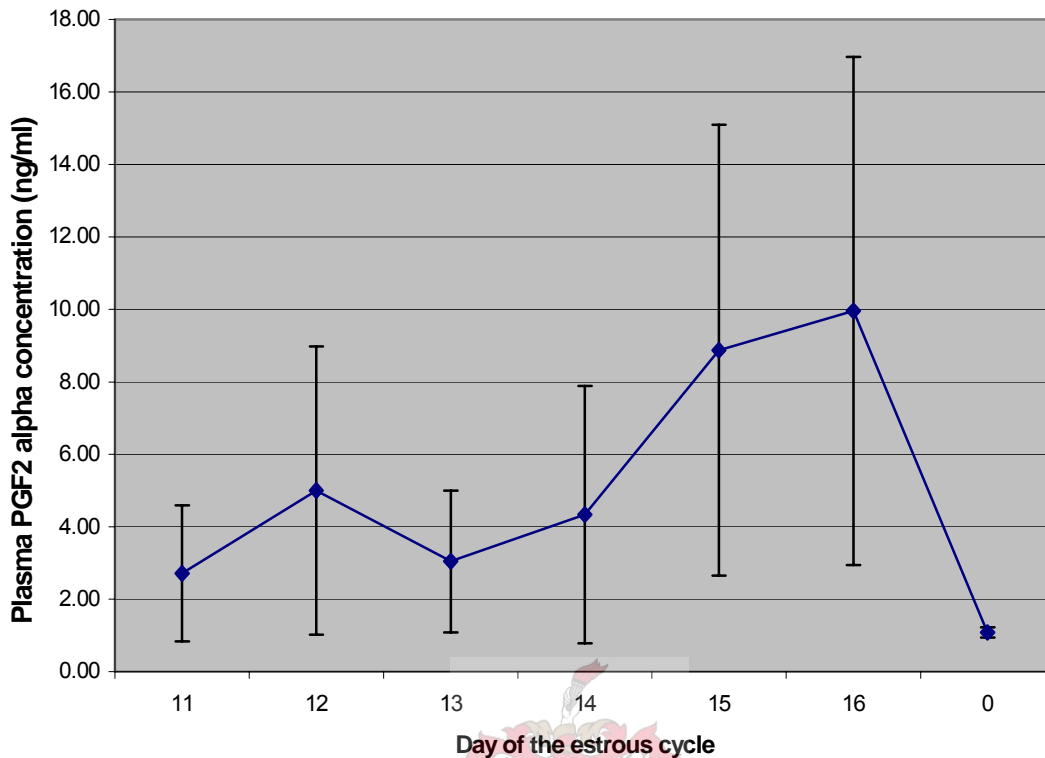
As mentioned earlier,  $^3\text{H-PGF}_{2\alpha}$  showed that 2% of the  $\text{PGF}_{2\alpha}$  in the uterine vein is transferred to the ovarian artery. Actual measurements of  $\text{PGF}_{2\alpha}$  in the ovarian artery confirmed this (see Figure 4). The same basic pattern is seen as in the uterine vein, but the amplitude of the pulses is much lower and varies between 80 to 400 pg/ml.



**Figure 3** Hourly PGF<sub>2α</sub> concentration in the uterine vein during luteolysis. Note that there are only 2 to 4 pulses from days 11 to 15, but 5 pulses occur between days 15 to 0 within 25 hours[131].



**Figure 4** Hourly PGF<sub>2α</sub> concentration in the ovarian artery during luteolysis. Note that there are only 2 to 4 pulses from days 11 to 15, but 5 pulses occur between days 15 to 0 within 25 hours[131].



**Figure 5** Average daily PGF<sub>2α</sub> concentration in the uterine vein of the ewe around the time of luteolysis[131].

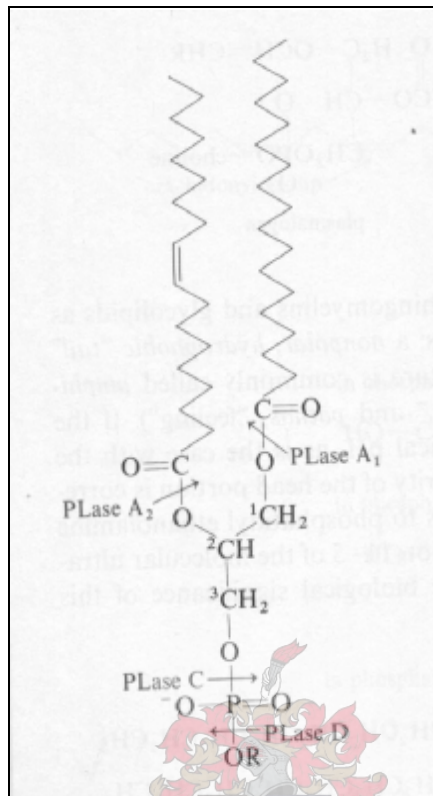
An interesting observation in these experiments was that between day 15 and 0, there were five pulses that occurred in 25 hours. Using the autotransplanted uterus and ovary model, it was shown that infusion of PGF<sub>2α</sub> using ultra low concentrations could bring about luteolysis in the ewe if the PGF<sub>2α</sub> is administered in pulses. By infusing PGF<sub>2α</sub> in five pulses of one hour at a rate of 0.5 μg/hour, 5 hours apart, complete luteolysis can be achieved. Four or less pulses would result only in a temporally decline in progesterone that could be reversed [132].

## 2.4 Synthesis of prostaglandins

Membrane lipids undergo “turnover”, that is, they are constantly being formed and degraded. Degradation involves the enzyme-catalysed hydrolysis of the various ester bonds to release the constituents. These enzymes are called phospholipases (P-lase) and are found in all types of cells. Before P-lase can act on amphipathic phosphor-acylglycerols, these components must be mobilized from the membrane into lipid droplets in the cytoplasm. This mobilization of amphipathic phosphor-acylglycerols is stimulated by  $P_4$ . Phospholipases is divided into different categories on the basis of their sites of hydrolysis of ester bonds in the structure of phosphor-acylglycerols (see Figure 6). After hydrolysis of ester bonds by P-lase  $A_2$  &  $A_1$ , fatty acids like arachidonic acid, are released. In general hydrolysis by P-lase  $A_2$  results in more free arachidonic acid than hydrolysis by P-lase  $A_1$ .

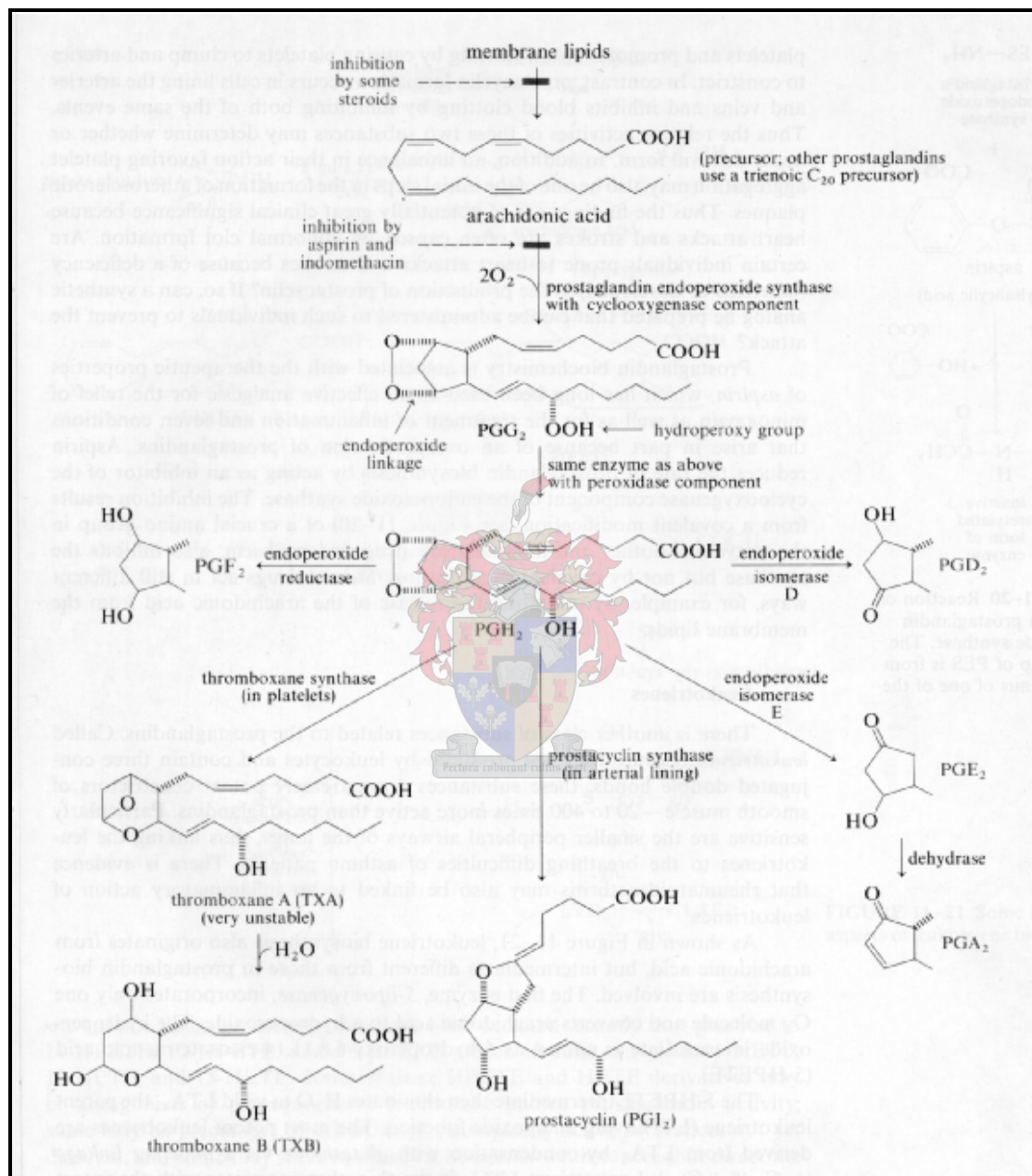
Prostaglandin biosynthesis begins with the conversion of arachidonic acid into two endoperoxide intermediates, first prostaglandin  $G_2$  ( $PGG_2$ ) and then prostaglandin  $H_2$  ( $PGH_2$ ). The reactions are catalysed by the same enzyme, prostaglandin endoperoxide synthase (PES), which has two separate catalytic components, prostaglandin endoperoxide synthase cyclooxygenase ( $PES_{cyc}$ ) ( $O_2$  incorporation and cyclopentane formation) and prostaglandin endoperoxide synthase peroxidase ( $PES_{per}$ ) (reduction of OOH group to OH group). The  $PGG_2$  species has an endoperoxide bridge (-O-O-) across the cyclopentane ring and a hydroperoxide (-OOH) group on a side chain. The  $PGH_2$  retains the (-O-O-) function, but the (-OOH) group on the side chain is reduced to (-OH).  $PGH_2$  serves as the precursor for the assembly of other prostaglandins (see Figure 7).





**Figure 6** Preferred sites of hydrolysis of different phospholipases (Plase) [133].

In the case of prostaglandin F<sub>2</sub> (PGF<sub>2</sub>), the (-O-O-) endoperoxide group is reduced to two (-OH) groups by the enzyme prostaglandin endoperoxide reductase (PES<sub>red</sub>). Only the  $\alpha$  isomer of PGF<sub>2</sub> is stable in nature. The other important prostaglandin as far as the reproductive cycle of the ewe is concerned is prostaglandin E<sub>2</sub> (PGE<sub>2</sub>). PGE<sub>2</sub> is converted from PGH<sub>2</sub> by the enzyme prostaglandin endoperoxide isomerase (PES<sub>iso</sub>) to a (-OH) and a (=O) group. Despite minor structural differences, PGF<sub>2 $\alpha$</sub>  and PGE<sub>2</sub> exert completely the opposite effects on vascular tissue. PGF<sub>2 $\alpha$</sub>  causes contraction and PGE<sub>2</sub> relaxation of smooth muscle in vascular tissue [133].



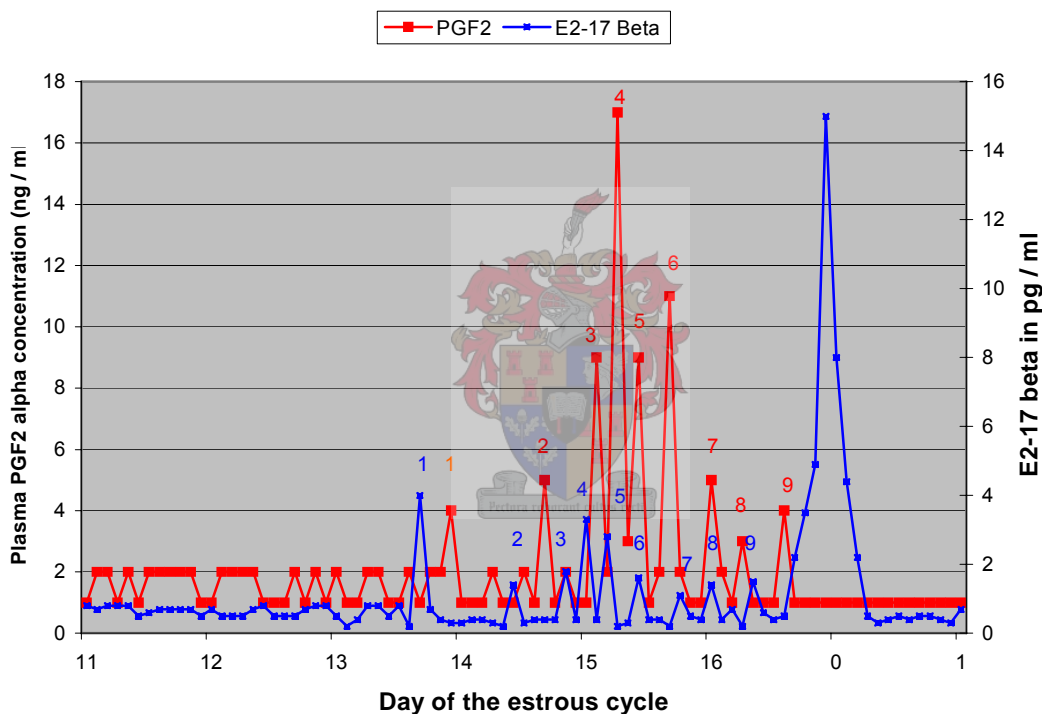
**Figure 7** Summary of some important aspects of prostaglandin biosynthesis. The path from PGH<sub>2</sub> differs from tissue to tissue [133].

## 2.5 Control of PGF<sub>2α</sub> secretion

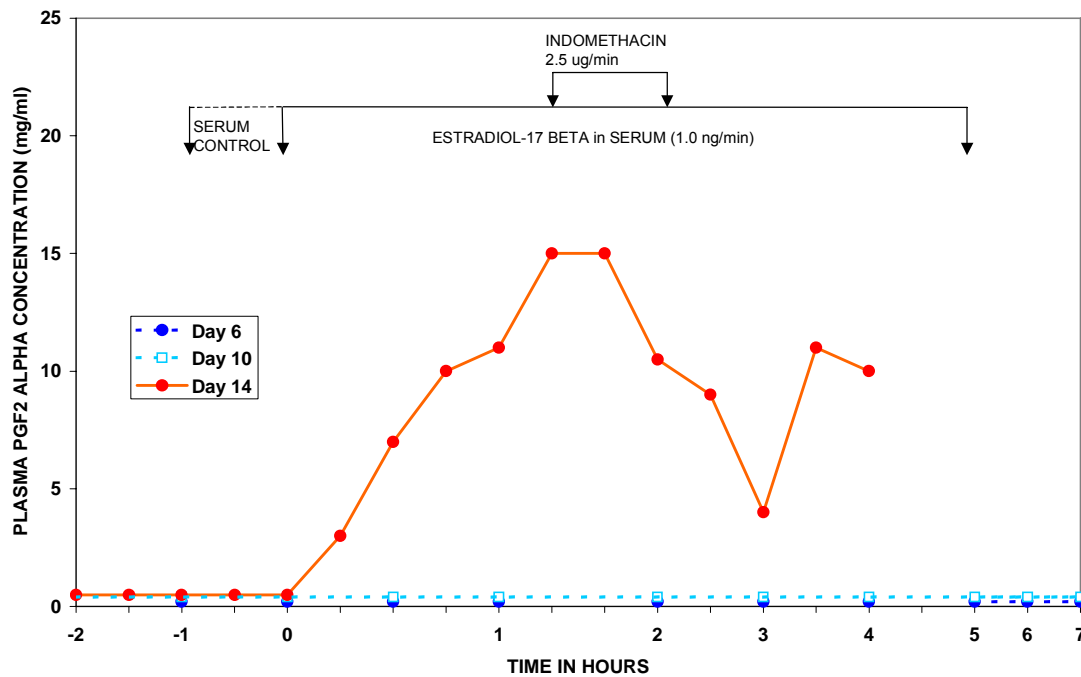
There is still considerable controversy as to which factors are responsible for the increase in PGF<sub>2α</sub> that causes luteolysis. It is generally agreed that uterine secretion of PGF<sub>2α</sub> is controlled by ovarian hormones, since little release occurs in ovariectomized ewes [134-138]. Three ovarian hormones have been implicated in regulation of uterine PGF<sub>2α</sub> namely P<sub>4</sub>, E<sub>2</sub>-17β and oxytocin (Ox), which are produced by the ovine corpus luteum [139-141] and ovarian follicle. In this section the effect of these hormones that can affect PGF<sub>2α</sub> secretion and physiological significance of each will be discussed. One general difficulty in evaluating the evidence, is the frequent use of peripheral concentrations of the major metabolite of PGF<sub>2α</sub>, 13,14-dihydro-15-keto-PGF<sub>2</sub> (PGFM) as an index of PGF<sub>2α</sub> release. The usefulness of PGFM measurement has been questioned [127] and continues to be a point of controversy in this field. However, for the sake of completeness, studies using both direct and indirect assessment of PGF<sub>2α</sub> secretion have been included in this section.

Around the time of corpus luteum regression, peaks of E<sub>2</sub>-17β precede peaks of PGF<sub>2α</sub> by approximately 6 hours (Figure 8) [116]. It has also been shown that inhibition of endogenous E<sub>2</sub>-17β secretion by follicle ablation [142-144] or x-irradiation [145, 146], prolongs luteal lifespan for a few days. Experiments were therefore conducted to examine the effect of exogenous E<sub>2</sub>-17β on PGF<sub>2α</sub> secretion by infusing physiological amounts of E<sub>2</sub>-17β into the arterial supply of the autotransplanted uterus at different times of the ovine estrous cycle [116]. It was observed that E<sub>2</sub>-17β has no effect on PGF<sub>2α</sub> secretion early in the cycle, whereas

on day 14 (the approximate time of onset of corpus luteum regression), there was a 50-to100-fold increase in  $\text{PGF}_{2\alpha}$ , an effect that could be decreased by indomethacin as can be seen in Figure 9. This suggests that  $\text{E}_2\text{-}17\beta$  promotes the *de novo* synthesis of  $\text{PGF}_{2\alpha}$ , rather than simply the release of stored material. From these results, it appears that prior exposure of the uterus to progesterone was necessary to evoke a maximal response to  $\text{E}_2\text{-}17\beta$  in terms of  $\text{PGF}_{2\alpha}$  secretion [116].



**Figure 8** Utero-ovarian vein plasma concentration of  $\text{PGF}_{2\alpha}$  and  $\text{E}_2\text{-}17\beta$  in a sheep bearing a utero-ovarian autotransplant sampled every 2 hours during the estrous cycle. (Note that peaks of  $\text{E}_2\text{-}17\beta$  precedes pulses of  $\text{PGF}_{2\alpha}$  by 6 hours from day 13 - 0 (17). Also the dramatic rise in  $\text{E}_2\text{-}17\beta$  on day 0 (17) is not followed by an rise in  $\text{PGF}_{2\alpha}$ ) [116].

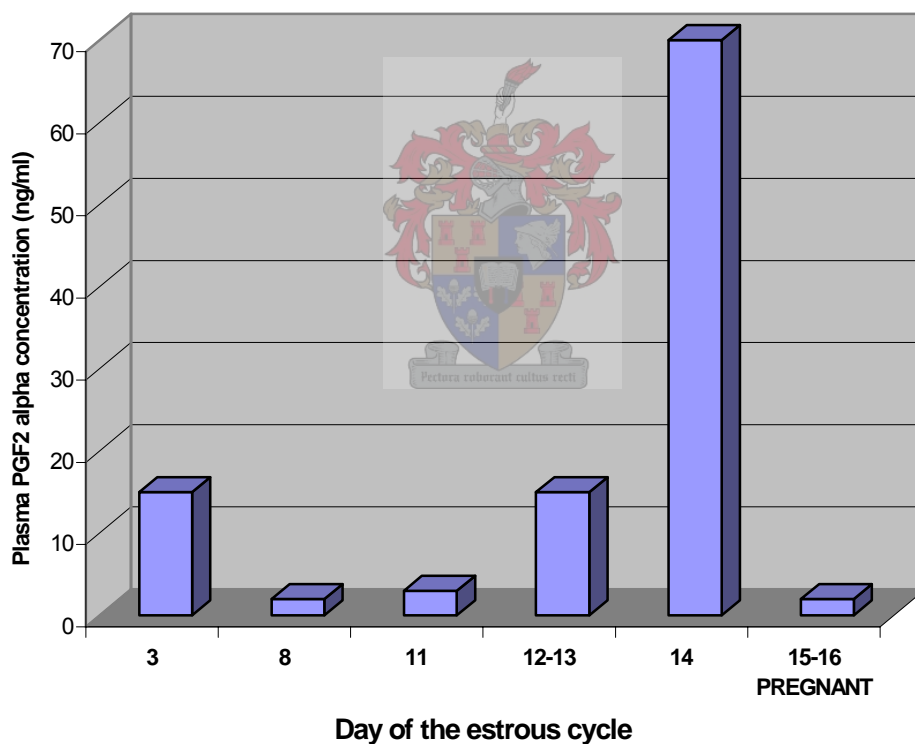


**Figure 9** Effect of intra-arterial infusions of E<sub>2</sub>-17β on PGF<sub>2α</sub> secretion from the utero-ovarian transplant model in the sheep. (Note that the superimposed infusion of indomethacin diminishes the synthesis of PGF<sub>2α</sub> on day 14) [116].

Other studies on intact animals have shown that E<sub>2</sub>-17β treatment increased PGF<sub>2α</sub> production and caused a slight advance of luteolysis [147-151], but it was not clear, especially when very large doses were given, whether this effect of E<sub>2</sub>-17β was a direct one on the uterus or an indirect effect e.g. via the pituitary gland. However, when E<sub>2</sub>-17β was infused systemically at a rate that was effective via the uterine artery (0.5 to 1.0 ng / hour), there was no effect on PGF<sub>2α</sub> secretion by the uterus [116]. This latter finding indicates that the stimulus of E<sub>2</sub>-17β in terms of PGF<sub>2α</sub> release is a direct one on the uterus.

In guinea pigs it was observed that mechanical stimulation of the isolated lung [152] or uterus [153] evoked release of prostaglandins. Furthermore, since the values reported for the concentration of PGF<sub>2α</sub> in uterine venous plasma of sheep

varies considerably among laboratories, it was suggested that this variation might be due to differences in manipulation of the uterus during blood sampling procedures. Experiments were therefore conducted to examine the effect of mechanical stimulation of the *in situ* ovine uterus on different days of the estrous cycle [154, 155]. The results are shown in Figure 10, where it can be seen that mechanical stimulation caused a small release of  $\text{PGF}_{2\alpha}$  early in the cycle, which had little or no effect during the luteal phase and produced a very marked increase in the secretion of  $\text{PGF}_{2\alpha}$  late in the luteal phase around day 14, which was abolished if the animal was pregnant.

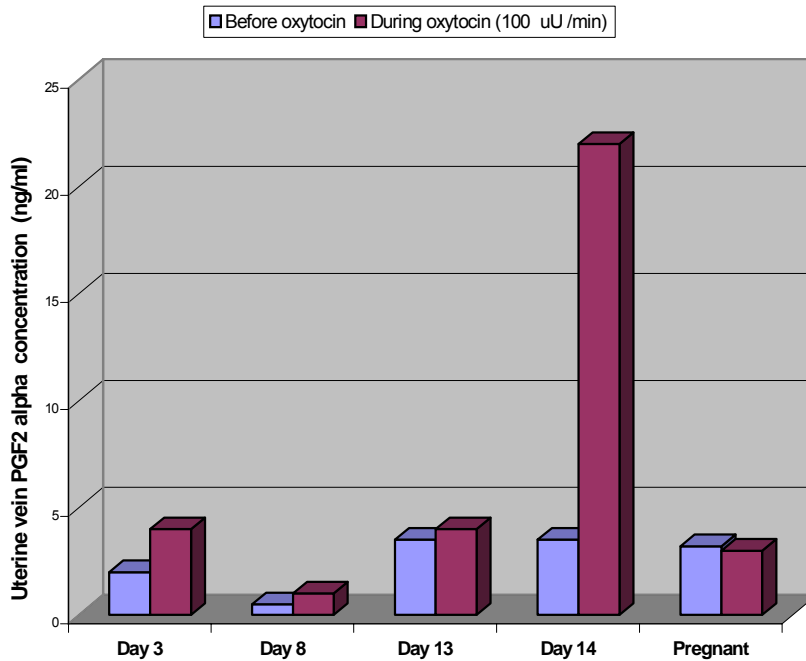


**Figure 10** Peak concentration of  $\text{PGF}_{2\alpha}$  in uterine venous plasma after mechanical stimulation of the *in situ* ovine uterus on different days of the estrous cycle [154, 155].

Since mechanical stimulation of the female reproductive tract causes an elevation of Ox in the peripheral blood of sheep and goats [156, 157] via the centrally acting Ferguson reflex [158], it was considered that Ox might play a role in the mechanically induced secretion of  $\text{PGF}_{2\alpha}$ . This proposal appeared quite plausible since exogenous Ox had been shown to curtail the estrous cycle of the cow [159], an effect which had been postulated to be due to Ox-induced stimulation of  $\text{PGF}_{2\alpha}$  secretion from the uterus.

Thus the ability of mechanical stimulation of the uterus to release  $\text{PGF}_{2\alpha}$  appears to depend on two distinct but simultaneously operating variables (i) the variation in the Ox releasing reflex during the cycle and (ii) the variation in the ability of the uterus to release  $\text{PGF}_{2\alpha}$  in response to Ox. According to Roberts (1975) *et al.* It is likely that both parameters are under ovarian steroid hormone regulation [154].

By infusing Ox on different days of the estrous cycle into the arterial supply of the autotransplanted uterus and ovary, a pattern similar to the effect of mechanical stimulation of the uterus was found. Ox infusions evoked small amounts of  $\text{PGF}_{2\alpha}$  secretion early in the cycle, had essentially no effect during the early luteal phase and caused a very pronounced increase in  $\text{PGF}_{2\alpha}$  secretion in the late-luteal phase around day 14, which is abolished once the animal is pregnant (Figure 11) [154, 155].



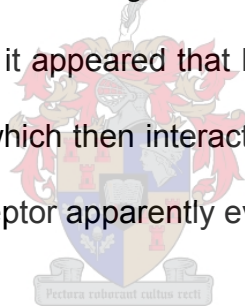
**Figure 11** Peak concentration of PGF<sub>2α</sub> in uterine venous plasma after intra-arterial infusion of Ox into the *in situ* ovine uterus on different days of the oestrous cycle [154, 155].

Because there was no evidence that Ox was elevated at the time of the luteolytic release of PGF<sub>2α</sub>, and since the autotransplanted uterus (which no longer had any connections to the CNS) exhibited spontaneous peaks of PGF<sub>2α</sub> secretion, it seemed likely that E<sub>2-17β</sub>, rather than causing Ox release, conditioned the uterus to make it responsive to endogenous levels of Ox and hence evoked secretion of PGF<sub>2α</sub>. Evidence for this was seen in the apparent association of peaks of E<sub>2-17β</sub> secretion and peaks of PGF<sub>2α</sub> from the transplanted uterus (Figure 11). Moreover, small amounts of E<sub>2-17β</sub> infused into the arterial supply of the uterus evoked PGF<sub>2α</sub> secretion, but not when the same amount of E<sub>2-17β</sub> was infused into the systemic circulation.

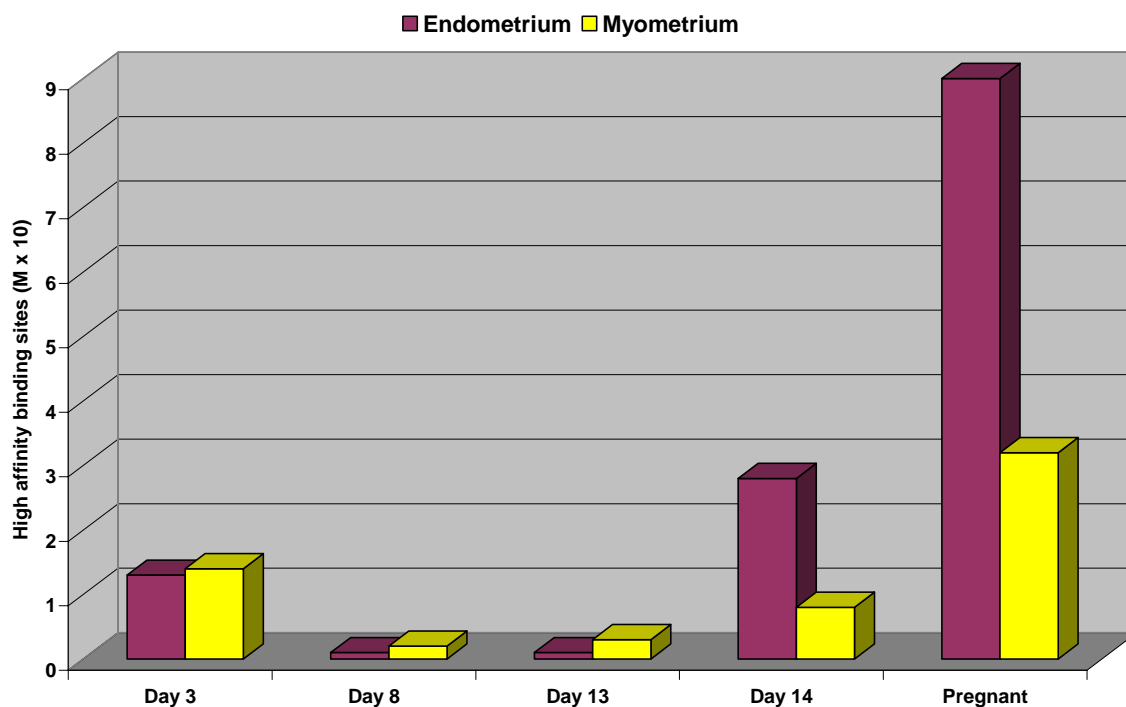


Furthermore, it had been demonstrated in anestrus sheep that Ox alone did not stimulate the release of  $\text{PGF}_{2\alpha}$  from the sheep uterus unless the animals were pretreated with  $\text{E}_2\text{-17}\beta$ . A slight increase in  $\text{PGF}_{2\alpha}$  was caused by  $\text{E}_2\text{-17}\beta$  treatment alone [160].

It therefore seemed likely that the concentration of oxytocin receptors (rOx) in the endometrium would change during the estrous cycle in parallel with the previously observed cyclical variation in the ability of Ox to evoke  $\text{PGF}_{2\alpha}$  secretion. This prediction proved to be correct in that the relative concentration of rOx in the endometrium, the principal site of  $\text{PGF}_{2\alpha}$  production in the ovine uterus [161], was found to vary cyclically [157] with the highest concentration appearing late in the estrous cycle (Figure 12). Thus, it appeared that  $\text{E}_2\text{-17}\beta$  acts directly on the uterus, causing the appearance of rOx which then interacted with endogenous levels of Ox. The interaction of Ox with its receptor apparently evoked  $\text{PGF}_{2\alpha}$  secretion.



McCracken (1980) investigated the relationship between the ovarian hormones in regulating the production and secretion of  $\text{PGF}_{2\alpha}$  [138]. This research led to the working hypothesis on the hormone-mediated events controlling  $\text{PGF}_{2\alpha}$  secretion from the endometrial cell.



**Figure 12** Concentration of high-affinity binding sites for oxytocin in the endometrium and myometrium obtained from *in situ* ovine uterus on different days of the estrous cycle. [157]

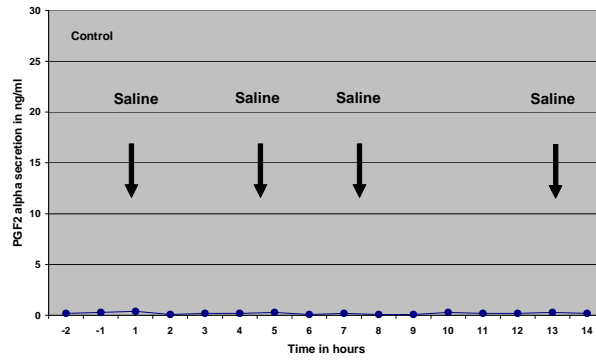
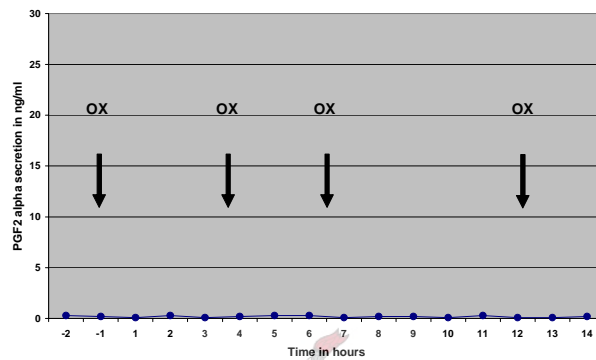
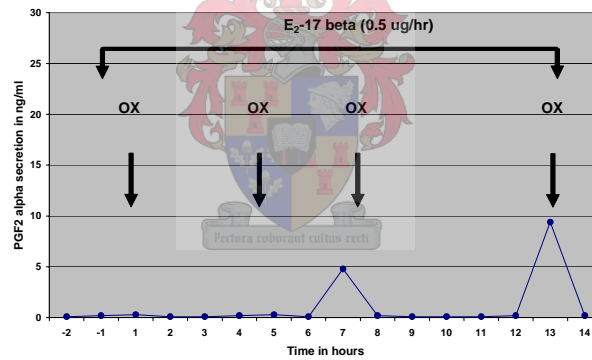
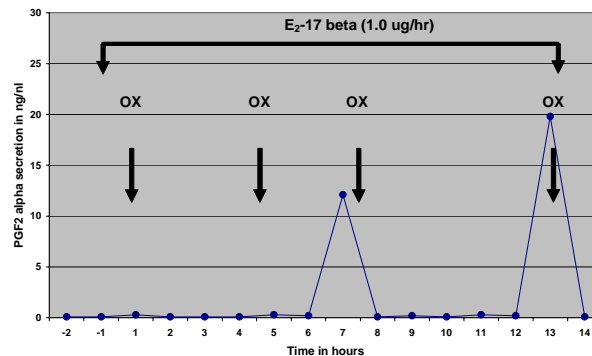
Since it appeared that Ox could evoke  $\text{PGF}_{2\alpha}$  secretion only late in the cycle, the only time when high-affinity binding sites for Ox were increasing in the uterus [157], it seemed likely that both  $\text{E}_2$ -17 $\beta$  and  $\text{P}_4$  might control  $\text{PGF}_{2\alpha}$  production by regulating the formation or availability of ROx in the endometrium. In his experiments McCracken (1980) used ovariectomized ewes with the uterus transplanted to the neck. The ability of arterial infusions of Ox to evoke  $\text{PGF}_{2\alpha}$  secretion from the transplanted uterus was utilised as an *in vivo* marker for the appearance of the ROx.

$\text{E}_2$ -17 $\beta$  and  $\text{P}_4$ , either alone or in combination, were infused intravenously (i.v.) into the systemic circulation in amounts that correspond to the ovarian secretion

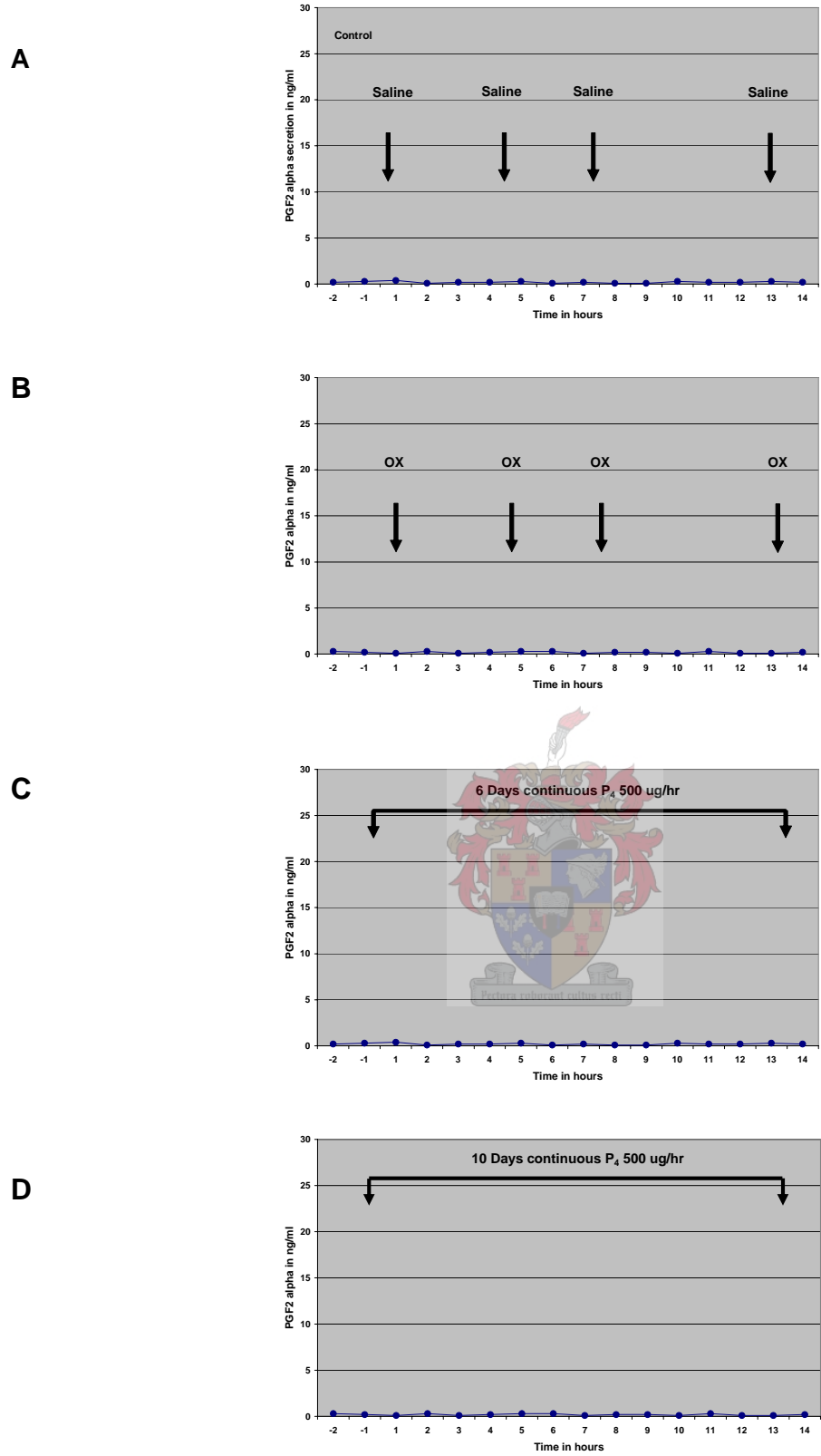
rate of these hormones in sheep [48]. At suitable intervals (-1, 3, 6 & 12 hours) the uterus was challenged intra-arterially with a 10 min infusion of Ox at 5.0 IU / min. This dose had previously been shown to evoke the maximum release of  $\text{PGF}_{2\alpha}$  from the *in situ* uterus under optimal conditions [154, 162].  $\text{PGF}_{2\alpha}$  was measured by RIA in timed samples of uterine venous blood collected throughout the experiments, and was expressed as secretion rate (ng/min).

In the first experiment, the uterus was infused with different concentrations of  $\text{E}_2\text{-17}\beta$  and then challenged with Ox. As can be seen in Figure 13 (A): there was no placebo effects, (B): Ox challenge without  $\text{E}_2\text{-17}\beta$  pre-treatment has no effect on  $\text{PGF}_{2\alpha}$  release because there are no rOx, (C): rOx appeared after 6 hours (approximate time needed for protein synthesis) induced by  $\text{E}_2\text{-17}\beta$  and then again after 12 hours based on the significant increase in Ox induced  $\text{PGF}_{2\alpha}$  secretion. The amplitude of the second increase was higher than the first. The minimum rate of  $\text{E}_2\text{-17}\beta$  needed to evoke the response was 0.5  $\mu\text{g}$  per hour.

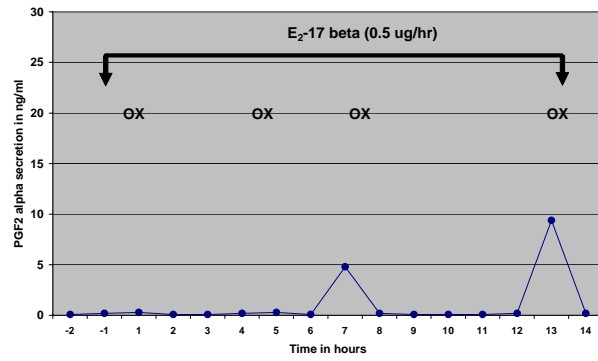
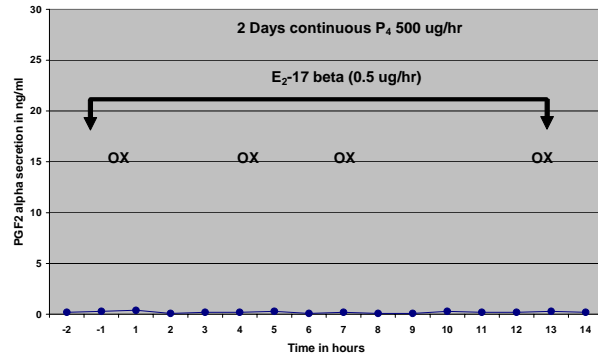
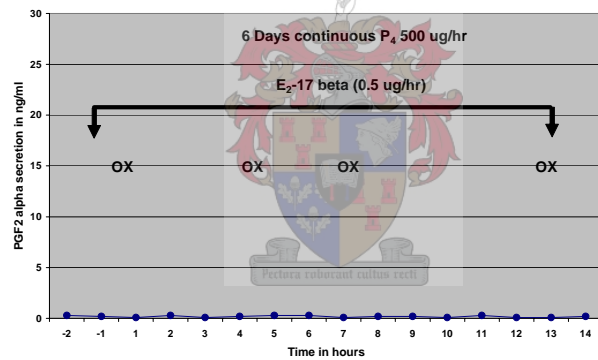
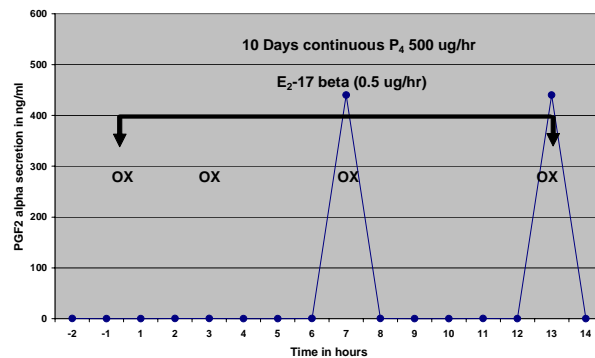
In the second experiment, the autotransplanted uterus was pretreated with  $\text{P}_4$  before the experiment and only challenged with Ox without the  $\text{E}_2\text{-17}\beta$  infusion. As can be seen in Figure 14 (A): there was no placebo effects, (B): Ox challenge without  $\text{P}_4$  pre-treatment has no effect on  $\text{PGF}_{2\alpha}$  release because there are no rOx, (C):  $\text{P}_4$  pretreatment for 0, 6 or 10 days could not evoke the synthesis of rOx based on the absence of Ox induced  $\text{PGF}_{2\alpha}$  release.

**A****B****C****D****Figure 13**

The effect of different 12 hour infusions of E<sub>2</sub>-17 $\beta$  on oxytocin-induced PGF<sub>2 $\alpha$</sub>  secretion from the autotransplanted uterus in ovariectomized ewes. A Control placebo, B only Ox, C 0.5 ug/hr E<sub>2</sub>-17 $\beta$  & Ox and D 1.0 ug/hr E<sub>2</sub>-17 $\beta$  & Ox . The ability of Ox to evoke PGF<sub>2 $\alpha$</sub>  secretion was used as an *in vivo* marker for the appearance of the ROx [138].



**Figure 14** The effect of different progesterone treatments on Ox-induced PGF<sub>2α</sub> secretion from the autotransplanted uterus in ovariectomized ewes. A Control placebo, B only Ox, C 6 days P<sub>4</sub> infusion & Ox and D 10 days P<sub>4</sub> infusion & Ox . P<sub>4</sub> alone cannot evoke Ox-induced PGF<sub>2α</sub> secretion [138].

**A****B****C****D**

**Figure 15** The effect of different P<sub>4</sub> pretreatments and E<sub>2</sub>-17β infusion on Ox-induced PGF<sub>2α</sub> secretion from the autotransplanted uterus in ovariectomized ewes. The combination of P<sub>4</sub> and E<sub>2</sub>-17β evoke a 50 to 100-fold greater response than when E<sub>2</sub>-17β is given alone. Also note that pretreatment with P<sub>4</sub> for 2 and 6 days abolishes the effect of E<sub>2</sub>-17β alone at the dose of 0.5 μg per hour [138].

The third experiment used the combination of P<sub>4</sub> pretreatment and E<sub>2</sub>-17β infusion at the minimum effective dose of 0.5 μg per hour (Figure 15). (A): ROx appeared after 6 hours (approximate time needed for protein synthesis) induced by E<sub>2</sub>-17β and then again after 12 hours based on the significant increase in Ox induced PGF<sub>2α</sub> secretion. The amplitude of the second increase was higher than the first. (B): Pretreatment with P<sub>4</sub> for 2 or (C): 6 days abolished the effect of E<sub>2</sub>-17β infusion alone. The interesting observation, however, was that (D): pretreatment with P<sub>4</sub> for 10 days caused a 50 to 100-fold increase on Ox induced PGF<sub>2α</sub> release after 6 hours and again after 12 hours (Figure 15).

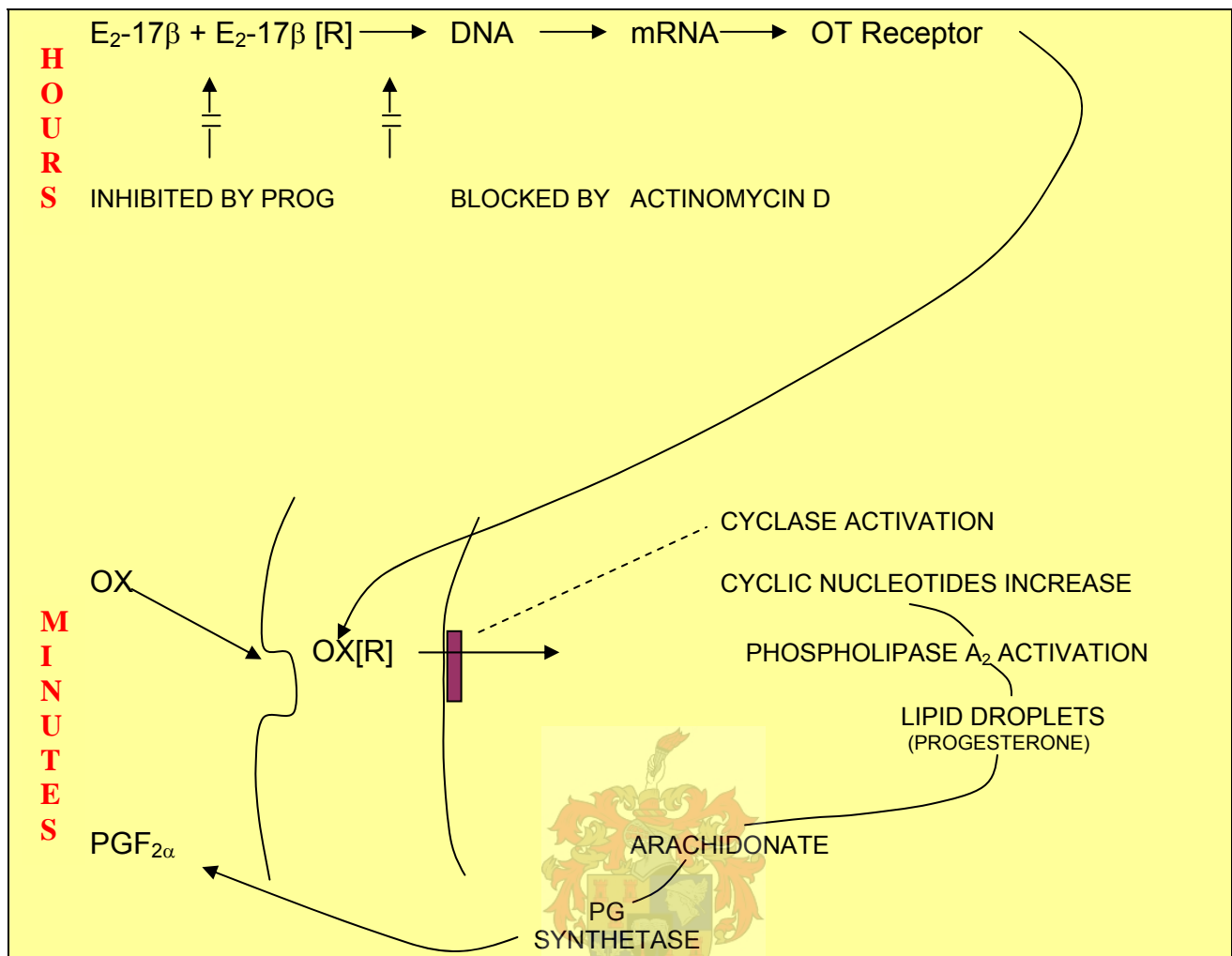
It seems that P<sub>4</sub> inhibits the action of E<sub>2</sub>-17β if it precedes infusion for 2 or 6 days, but enhances the effect of E<sub>2</sub>-17β if it precedes an E<sub>2</sub>-17β infusion by 10 days. Thus, for the release of a luteolytic amount of PGF<sub>2α</sub> a 10 day P<sub>4</sub> prime followed by E<sub>2</sub>-17β exposure for at least 30 hours was needed to produce 5 pulses in 25 hours that could bring about complete irreversible luteolysis.

McCracken *et al* (1980) [138] constructed the following hypothesis to explain the observations they observed:

- i) E<sub>2</sub>-17β receptors (rE<sub>2</sub>-17β) (cytoplasmic) in the endometrium, when activated by E<sub>2</sub>-17β, appear to elaborate the rOx after a 6 hour delay (activation of protein synthesis) i.e. using Ox-induced PGF<sub>2α</sub> secretion as an *in vivo* marker for the appearance of the rOx.

- ii) The interaction of Ox with its receptor (plasma membrane-bound) immediately appears to evoke  $\text{PGF}_{2\alpha}$  secretion. The latter event is thought to involve the prostaglandin synthetase system (microsomal) probably through the release of precursor fatty acid via phospholipase action.
  
- iii)  $\text{P}_4$  inhibits R- $\text{E}_2$ - $17\beta$  replenishment, thus probably explaining why  $\text{E}_2$ - $17\beta$  could not cause the formation of the ROx after  $\text{P}_4$  administration for 2 and 6 days. This explains why the system becomes unresponsive to Ox after pretreatment for 2 or 6 days.
  
- iv) Eventually, (after 10 days), R- $\text{E}_2$ - $17\beta$  replenishment is no longer inhibited by  $\text{P}_4$  and ROx are once again induced by  $\text{E}_2$ - $17\beta$  after the usual 6 hour delay. In their article they explain this result by the report that  $\text{P}_4$  appears to catalyse the destruction of its own receptor [163], so that  $\text{P}_4$  can no longer inhibit R- $\text{E}_2$ - $17\beta$  replenishment. When the uterus is now challenged with Ox, there is a 50- to 100-fold greater enhancement of  $\text{PGF}_{2\alpha}$  secretion than when  $\text{E}_2$ - $17\beta$  alone is infused. This enhancement may be due either to an increase in the number of ROx, or more likely, to an amplification of the prostaglandin synthesizing system. This latter event may be due to an arachidonic acid cascade effect from the  $\text{P}_4$  induced accumulation of lipid droplets in endometrial cells as reported by Brinsfield & Hawk [164] (Figure 16).





**Figure 16** Working hypothesis on the hormone-mediated events controlling PGF<sub>2α</sub> secretion from the endometrial cell [138]

## 2.6 The Mechanism of PGF<sub>2α</sub> action

Several different mechanisms have been proposed to explain the luteolytic effects of PGF<sub>2α</sub>. These include:

- a rapid and dramatic decrease in luteal blood flow [165, 166],
- a reduction in the number of receptors for Luteinizing Hormone (LH) [167],
- an uncoupling of the LH receptor from adenylate cyclase [168],
- activation of protein kinase C (PK-C) [169],

- influx of high levels of calcium [18, 170] and
- and a cytotoxic effect [171].

Although there is good evidence for all these actions, the effects of  $\text{PGF}_{2\alpha}$  appear to differ among species, and the actions demonstrable *in vivo* differ from those observed *in vitro*. Two important destinations that must be made in studying the effects of  $\text{PGF}_{2\alpha}$  are (a) the effect on progesterone synthesis and (b) the so called cytotoxic effect or cell lysis. These two steps are not necessarily caused by the same stimulus. In this section these two processes will be dealt with separately.

## **2.6.1 Inhibition of Progesterone synthesis**

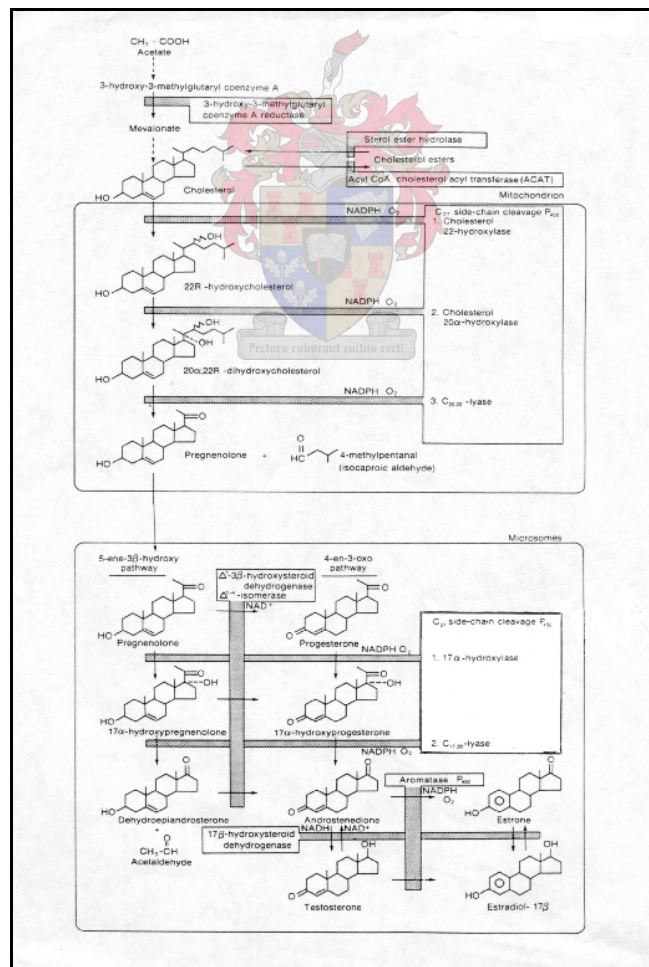
### **2.6.1.1 Progesterone biosynthesis**

Progesterone is produced from cholesterol derived from one of three possible sources (i) cholesterol taken up from the blood, primarily in the form of circulating lipoproteins, (ii) cholesterol stored within the ovarian cell, either as free cholesterol, a constituent of cell membranes, or liberated from cholesterol esters stored within cytoplasmic lipid droplets and (iii) cholesterol synthesized *de novo* in the ovarian cell from 2-carbon components derived from metabolism of carbohydrate, fat or protein within the cell. In the corpus luteum cholesterol in circulating lipoproteins appears to be the most important source of steroidogenic cholesterol [172]. Considerable evidence has been accumulated supporting a mechanism of uptake that involves binding of extracellular lipoproteins via their apoprotein component to specific receptors located on the cell membrane, followed by internalization of the lipoprotein-receptor complex, uptake of the complexes by lysosomes, degradation of lipoproteins by lysosomal esterases, and release of free cholesterol, which is then able to gain access to the steroidogenic enzymes. Cholesterol from both low-density

lipoproteins (LDL) and high-density lipoproteins (HDL) has been implicated as a steroidogenic precursor in the corpus luteum[173]. Species differences occur in the relative importance of the two classes of lipoproteins. HDL appears to be of greater quantitative importance in rodents, whereas cholesterol associated with LDL is the major circulating form of steroidogenic cholesterol in other species including humans and sheep.

The first step in the conversion of cholesterol to progesterone, and the step generally believed to be rate limiting in progesterone biosynthesis under most conditions, is cleavage of the C-20,22 bond resulting in the C<sub>21</sub> compound pregnenolone, and a 6-carbon fragment, isocaproic aldehyde. The enzyme system that catalyzes this reaction C<sub>27</sub> side chain cleavage cytochrome P-450 (P-450<sub>SCC</sub>), is located on the matrix side of the inner mitochondrial membranes[174]. It is a multi-enzyme complex comprising three components (i) cholesterol 22-hydroxylase, (ii) cholesterol 20 -hydroxylase and (iii) C<sub>20,22</sub> lyase. The reaction utilizes nicotinamide adenine dinucleotide phosphate hydrogenase (NADPH), generated within the mitochondria by oxidation of Krebs cycle intermediates or fatty acids. Three moles of NADPH and three moles of oxygen are utilized per mole of cholesterol undergoing side-chain cleavage. The generally accepted pathway for biosynthesis of pregnenolone from cholesterol undergoing side chain cleavage is shown in Figure 17. Evidence summarized by Lieberman *et al* (1984) suggests that the overall reaction *in vivo* probably does not involve stable, free hydroxylated intermediates as shown here[175]. Instead, it has been proposed that the substrate cholesterol and subsequent transient hydroxylated intermediates remain bound to the P-450<sub>SCC</sub> until the ultimate product, pregnenolone, is formed and released.

Pregnenolone is the key steroidogenic intermediate of progesterone produced by the corpus luteum. It is converted to progesterone by a microsomal enzyme, or enzyme complex,  $\Delta^5$ - $3\beta$ -hydroxysteroid dehydrogenase:  $\Delta^5$ - $4$ -isomerase ( $3\beta$ -HSD). Although separation of the dehydrogenase from the isomerase activities has been achieved for a bacterial enzyme system, the two enzyme activities in mammalian steroidogenic tissues, including the ovary, have not been separated and appear to function physiologically as a single entity. The enzyme utilizes nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ) as an electron acceptor, and the reaction is essentially irreversible under physiological conditions[176].



**Figure 17** Pathway of the biosynthesis of steroid hormones [176].

### 2.6.1.2 Regulation of progesterone biosynthesis by gonadotropins

After the LH surge and luteinization, both granulosa and theca cells differentiate to become progesterone secreting large and small lutein cells respectively. The enzyme systems of these cells undergo modification and this is mediated by gonadotropins and other factors.

Before the LH surge, the theca cells in the ovarian follicle produce almost exclusively androstenedione. In the mitochondria of the theca cell, cholesterol is converted to pregnenolone by the  $C_{27}$  P-450<sub>SCC</sub> complex. A low-molecular-weight activator peptide increase the supply of cholesterol to the mitochondria via a cytoskeleton-mediated process involving tubulin [176]. Pregnenolone is converted to androstenedione by the  $C_{21}$  P-450<sub>SCC</sub> and  $3\beta$ -HSD, two enzymes present in the microsomes. This androstenedione is then transported to the avascular granulosa cells where it is converted to  $E_2-17\beta$  by aromatase P-450 (P-450<sub>AR</sub>) and  $17\beta$ -hydroxysteroid dehydrogenase ( $17\beta$ -HSD) present in the microsomes. Granulosa cells lack  $C_{21}$  P-450<sub>SCC</sub>, and it is uncertain if it has active  $C_{27}$ -P-450<sub>SCC</sub>. Thus, for granulosa cells to be able to produce  $E_2-17\beta$ , androstenedione from the theca cells is needed (Figure 17). In order for these cells to produce  $P_4$  exclusively after the LH surge, the following modifications in the enzyme system are necessary for theca and granulosa cells respectively.

In small lutein cells (theca cells) there must be down regulation of  $C_{21}$  P-450<sub>SCC</sub>. With all the other enzymes in place, this will ensure production of only  $P_4$  as the final product. Binding of LH to its membrane receptor produces an increase in

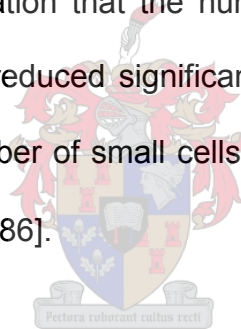
adenosine 3'-5' cyclic monophosphate (c-AMP) that stimulates phosphokinase A (PK-A). It appears that PK-A downregulate C<sub>21</sub> P-450<sub>SCC</sub> in these cells [177, 178].

Large lutein cells (granulosa cells) need more extensive changes. There is evidence that follicle stimulating hormone (FSH) stimulate the activity of C<sub>27</sub>-P-450<sub>SCC</sub> [179]. It is not clear if it is up-regulation of the enzyme-complex or increased supply of cholesterol to the mitochondria that mediate the stimulus to produce more pregnenolone. Additional actions of LH in luteal tissue of various species, include enhancement of cholesterol availability by (a) stimulating 3-hydroxy-3-methylglutaryl coenzyme A reductase, which is rate limiting for cholesterol biosynthesis, (b) acutely stimulating cholesterol esterase, thereby increasing the availability of free cholesterol from intracellular stores or fatty acid esters and (c) increasing the number of lipoprotein receptors, thereby enhancing uptake of cholesterol in the form of lipoprotein. FSH [180] and subsequently LH [181-184], greatly stimulate transcription of 3 $\beta$ -HSD. With no C<sub>21</sub> P-450<sub>SCC</sub> present in granulosa cells and in the absence of androstenedione from theca cells, this will make progesterone the exclusive endproduct of steroid synthesis. It is not clear if the enzymes aromatase P-450 and 17 $\beta$ -hydroxysteroid dehydrogenase is down regulated.

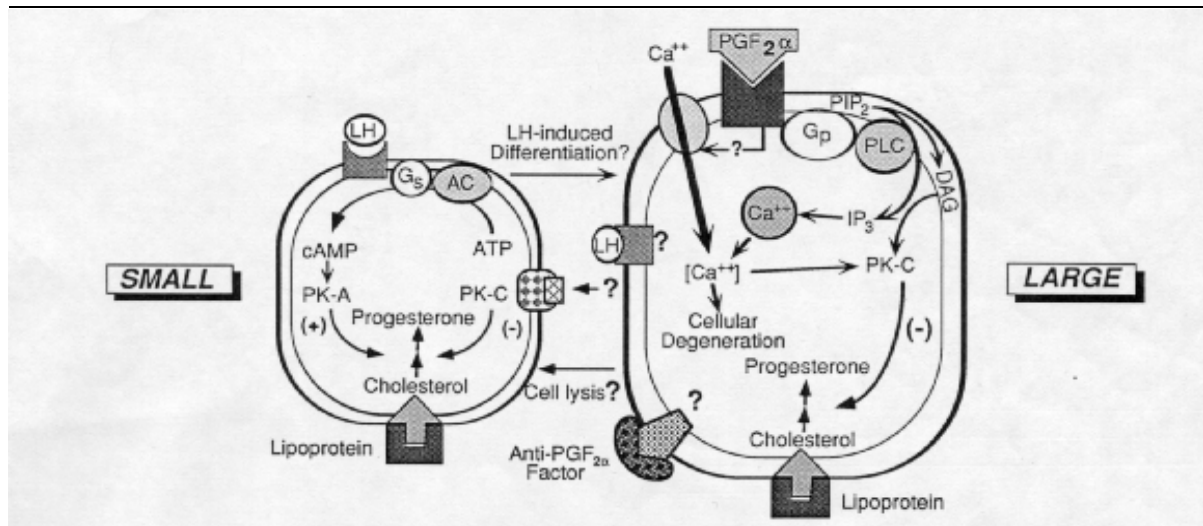
### **2.6.1.3 The effect of luteolysis on progesterone biosynthesis**

In ewes, the first effect of PGF<sub>2 $\alpha$</sub>  appears to be a rapid decrease in luteal blood flow [168, 185, 186]. The decrease in blood flow is highly correlated with decreased levels of P<sub>4</sub> in the serum. The result of the drop in blood flow, will be decreased oxygen delivery to the luteal cells. The production of P<sub>4</sub> is very sensitive

to oxygen tension. The production of pregnenolone needs 3 moles of oxygen as well as NADPH from the oxygen dependant Krebs cycle. The supply of cholesterol from all the sources is also oxygen dependant [166]. There is no drop in the number of LH receptors (RLH) in ewes administered a luteolytic dose of  $\text{PGF}_{2\alpha}$ , until well after a dramatic decrease in serum levels of  $\text{P}_4$  has occurred [185]. Several effects of  $\text{PGF}_{2\alpha}$  have also been demonstrated *in vitro*. There is a total block of LH-induced increases in the production of c-AMP and  $\text{P}_4$  secretion [168, 187]. This observation is particularly interesting because most receptors for LH are on small steroidogenic cells, whereas receptors for  $\text{PGF}_{2\alpha}$  are on large luteal cells. This suggests that there must be large cell - small cell communication in the corpus luteum. This concept is further sustained by the observation that the number of large luteal cells and the levels of serum cholesterol are reduced significantly within 12 hours of a luteolytic dose of  $\text{PGF}_{2\alpha}$ , but that the number of small cells does not decrease until 24 hours after the introduction of  $\text{PGF}_{2\alpha}$  [186].



$\text{PGF}_{2\alpha}$  activates phosphokinase C (PK-C) in large luteal cells which inhibits secretion of  $\text{P}_4$  in large luteal cells and possibly indirectly in small luteal cells as can be seen in Figure 18. However, PK-C does not induce cytotoxic effects [188] or cause luteolysis [178]. Interestingly, treatment of ewes with a luteolytic dose of  $\text{PGF}_{2\alpha}$  also results in a rapid decrease in messenger ribonucleic acid (mRNA) encoding  $3\beta$ -HSD without an effect on levels of mRNA encoding  $\text{C}_{27}\text{-P-450}_{\text{SCC}}$  or tubulin [189]. Thus, it appears that  $\text{PGF}_{2\alpha}$  acting via PK-C may have a specific effect on transcription of the gene for  $3\beta$ -HSD or influence the rate of degrading of this mRNA.



**Figure 18** Schematic diagram of some hormonal and second-messengers systems that interact to regulate secretion of progesterone from small and large ovine luteal cells [176].

## 2.6.2 Cell lysis

### 2.6.2.1 Morphological changes

During regression of the corpus luteum, several distinct morphological changes occur. The first morphological alteration that can be distinguished is a decrease in the percentage volume occupied by vascular elements [166]. There is a rapid decrease in the number of red blood cells and luminal volume of capillaries in the corpus luteum after treatment with  $\text{PGF}_{2\alpha}$  [165]. Swelling of endothelial cells appears to be responsible for the initial reduction in luminal volume. The decreased luminal volume most likely results in decreased perfusion of luteal tissue and probably causes blood to be shunted away from the corpus luteum [166, 190, 191].

As luteal regression continues, there is accumulation of lipid droplets in cells and steroidogenic cells decrease in size. Shrinkage of cells results in clumping of



sub-cellular components, particularly mitochondria. Normally there is little, if any, evidence of lipid accumulation in cells of the ovine corpus luteum, however, during regression there is a striking increase in intracellular lipid, with the lipid droplet becoming the most prominent organelle near the end of the estrous cycle. It has been shown that triglycerides increase tenfold during luteal regression [192-195]. This might be due to inactivation of the production of pregnenolone due to insufficient oxygen supply, while there is still normal recruiting of cholesterol.

The plasma membrane of cells from the regressing corpus luteum contain membrane specializations, including gap junctions, maculae adherens, coated invaginations, and microvilli similar to those of the corpus luteum of pregnancy. This is consistent with the fact that only large luteal cells contain receptors for  $\text{PGF}_{2\alpha}$ . The  $\text{PGF}_{2\alpha}$  induces inhibition of progesterone via PK-C, which inhibits  $3\beta$ -HSD either via reduced transcription or increased breakdown of this mRNA. This is communicated via these membrane specializations to the small luteal cells. Also, the signal for cell lysis seems to be communicated via these specializations, because the small luteal cells decrease 12 hours later than the large luteal cells.

The densely staining secretory granules observed during periods of maximal secretion of  $\text{P}_4$  decrease in number throughout the period of luteal regression. Concurrent with the decrease in numbers of secretory granules is the appearance of autophagosomes [165]. Autophagosomes appear to be sites of cytoplasmic involution, and their numbers increase as regression of the corpus luteum proceeds. Lysosomes are present in luteal cells throughout the estrous cycle, however, they appear to increase in number as regression of the corpus luteum progress [196].

During the midluteal phase of the cycle, acid phosphatase activity is restricted to lysosomes and inner cisternae of the Golgi apparatus. During regression, an increase in the number of primary lysosomes appears to occur as a result of pinching off from the lateral margins of inner Golgi cisternae. The quantity of smooth endoplasmic reticulum (SER) decrease as regression progresses, intracellular organelles become increasingly disorganized, and acid phosphatase activity is found with degenerating organelles. The histological hallmark of the regressing corpus luteum, however, is the presence of discrete, well-preserved apoptotic bodies. During apoptosis chromatin is segregated and sharply circumscribed masses align against the nuclear envelope. Due to the action of a  $\text{Ca}^{2+} / \text{Mg}^{2+}$  - dependent endonuclease, nuclear fragmentation takes place and the DNA helix is broken down into fragments of different sizes. Condensation of the cytoplasm and convolution of the nuclear and cell outlines takes place. In the next phase, the nucleus fragments and the cell as a whole buds to produce membrane-bounded apoptotic bodies of varying size and structure, which are phagocytosed by nearby cells and degraded within lysosomes. Finally, the bodies are reduced to unrecognizable residues [197-201].

#### **2.6.2.2 Mechanism of apoptosis in the corpus luteum**

Apoptosis is the cellular mechanism of ovarian follicular atresia and luteal regression [202-204]. A common biochemical feature of apoptosis, the degradation of genomic DNA by the action of a  $\text{Ca}^{2+} / \text{Mg}^{2+}$  - dependent endonuclease [205], has been demonstrated in atretic follicles and regressing luteal tissue of diverse species [206, 207]. It was shown that the  $\text{Ca}^{2+} / \text{Mg}^{2+}$  - dependent endonuclease responsible for apoptotic DNA degradation in the ovary is Dnase I [208-210].

Immunohistochemistry revealed that DNase I was present in nuclei of granulosa and luteal cells in healthy follicles and corpora lutea [210]. This suggests that DNase I is present, but inactive, in granulosa and luteal cells and that a signal to activate DNase I is necessary for apoptotic DNA degradation during follicular atresia or luteal regression. However, the signal that activates DNase I during ovarian apoptosis, has not been identified.

Recently it has become evident that apoptosis is triggered by the activation of cysteine aspartate-specific proteases (caspases), a family of enzymes related to interleukin-1 $\beta$  converting enzyme (ICE, caspase-1) [211]. A cascade of proteolysis beginning with activation of caspase-8 leads to the activation of caspase-3, which is the principal downstream effector of cell death [212-217]. Caspase 3 is activated by a variety of apoptotic signals including serum withdrawal, binding of Fas ligand/CD95 ligand to the Fas/CD95 membrane receptor protein, radiation and pharmacological agents [218]. Specific tetrapeptide inhibitors of caspase-3 block activation of the apoptotic program (nuclear fragmentation and endonuclease activation) in intact cells and cytosolic extracts [218, 219], whereas addition of active caspase-3 to normal cytosol activates the apoptotic program [218, 219]. These findings point to a central role for caspase-3 in apoptosis and demonstrate that caspase-3 activity results in the activation of the endogenous endonuclease.

A limited number of substrates are cleaved by activated proteases during apoptosis, including poly (ADP-ribose) polymerase (PARP), sterol regulatory element binding protein (SREBP), U1-70 small nuclear riboprotein, DNA dependent protein kinase, and structural proteins such as actin, fodrin, and lamins [220]. Both

PARP and actin are inhibitors of DNase I [221-223]. Therefore cleavage of these proteins might be one mechanism where by DNase I is activated during apoptosis in the ovary. This was investigated by Bone & Tsang but they concluded that although actin and PARP can be degraded during ovarian apoptosis, extensive cleavage of these proteins is not necessary for the activation of DNase I [224].

A novel heterodimeric protein, DNA fragmentation factor (DFF), has been identified as a potential link between caspase-3 activation and DNA fragmentation [219]. Caspase-3 cleaves DFF to produce an active factor that induces DNA fragmentation in isolated nuclei. Cleavage of DFF in the absence of PARP cleavage, is sufficient to reproduce all the events of nuclear fragmentation observed during apoptosis [219]. Whether DFF is present and cleaved during apoptosis, in ovarian cells, is not known.

Although PARP and actin cleavage are not necessary for the activation of DNase I, it is possible that the degradation of these proteins have other functions during luteal regression. For example, since steroidogenesis is dependent on the cytoskeleton [225], actin degradation during apoptosis could account for the rapid loss of steroidogenesis associated with luteal regression [226]. SREBP has also been identified as a substrate of caspase-3 [227]. SREBP is a membrane-bound transcription factor that is released and activated by proteases in response to low cellular cholesterol levels [227, 228]. The cleaved SREBP acts as a transcription factor to increase the synthesis of low-density lipoprotein receptor (LDL), cholesterol-biosynthetic enzymes and enzymes of fatty acid synthesis [228]. The

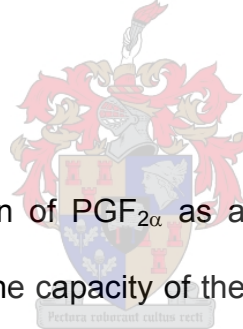
cleaved SREBP may be responsible for the accumulation of large amounts of cholesterol esters in lipid droplets in the regressing corpus luteum.

## 2.7 Maternal recognition of pregnancy

In most mammalian species,  $P_4$  must be secreted throughout gestation to maintain a uterine environment conducive to pregnancy [229]. In some species (e.g. carnivores and marsupials), gestation lasts no longer than a normal luteal phase [229]. Therefore, there is no need for the conceptus to alter luteal function because the corpus luteum provides adequate progesterational support. Most other mammalian species have developed a means of shortening the life span of the corpus luteum in event of a non-fertile cycle to increase reproductive efficiency. Along with development of a uterine luteolytic mechanism, some means of maintaining the uterus in a progesterational state during pregnancy must also be developed if successful reproduction is to occur. The simplest solution would be for the conceptus itself to secrete progesterone and ensure its own survival [229].

In many species, the placenta is a very active endocrine organ, secreting several hormones, including progesterone. However, in most species, ability of the conceptus to secrete progesterone is not developed until after the corpus luteum of the estrous cycle would have normally regressed (e.g. sheep) [229]. Thus, to maintain adequate progesterational support for developing pregnancy, it is necessary to prevent luteal regression. The process by which the conceptus acts to maintain luteal secretion of  $P_4$  has been termed maternal recognition of pregnancy. The mechanism by which this process is accomplished differs from species to species. In this section the mechanism in sheep will be considered.

In the ewe, the corpus luteum must be present through the first 50 days of gestation to maintain pregnancy [230]. Pregnancy can be maintained in ovariectomized ewes by treatment with  $P_4$  [32, 231]. The placenta begins to secrete  $P_4$  by approximately the 50<sup>th</sup> day of gestation [229] and provides adequate progestational support for the remainder of gestation. After removal of embryos on day 13 or thereafter, the lifespan of the corpus luteum is extended [232], indicating that the embryo has a luteotropic effect by this time. However early studies in the sheep by Moor & Rowson (1966) [43] demonstrated that the transfer of an embryo to the uterus of a synchronized non-pregnant recipient sheep would prevent cyclic regression of the corpus luteum in the recipient even if transferred as late as day 12 of the cycle. Thus the critical period for maternal recognition of pregnancy is days 12 to 13.



Following the identification of  $PGF_{2\alpha}$  as a luteolytic hormone in the sheep [233-235], it seemed likely that the capacity of the embryo to prevent corpus luteum regression might be related to either (a) suppression of  $PGF_{2\alpha}$  release from the uterus by the embryo or its products (anti-prostaglandin secreting effect) or (b) the ability of a secretory product of the embryo to protect the corpus luteum against potentially luteolytic releases of  $PGF_{2\alpha}$  from the uterus (luteoprotective effect). One or both of these mechanisms would allow the corpus luteum to continue to secrete the  $P_4$  which is necessary for the maintenance of early gestation in most species. These two different mechanisms have been loosely termed “antiluteolytic effect of the embryo”, which is an unfortunate phrase, since it does not indicate the nature of the effect, i.e. an indirect one via suppression of  $PGF_{2\alpha}$  secretion from the uterus or a direct one on the corpus luteum by protecting it against the effects of  $PGF_{2\alpha}$ . In my

opinion the terms (a) “antiprostaglandin secreting” and (b) “luteoprotective” respectively would seem more appropriate.

### **2.7.1 Antiprostaglandin secreting effect**

Until recently reports on the effect of the presence of an embryo on  $\text{PGF}_{2\alpha}$  secretion from the uterus in early pregnancy have been conflicting. The levels of  $\text{PGF}_{2\alpha}$  at this time have been reported to be absent [116], diminished [117], unchanged [119-121, 236-238] or higher [235, 239]. The reports used both  $\text{PGF}_{2\alpha}$  and its major metabolite PGFM as an indicator of  $\text{PGF}_{2\alpha}$  synthesis. If reports that used PGFM and reports where samples were taken once a day were excluded because of the controversial nature of these methods, then there would be a consensus on  $\text{PGF}_{2\alpha}$  secretion during the time of maternal recognition of pregnancy. The basal concentrations of  $\text{PGF}_{2\alpha}$  are greater in pregnant animals, but the main episodic peaks of  $\text{PGF}_{2\alpha}$  which appear to be associated with luteolysis in cycling ewes, do not occur [116, 117, 240-242]. It must be mentioned, however, that there are some concerns that the method of analysis of  $\text{PGF}_{2\alpha}$  may pick up some interferences from  $\text{PGE}_2$ .

Secretion of ovine trophoblast protein 1 (o-TP-1) from the day 13 - 17 sheep conceptus appears to be responsible for the lack of major peaks of  $\text{PGF}_{2\alpha}$  in pregnant ewes [243, 244]. o-TP-1 binds to the oxytocin receptor thus preventing the production of c-AMP and the activation of Phlase- $\text{A}_2$  and endoperoxide reductase necessary for the production of  $\text{PGF}_{2\alpha}$ .

### 2.7.2 Luteoprotective effect

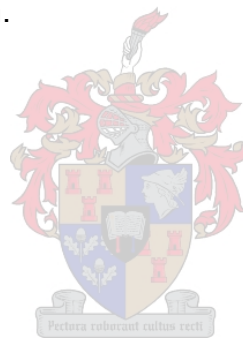
Sensitivity of the corpus luteum to the luteolytic action of  $\text{PGF}_{2\alpha}$  appears to be altered in early pregnancy. Intrafollicular or periarterial injections of  $\text{PGF}_{2\alpha}$  were less effective in inducing luteal regression in pregnant than in nonpregnant ewes on day 12 or 13 [64, 245, 246]. Also, the dose of  $\text{PGF}_{2\alpha}$  required to induce continued luteolysis when given intramuscularly, was less in cycling than in pregnant ewes [247]. Luteal resistance to  $\text{PGF}_{2\alpha}$  was absent on day 10 and 20 after mating, but the strongest between days 13 to 16 after mating. Thus, resistance to  $\text{PGF}_{2\alpha}$  was transient and coincided with the time for maternal recognition of pregnancy. The two factors that have been implicated in the luteoprotective effect of the embryo on the corpus luteum is  $\text{PGE}_2$  and a proteinaceous factor secreted by the embryo.

Increased concentrations of  $\text{PGE}_{2\alpha}$  are observed in pregnant ewes in the critical period for maternal recognition of pregnancy. It has been reported that  $\text{PGE}_{2\alpha}$  is secreted by the embryo and the endometrium of the gravid uterus. The shift in production from  $\text{PGF}_{2\alpha}$  to  $\text{PGE}_2$  by the uterus can be achieved by down regulation or inactivation of endoperoxide reductase and up-regulation or activation of endoperoxide isomerase.  $\text{PGE}_2$  is a potent vasodilator and antagonizes the effect of  $\text{PGF}_{2\alpha}$  which is a potent vasoconstrictor. Thus,  $\text{PGE}_2$  will cause a shunt of blood to the corpus luteum and better oxygen delivery. This, unlike  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$  will be conducive to the early stages of  $\text{P}_4$  synthesis. A direct effect for  $\text{PGE}_2$  on the luteal cell acting via a receptor remains speculative at this stage. There are no conclusive studies that could show the presence of receptors for  $\text{PGE}_2$  present on these cells.



However incubation of luteal cell cultures with PGE<sub>2</sub> prevents PGF<sub>2α</sub> induced regression [248].

Wiltbank *et al* (1992) demonstrated that the day-15 sheep conceptus secretes a proteinaceous factor that is capable of preventing both the antisteroidogenic and cytotoxic effects of PGF<sub>2α</sub>. This factor does not bind PGF<sub>2α</sub> or prevent it from binding to its luteal receptor [249]. A similar factor is secreted from cow embryos. Because the molecular weight of this factor is approximately 30 000, it is hard to envision its being transported locally from the uterus to the ovary via the uterine vein and ovarian artery similar to PGF<sub>2α</sub>. It is thus not clear how this proteinaceous factor provides its luteoprotective action.



## SECTION B      PREMATURE LUTEAL REGRESSION

### 2.8      The phenomenon of premature luteal regression

Premature luteal regression can be defined as: “Regression of the corpus luteum earlier in the estrous cycle before the onset of natural luteal regression between day 13 and 14 of the cycle”. This phenomenon has been observed in various domesticated species including sheep [250] , goats [251], cows [252] and also wild deer species like the red deer (*Cervus elaphus*) [253]. This phenomenon is found in a diversity of stages in these animals life cycles. The occurrence of subnormal luteal phases is particular significant in conditions where ovulation is induced [251, 252, 254] (anestrus, pre-puberty, postpartum) and stimulated 250, 253, 255] (superovulation).

Superovulation is an integral part of embryo transfer. The term “embryo transfer” encompasses a variety of procedures which may include superovulation, embryo recovery, short-term culture of embryos *in vitro* and transfer of embryos into the recipient. The success rates of embryo transfer are highly variable in small stock like sheep, because of sub-optimal conditions associated with one or more of the above procedures. One of these sub-optimal conditions is a short luteal phase.

The processes of fertilization and early embryonic development are P<sub>4</sub> independent *in vitro* [256]. However, luteal P<sub>4</sub> is essential to create a feasible environment for these processes to take place *in vivo* [257]. Premature regression of corpora lutea in superovulated sheep ewes prior to the time of embryo collection (7<sup>th</sup> day of the cycle) results in very low rates of recovery. In the majority

of animals with premature regression no ova / embryos are found. In some cases only unfertilized ova were found, and in the exceptional cases where embryos were recovered they were of poor quality. This might be attributed to expulsion of embryos / unfertilized ova via the cervix as a result of endocrine abnormalities associated with luteal failure and embryo toxicity of a regressing corpus luteum [250-252].

The reported incidence of premature regression in the literature varies from as low as 2% to as high as 100% of superovulated ewes. The incidence is influenced by season, nutrition, superovulatory hormone(s) used, timing of administration and dose of superovulatory hormones and method of synchronisation. These issues will be dealt with separately in the following sections. To date, the cause of premature regression has not been elucidated.

## **2.9 The effect of season**

### **2.9.1 Neuro-endocrine mechanisms mediating photoperiodic regulation of gonadotropin secretion**

In most breeds of sheep [258-262], normal estrous cycles occur in the fall and winter (breeding season), while cycles cease in the spring and summer. From an adaptive point of view, this limitation of ovarian cycles to the fall and winter ensures that lambs are born in the spring, when environmental conditions are usually favourable for their survival. There is, however, considerable variation in seasonal reproductive patterns. At some extremes, some primitive mountain sheep have only one estrous cycle during the breeding season, whereas in Merino ewes, estrous cycles occur throughout most of the year [263, 264]. The seasonal ovarian cyclicity

in the ewe is mainly controlled by the photoperiod involving LH, E<sub>2</sub>-17β and melatonin (MT) [263].

In sheep, the mechanisms by which a change in photoperiod is transformed into an alteration in LH secretion can be divided into three major steps. First the day length is perceived, and an appropriate neural signal is transmitted to the pineal gland. The second step is transduction of this neural information into an endocrine signal by the pineal gland. Finally, the endocrine signal from the pineal is transformed into a change in gonadotropin secretion via the hypothalamo-hypophyseal axis. A detailed analysis of these steps in the sheep has been presented in several reviews [265-267]. This section is intended as a brief overview, with particular emphasis on current developments.

Perception of day length requires a photoreceptor and a mechanism for measuring the hours of daylight. The most important photoreceptor in sheep, as in other mammals, is the eye [268]. Following orbital enucleation, changes in photoperiod no longer alter either E<sub>2</sub>-17β negative feedback or ovarian cycles[269]. The mechanism by which sheep measure day length, most likely involves endogenous circadian rhythm in photosensitivity [270, 271]. In its simplest form, this endogenous rhythm is set that its photosensitive phase occurs more than 12 hours after the start of the light phase. If light is on during this photosensitive phase, the day is perceived as long (>12 hours of light); if no light occurs during that period, the day is perceived as short. In sheep, however, the situation is more complex because the animal's photoperiodic history can influence her response to day length. For example, an intermediate day length (13 hours light / day) is perceived as a

“long day” when ewes are switched from 10 hours to 13 hours of light per day, and as a “short day” following a switch from 16 hours to 13 hours of light per day [272].

After the photoperiod is converted into a neural signal by the retina, the information is transmitted to the pineal gland. The pathway for transmission of this information in the ewe remains largely unknown. In the hamster, this pathway includes the suprachiasmatic nucleus, paraventricular nucleus, and the superior cervical ganglion, which innervates the pineal [273, 274]. The little information available in sheep is consistent with a similar pathway. Lesions of the suprachiasmatic nucleus produce ovulatory cycles during anestrus [275-277] and removal of the superior cervical ganglion blocks the photoperiodic response of rams [278, 279].

The crucial role of the pineal gland in mediating the effects of photoperiod in sheep was demonstrated by classic ablation experiments. Pinealectomy prevents photoperiod-induced shifts in reproductive function in ewes [280-282]. Furthermore, the pineal is clearly required for both the stimulatory effects of short days [280, 281] and the inhibitory effects of long days [280, 282] on ovarian function. Because of the large amount of work implicating melatonin as an important pineal hormone in other species [283], much of the work on the role of the pineal in sheep has focused on this indoleamine.

Secretion of MT shows a circadian rhythm [284] that is influenced in two ways by photoperiod: (a) the rhythm is synchronized by light-dark cycle [281, 283], and (b) MT is directly inhibited by light [283, 285, 286]. The net effect of these actions are

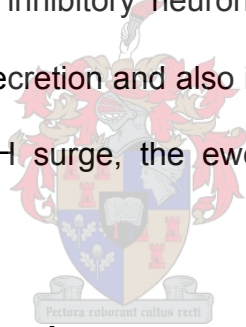
that under both natural [286-289] and artificial photoperiod [280, 281], MT secretion increases shortly after darkness and remains elevated until the beginning of the light phase. Thus, the serum MT pattern can be considered a hormonal analogue of the light-dark cycle.

Information on the possible sites where MT acts to control this endogenous rhythm has come from recent studies on the localization of melatonin receptors (R-MT). Surprisingly, the highest concentrations of R-MT are observed in the pars tuberalis [290-293], which is the portion of the adenohypophysis in close apposition to the median eminence. Little, if any, binding occurs in the median eminence or pars distalis [290-293], but R-MT are distributed elsewhere throughout the ovine central nervous system, including the rostral hypothalamic areas, that contain gonadotropin releasing hormone (GnRH) cell bodies [291-293], and the medial basal hypothalamus [290]. Which of these R-MT are important for the control of reproductive function, remains to be determined.

Information on the possible mechanisms of action of MT has come from studies on the changes in the hypothalamo- hypophyseal function that underlie seasonal breeding in the ewe. Studies on the effects of pentobarbital anaesthesia have raised the possibility that these actions of photoperiod reflect activation of inhibitory neural systems [294]. In intact anestrus ewes, pentobarbital produces a dramatic increase in pulsatile LH secretion [294]. Based on the assumption that pentobarbital anaesthesia decreases neural activity [295], these data imply that during anestrus, LH pulse frequency is inhibited by a set of inhibitory neurons. Pentobarbital decreases the activity of these inhibitory neurons, allowing pulsatile LH

secretion to increase. This interference, in turn, led to a model for seasonal control of LH secretion in the ewe.

This model proposes that the long-day photoperiod of anestrus activates a set of  $E_2$ - $17\beta$  sensitive neurons that inhibit the GnRH pulse generator.  $E_2$ - $17\beta$  suppresses GnRH pulse frequency by increasing the activity of these neurons, but can only do so in anestrus. The reason for this is because these inhibitory neurons are only sensitive for the effects of  $E_2$ - $17\beta$  during anestrus. The most logical mechanism to explain this is that estradiol- $17\beta$  receptors ( $R$ - $E_2$ - $17\beta$ ) is controlled by MT. In the summer with its long days, there is a decrease in MT secretion and an upregulation in  $R$ - $E_2$ - $17\beta$  in the inhibitory neurons of the GnRH pulse generator. This leads to a decrease in LH secretion and also in  $E_2$ - $17\beta$  secretion. The result is that there is no preovulatory LH surge, the ewe doesn't ovulate and goes into anestrus.



### **2.9.2 The effect of season on the occurrence of premature regression in the superovulated sheep ewe**

The incidence of premature luteal regression in the superovulated sheep ewe is greater in autumn than in spring [250, 296]. In two reports it also seems that the incidence may differ from year to year because the incidence was higher in the study by Ryan *et al* (1987) [250] than in the study by Jabbour *et al* (1991) [296]. Both studies were conducted in the same region in Western Australia (see Table 1).

**Table 1** The incidence of superovulated ewes with premature regressed corpora lutea as affected by season [250, 296]

	Autumn	Spring
<b>Study 1[250]</b>	<b>53/143 (37%)</b>	<b>32/156 (21%)</b>
<b>Study 2[296]</b>	<b>21/89 (24%)</b>	<b>5/88 (6%)</b>

number of ewes with PLR / number of ewes superovulated

During the non-breeding season (spring) there is active suppression of the GnRH pulse generator by  $E_2$ - $17\beta$  sensitive inhibitory neurons [297]. This effect seems to be more obvious in some breeds, and less so in breeds like the Merino. Theoretically this would influence the formation and the normal functioning of the corpus luteum because of inadequate LH release. Randel *et al* (1982) and Sumbung *et al* (1987) reported that season can alter the timing and magnitude of the LH surge [297, 298].

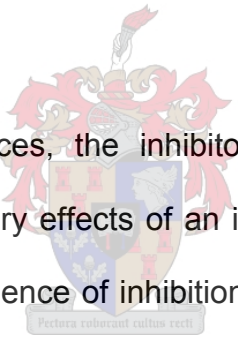
In the superovulated sheep ewe with more follicles ovulating and more corpora lutea formed, one would expect this effect to be more pronounced. Theoretically there is the possibility that short luteal phases are not because of premature regression, but merely because of inadequate function. The results from the studies by Ryan *et al* (1987) [250] and Jabbour *et al* (1991) [296], however, do not support this hypothesis. They found that there is a lower incidence of premature regression in the non-breeding season when the inhibition of the GnRH pulse generator, and thus also the LH production, is at its strongest. This finding raised the question of whether pastures with higher nutritional value in the spring and summer (non-breeding season) could influence the incidence of premature regression rather than photoperiod.



## 2.10 The effect of nutrition

### 2.10.1 The effect of nutrition in reproductive performance

There is considerable evidence in the literature that the ovulation rate in ewes is stimulated in response to increased feeding [263, 264, 299]. An increase in the amount of protein digested post-ruminally [300] or increased availability of energy-yielding substrates like carbohydrates [301], may be responsible for increased ovulation rates in ewes. Increased secretion of FSH [302] and increased ovarian sensitivity to gonadotropins [303] have been suggested as a mechanism for the higher ovulation rates and higher fecundity seen in animals with increased levels of nutrition.



Under normal circumstances, the inhibitory effect of photoperiod on LH secretion overrides the stimulatory effects of an increased nutritional state in most breeds of sheep [302]. In the absence of inhibition of the GnRH pulse generator by  $E_2-17\beta$  sensitive inhibitory neurons, better nutrition will increase reproductive performance. This can clearly be seen in the practice of “flushing” where animals are fed a high-energy supplement prior to mating to improve their reproductive performance. This practice is effective even when supplementation starts within six days prior to ovulation [299]. According to the same author, in breeds like the Merino where the effects of photoperiod is minimal, this effect is also seen in the non-breeding season.

The exact mechanism whereby increased nutrition increases reproductive performance, has not been explained. One proposed theory, is that a higher protein

intake increases the availability of essential amino acids being the building blocks of protein hormones like LH, FSH and GnRH [302, 303]. If the specific amino acids necessary to produce FSH are limited, FSH, LH and GnRH secretion will be decreased which will influence ovulation rate and reproductive performance. Also, a decrease in LH secretion can lead to luteal failure and embryonic death. For this theory to be valid, there has to be a deficiency in essential amino acids. At least one study proved that increased protein intake do not increase the amplitude of the LH surge, however, it did advance the LH surge by  $\pm 10$  hours in that particular study [296].

Another argument that is becoming increasingly popular among scientists, is the role of the intra-ovarian insulin like growth factor (IGF) system. A large body of evidence now suggests the existence of an intra-ovarian IGF system complete with ligands, receptors and binding proteins [304]. Although IGFs may be acting in their own right, their most important role appears to be their ability to synergise with the pituitary gonadotropins, and to amplify their impact thus making the ovary more sensitive for pituitary gonadotropins. It has now been clearly established in several species that the ovary is the site of hormonally regulated IGF-I gene expression [305-308]. The ovary displays the third highest level of IGF-I gene expression, with only the uterus and liver showing higher expression [305].

IGF-I and IGF-II are single chain polypeptides that have 70 and 67 amino acid residues, respectively, and structural features similar to those of insulin. The regions of structural similarity of IGF-I and IGF-II to insulin are confined to the A-chain and B-chain portions of the molecules where they are more homologous to insulin than

relaxin, another member of the insulin-like super-family of molecules. Due to their structural similarity, insulin will bind to IGF-I receptors (R-IGF), but the receptor's affinity for insulin is not as high as for IGF-I [309, 310].

Besides ligands and receptors, the ovary is well endowed in IGF binding proteins (IGFBPs), the exact role which remains largely unknown. IGFBPs constitute a heterogeneous group of at least six distinct proteins capable of binding IGFs (but not insulin), with affinities in the  $10^{-10}$  to  $10^{-9}$  M range. The six known rat IGFBPs range in size from 21.5 to 29.6 kDa. It is assumed, although by no means proven, that the IGFBPs subserve different functions, thereby justifying the existence of multiple IGFBPs types. Although the exact role of the IGFBPs remains a matter of study, general consensus supports a role in the transport of IGFs and regulation of their bio-availability [311-314].

It is known that high energy supplementation due to increased carbohydrates in the diet, increases blood levels of insulin. There is a distinct possibility that these high levels of insulin react with R-ILGF and mediate the effects on the level of the ovary. It is known that sheep have a higher lambing rate if exogenous insulin is administered. This effect contributes to more follicles ovulating. The effect of insulin or IGF on luteal function, however, has not been studied [314].

### **2.10.2 The effect of nutrition on the occurrence of premature regression in the superovulated sheep ewe**

Jabbour *et al* (1991) [296] investigated the effect of a high energy, high protein supplement like lupin grain on the incidence of premature regression in Autumn (Table 2). It was found that feeding lupin grain reduces the incidence of

premature regression to levels normally experienced in spring [250, 296]. This supports the hypothesis that the higher incidence of premature regression in autumn is caused by lower nutrition levels. Nutritional values of pastures vary from year to year, depending on environmental conditions. Thus, the fact that nutrition has an influence on the incidence of premature regression could also explain why a higher incidence was seen in the study by Ryan [250], than the study by Jabbour [296] in the same region in Western Australia, because their studies were conducted in different years.

**Table 2** The effect of supplementary lupin grain feeding on the endocrine and steroidogenic responses and luteal function of superovulated Merino ewes [296]

	+ Lupin Grain	- Lupin Grain
<b>Incidence of premature luteal regression</b>	<b>1/44 (2%)</b>	<b>20/45 (44%)</b>
<b>Time to onset of estrous from sponge removal (hours) (mean ± SD)</b>	<b>32.9 ± 1.1 (n=24)</b>	<b>35.4 ± 1.2 (n=24)</b>
<b>Peak E<sub>2</sub> concentration (pg/ml) (mean ± SD)</b>	<b>14.2 ± 4.7 * (n=5)</b>	<b>11.5 ± 2.4 (n=4)</b>
<b>Peak E<sub>2</sub> concentration per follicle (pg/ml/follicle) ** (mean ± SD)</b>	<b>1.2 ± 2.9 * (n=5)</b>	<b>0.5 ± 0.3 (n=4)</b>
<b>Time to LH peak from sponge removal (hours)(mean ± SD)</b>	<b>36.0 ± 4.0 * (n=2)</b>	<b>46.6 ± 1.3 (n=3)</b>
<b>Peak LH concentration (ng/ml) (mean ± SD)</b>	<b>6.3 ± 1.3 (n=2)</b>	<b>5.3 ± 0.9 (n=4)</b>

\* Values between the two groups are significantly different (p<0.05)

\*\* The peak E<sub>2</sub>-17β concentration in plasma is corrected for the number of preovulatory follicles in each group, i.e. the peak E<sub>2</sub>-17β concentration is divided by the number of preovulatory follicles. The total of the corpora lutea and large unruptured follicles are assumed to represent the total number of preovulatory follicles.

In the study by Jabbour *et al* (1991) [296] the endocrine and steroidogenic responses of superovulated ewes were also measured. Unfortunately, the authors used a small number of subjects for the E<sub>2</sub>-17β and LH determinations, but it seems that although the synthesis of protein and peptide hormones are not influenced, the production of steroids are affected. The advancement of the LH surge can also be

explained by the effect on  $E_2-17\beta$  synthesis. Thus, the argument that high protein supplementation abolishes the shortage of essential amino acids does not seem to play a role in the incidence of premature luteal regression (Table 2).

## 2.11 Superovulatory hormones used and the influence of the dosage

### 2.11.1 Superovulation theory and methods of inducing superovulation

Superovulation can be defined as “an increase in the normal ovulation rate by affecting levels of gonadotropins from exogenous or endogenous sources. Three mechanisms of actions have been suggested for the effects of gonadotropins on follicular development.

- i) follicles already undergoing atresia are “rescued” presumably as a result of vigorous mitotic activity in granulosa and/or thecal compartments.
- ii) smaller healthy follicles are recruited into a more active growth phase.
- iii) the rate of follicular atresia is reduced.

These mechanisms are not exclusive and indeed the most likely explanation for superovulation is a combination of the mechanisms mentioned. Methods of inducing superovulation in the sheep include: [315]

1. Administration of equine chorionic gonadotropin (eCG)
2. Administration of follicle stimulating hormone (FSH)
3. Active and passive immunization against androstenedione and  $E_2-17\beta$
4. Administration of bovine follicular fluid
5. Administration of GnRH in a pulsatile fashion

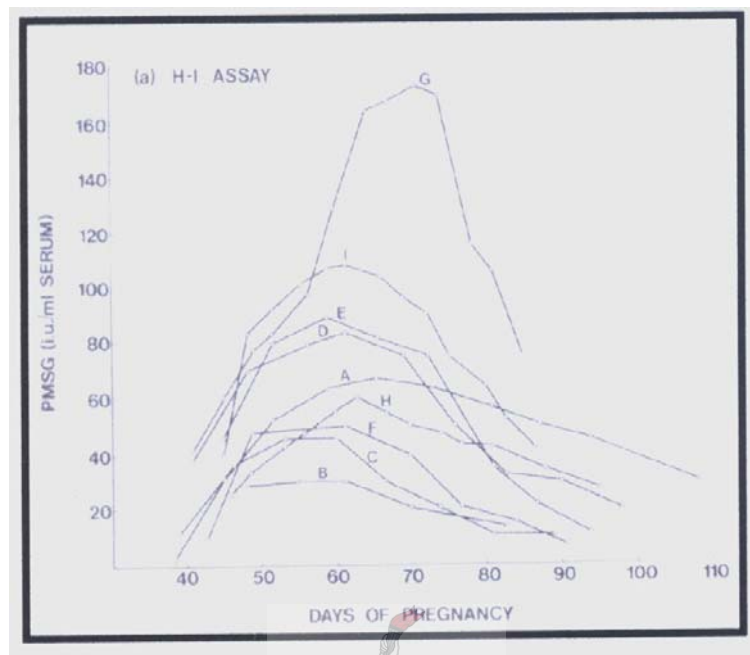
6. Administration of human menopausal gonadotropin (hMG)
7. Administration of horse LH
8. Administration of anti-LH.

The following discussion will be limited to the two most commonly used hormones used to achieve superovulation in sheep, namely eCG and FSH.

#### **2.11.1.1 Equine chorionic gonadotropin**

In 1930 equine chorionic gonadotropin was discovered at the Department of Animal Science of the University of California by the late Professors Harold Cole and George Hart [316]. Until recently, their discovery was known as Pregnant Mare Serum Gonadotropin (ECG)[316]. In their classic paper announcing the discovery of equine chorionic gonadotropin (eCG), Cole & Hart (1930) demonstrated, with remarkable accuracy, the general pattern of secretion of the hormone during pregnancy [316]. Many other similar studies undertaken since then to monitor eCG secretion patterns in pregnant mares and other equids, using a wide variety of *in vivo* biologic [317-321], hemagglutination-inhibition [316, 322], radioimmunologic [323, 324], and *in vitro* radioreceptor [325, 326] assay methods, have all confirmed the essential shape of the secretion curve and the variability between individual animals in terms of peak concentration of eCG measurable in blood (Figure 19). Soon after their discovery of eCG in 1930, Cole and his colleagues drew attention to the close relationship that exists between secretion of gonadotropic hormone and the considerable degree of secondary luteal development that occurs in the mare's ovaries [327]. They noted that on or soon after the first appearance of eCG in maternal blood around day 40 after conception, one or more accessory ovulations

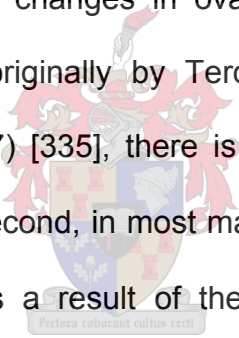
occurred in the maternal ovaries, with further secondary structures appearing in an accumulative fashion during the next 100 or more days of gestation.



**Figure 19** Concentrations of eCG (ECG) measured by hemagglutination-inhibition (H-I) assay in the serum of nine pony mares between 40 and 110 days of gestation. Note the individual variation between mares in the peak concentrations (31 to 173 IU/ml) reached between days 54 and 73 [322].

Amoroso *et al* (1948) [328] described the same phenomenon, and Allen (1970) [329] counted as many as 35 distinct luteal structures in the ovaries of one mare at day 140 of pregnancy. This led to the general assumption that eCG provided both gonadotropic and luteotropic stimuli. Serial measurements of the concentrations of pituitary FSH and LH in the serum of cycling and pregnant mares throughout the spring and summer months of the physiologic breeding season showed FSH is released from the pituitary gland in mares at intervals of approximately 10 to 12 days [330, 331]. The rhythmic pattern of release is controlled essentially by day length

and the secretion of follicular inhibin and / or  $E_2-17\beta$ , but it is not influenced by negative feedback effects of  $P_4$ . Thus, releases of pituitary FSH continue unchanged during early pregnancy just as in the estrous cycle. This finding coincides well with the earlier observations of Bain (1967) [332], and Van Rensburg & Van Niekerk (1968) [333], that considerable follicular growth occurs in the mare's ovaries around days 18 to 23 of gestation. This corresponds with the time the mare could have been expected to have returned to oestrus and re-ovulate had she not been pregnant. However, these large follicles do not ovulate, and they tend to regress and make way for a further wave of follicular growth some 10 to 12 days later. This normally coincides with the initial onset of eCG secretion between days 36 and 40 when two significant changes in ovarian steroid hormone production occur. First, as demonstrated originally by Terqui & Palmer (1979) [334], and confirmed by Jeffcott *et al* (1987) [335], there is a sharp and pronounced rise in plasma  $E_2-17\beta$  concentration. Second, in most mare's a pronounced rise in plasma  $P_4$  concentration is observed as a result of the first secondary ovulation [329].

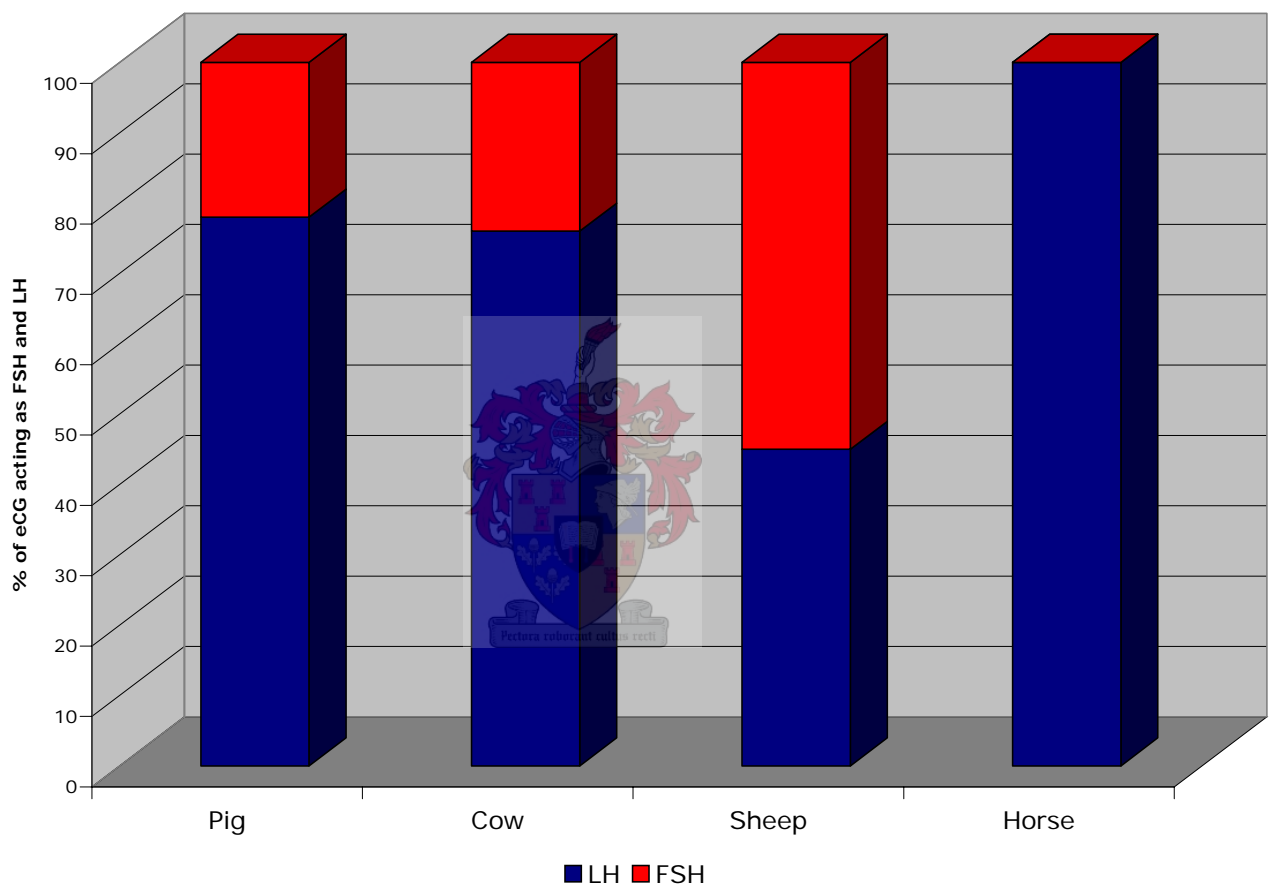


Clearly the continuing secretion of pituitary FSH, not eCG, stimulates the waves of follicular growth in the pregnant mare. The LH-like action of eCG, in the absence of pituitary LH, merely acts to induce final maturation of the dominant follicle in the wave and to either ovulate or luteinize it.

Perhaps the most unusual and interesting feature of the eCG molecule is its unique ability to express both FSH-like and LH-like biological activities when administered to other mammalian species [316, 336] (Figure 20). This is in contrast with the mare and stallion where it only has an LH-like action [337, 338]. This dual capacity of eCG as an FSH and LH-like molecule makes it the ideal candidate to



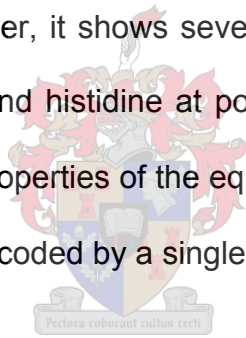
induce superovulation in other mammalian species. Not only would it stimulate excessive follicular development and recruitment and protect the non-dominant follicles from the lack of FSH caused by negative feedback via inhibin from the FSH independent dominant follicle, but the LH properties would also ovulate the follicles and give luteotropic support to the newly formed corpora lutea.



**Figure 20** An comparison of the dual capacity of eCG as an FSH and LH like hormone when administered to various domesticated animals [316, 336].

This unique molecule shows structural similarities to human chorionic gonadotropin (hCG) [339]: It is a high molecular weight (70 000 Da) acidic glycoprotein with an abnormally high carbohydrate content (45 %) consisting mainly of sialic acid, galactose and glucose amine [339]. The high sialic acid content has

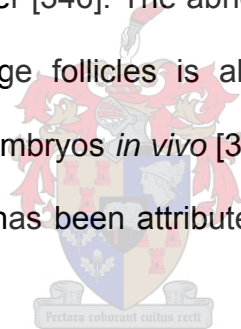
been linked with the long biological half life of this molecule (7 days). Like the pituitary and chorionic gonadotropins in all the mammalian species, eCG is a heterodimeric glycoprotein that consists of an  $\alpha$  and a  $\beta$ -subunit joined noncovalently [340]. The  $\alpha$ -subunits are identical in pituitary and chorionic gonadotropins within each species, whereas the  $\beta$ -subunit differ, indicating that the specificity of action must reside in the  $\beta$ -subunit. This has been experimentally confirmed by interchanging the  $\alpha$ -subunits between different species, without altering their specificity of action. The amino acid sequence of the  $\alpha$ -subunit is highly homologous across species and the horse sequence show 68 to 79% homology with the other available mammalian  $\alpha$ -subunits sequences from both pituitary and chorionic gonadotropins. However, it shows several unique substitutions, including transposition between tyrosine and histidine at positions 87 and 93, which may be related to some of the unusual properties of the equine gonadotropins [340]. The  $\alpha$ -subunit has been shown to be encoded by a single gene across species.



In general, each  $\beta$ -subunit is also encoded by a single gene. Across species these genes form a family of closely related genes that have clearly arisen through gene duplication. It was discovered some time ago that both horse and human CG  $\beta$ -subunits are longer than all pituitary  $\beta$ -subunits by 30 residues at the C-terminal. This implied a possible evolutionary linkage between primate and equine CG, but has now been established that these hormones evolved independently. The primate CG  $\beta$ -subunit genes have apparently arisen via duplication of the LH gene, followed by a mutation leading to the C-terminal extension and further gene duplication [341, 342] probably before divergence of baboons and humans [343]. Bousfield and colleagues (1987) showed that the horse LH  $\beta$ -subunit is, in fact, identical in amino

acid sequence to the horse CG  $\beta$ -subunit except for the C terminal extension [344]. This provided an explanation for the previous somewhat surprising observation that horse LH exhibits the same intrinsic FSH-like activity when administered to non-equine species [345]. Thus the FSH-like properties is most likely not linked to the C terminal extension but linked to minor amino acid sequence differences between hCG and eCG in the  $\beta$ -subunit.

The use of the hormone in large and small domestic species has unfortunately been associated with the formation of follicles that fail to ovulate and lutenize to form cysts. This results in a decrease ovulation rate and a lower yield of viable quality embryos for transfer [346]. The abnormal endocrine status associated with the presence of these large follicles is also thought to be detrimental to fertilization and development of embryos *in vivo* [347, 348]. The occurrence of cysts increase with dose of eCG and has been attributed to the prolonged half-life of the gonadotropin [349].



Generally superovulation with 1600 IU eCG can yield 230 grade 1 embryos per 100 ewes superovulated [350]. Table 3 and 4 represents the typical response to superovulation with eCG in sheep ewes, with and increased dose of eCG.

**Table 3** Numbers of corpora lutea (Cl) and total ovarian response (Cl + persistent large follicles (< 5 mm)) (mean  $\pm$  SD) assessed on day 6 of the oestrous cycle for ewes treated with eCG [350]

Dose eCG IU	No ewes	Cl's	Total ovarian response
800 IU	36	2.7 $\pm$ 0.2	3.5 $\pm$ 0.3
1600 IU	35	9.0 $\pm$ 0.8	11.8 $\pm$ 1.1

**Table 4** Proportion of ewes that fail to superovulate in response to treatment with eCG [350]

Dose of eCG IU	Proportion of ewes that fail to superovulate (< 3 CL's or large follicles)
800	75 %
1200	12%
1600	10%

### 2.11.1.2 Follicle stimulating hormone

Follicle stimulating hormone (FSH) stimulates follicular development in the ovary, and in particular the granulosa cells of the follicle. FSH along with LH prepares the follicle for ovulation and luteinization. FSH specifically stimulates the production of the enzyme system aromatase  $P_{450}$ , for converting androstenedione and testosterone to  $E2-17\beta$ . It also stimulates the production of the enzymes involved in plasminogen activation which plays an important role in ovulation. Studies on the induction of follicular development by exogenous gonadotropins in women and domestic species have been accelerated by their application in *in vitro* fertilization and embryo transfer [351].

In most species the pituitary contain 5% FSH and 95 % LH during most of the estrous cycle. The highest FSH:LH ratio is achieved just after the LH surge when the pituitary LH store is almost depleted [315, 350].

There are three types of commercial FSH available including :

- a) FSH-P : The earliest form of commercially available FSH was a crude extract from the pituitary known as FSH-P. The biggest problem with this source of

commercial FSH is that usually the amount of LH contamination is extensive and undisclosed. If the commercial company producing this type of FSH would use only pituitaries from animals just after the LH surge, LH contamination could be limited. From experience in the field, researchers have found that LH contamination varies from batch to batch.

- b) Purified FSH extracts like Ovagen™: More purified forms of FSH became available during the later years. One of the first methods to “purify” FSH from LH contamination was with immunoabsorption with antiserum to the  $\beta$ -subunit of LH. Today there are various methods used but even purified preparations still contains 1 - 10 % LH contamination[351].
- c) Recombinant FSH: The purest form of FSH is recombinant FSH (recFSH). The genes for FSH is transferred into bacteria like *Escherichia coli* , which then produce the FSH in a culture medium. The FSH can then be recovered and purified from the culture medium. This is the purest form of FSH (<0.01 % LH contamination), but expensive to produce.

The biggest problem with FSH is the relative short half-life of pituitary gonadotropins. FSH has a half-life of approximately 2.5 hours, which means that after 6 hours almost all of the FSH is catabolised. To achieve superovulation, FSH must therefore be administered frequently. A significant proportion of ewes treated with FSH alone fail to show a superovulatory response, thus reducing yields of embryos available for transfer or cryopreservation[315, 352].

Generally superovulation with 18 mg FSH can yield 230 grade 1 embryos per 100 ewes superovulated. Table 5 and 6 represent the typical response to superovulation with an increased dosage of FSH-P.

**Table 5** Numbers of corpora lutea (Cl) and total ovarian response (Cl + persistent large follicles (> 5 mm)) (mean  $\pm$  SD) assessed on day 6 of the oestrous cycle for ewes treated with FSH-P[317]

Dose FSH-P	No ewes	Cl's	Total ovarian response
12 mg	37	6.7 $\pm$ 1.0	8.2 $\pm$ 1.0
18 mg	37	10.8 $\pm$ 1.3	12.8 $\pm$ 1.3

**Table 6** Proportion of ewes that fail to superovulate in response to treatment with FSH-P[317]

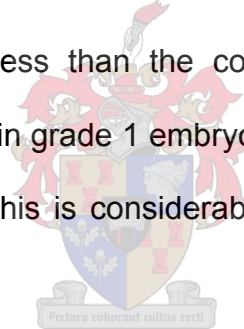
Dose of FSH-P mg	Proportion of ewes that fail to superovulate (> 3 CL's or large follicles)
12	32%
18	19 %

### 2.11.1.3 Combination of pregnant mare serum gonadotropin and follicle stimulating hormone

In an attempt to increase the amount of normal grade 1 embryos for transfer or cryopreservation, a lower dose of eCG is sometimes used in combination with a high dose of FSH. This is done to decrease the amount of animals that do not superovulate with FSH alone. This approach will also decrease the incidence of large unruptured follicles observed in animals superovulated with a high dose of eCG. A moderate dose of eCG (800 IU) in conjunction with 18 mg of FSH-P

increased the ovulatory response above that observed when FSH-P was used alone [350].

By using a purified extract of FSH like Ovagen™ with LH contamination limited to 1 - 10 % of protein mass, resulted in a reduced variability in ovulatory response [351]. Commercial purified extracts of FSH like Ovagen™ are, however, still extremely expensive resulting in a limited application. The conventional method of superovulating small stock with Ovagen™ is by injecting a total dose of 7 to 10 mg at 12 hour intervals in decreasing doses for 3 to 4 days. It was reported by King *et al* (1997) [350] that the total dose of this treatment can be reduced by 55 % if the FSH is dissolved in a 3.2 % gelatin-saline vehicle and combined with 400 IU eCG. This dosage of eCG is even less than the conventional 800 IU eCG used in combination the FSH. The yield in grade 1 embryos by using this approach was 444 per 100 ewes superovulated. This is considerably better than either eCG or FSH alone.



### **2.11.2 The influence of the type and dose of superovulatory hormones on the incidence of premature luteal regression**

It is difficult to exclude the influence of nutritional factors when accessing the influence of the type and dose of superovulatory gonadotropins on the occurrence of premature luteal regression, since nutritional status may vary from animal to animal. However, it is generally accepted that the use of eCG and FSH in combination is associated with a higher incidence of premature regression than if only eCG or FSH is used alone. Irrelevant of the type of gonadotropin used, the incidence of

premature regression increase with an increase of dose of the particular gonadotropin (see Tables 7 - 9).

**Table 7** The dose responsive increase in the incidence of premature luteal regression in eCG treated ewes [316, 350]

Dose of eCG in IU	Incidence of premature regression / ewes superovulated (%)	Average
0	1/32 (3%), 17/98 (17 %),	10 %
400	2/15 (13%)	13 %
800	0/35 (0%), (30/104) (29%),	14.5 %
1000	6/12 (50%), 1/10 (10%)	30 %
1200	4/4 (100%)	100 %*
1600	12/34 (35 %), (38/97) (39%)	37 %

This study by Cole (1975)[316] used a very small amount of experimental animals & and also used 16 day progesterone priming instead of the usual 10 or 12 days.

**Table 8** The dose responsive increase in the incidence of premature luteal regression in FSH treated ewes [316, 350]

Dose FSH (mg)	Incidence of premature regression / ewes superovulated (%)	Average
0	13/101 (13%), 1/32 (3%)	8 %
12	32/99 (32%), 7/32 (22%)	27 %
18	40/99 (40%), 9/34 (26%)	33 %

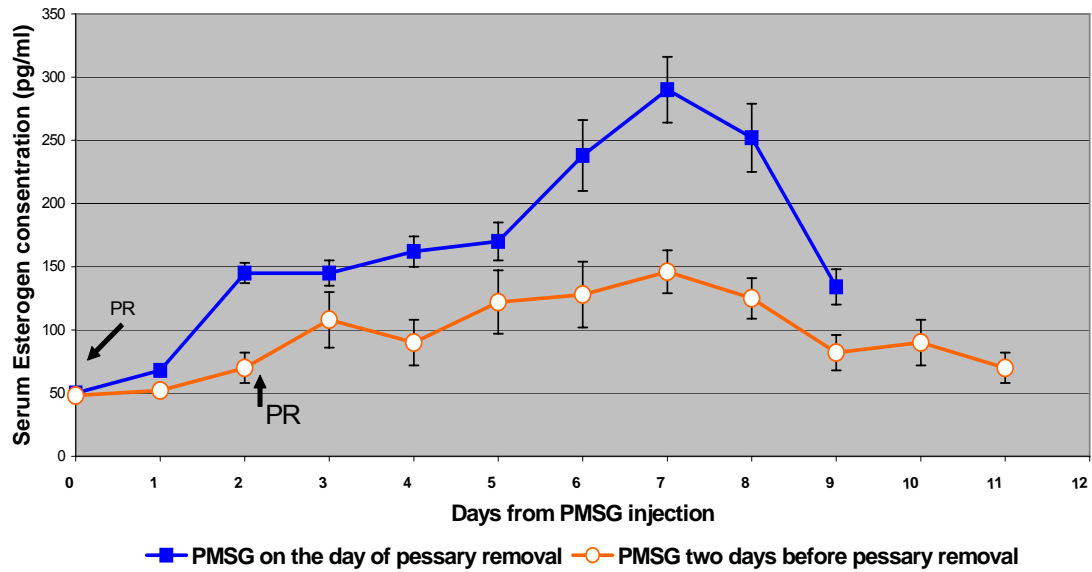


**Table 9** The dose responsive increase in the incidence of premature luteal regression in ewes treated with a combination of eCG and FSH[316, 350]

Dose of eCG (IU)	Dose of FSH-P (mg)		
	0	12	18
0	1/32 (3%)	7/32 (22%)	9/34 (26%)
800	0/35 (0%)	16/35 (46%)	14/34 (41%)
1600	12/34 (35%)	9/32 (28%)	17/31 (55%)

### 2.11.3 The influence of the day of administration of superovulatory hormones on the incidence of premature luteal regression

In a study on ferral goats (*Capra hircus*), Armstrong *et al* (1982) studied the effect of the timing of the administration of eCG with regard to synchronisation with P<sub>4</sub> pessaries [348]. In that study it was found that administration of eCG on the day of P<sub>4</sub> pessary removal, the incidence of premature luteal regression was reduced compared to the traditional introduction of eCG two days before P<sub>4</sub> pessary removal. Interestingly in this study, the serum E<sub>2</sub>-17β values of the does where eCG was administered on the day of P<sub>4</sub> pessary removal exceeded the values where eCG was administered two days before removal of the pessaries (Figure 21). These results were unexpected since E<sub>2</sub>-17β plays an important role in the initiation of luteal regression at the end of the normal cycle if no embryo is present in the uterus.



**Figure 21** Serum E<sub>2</sub> - 17 $\beta$  levels (mean  $\pm$  SD) in ferral does given 1000 IU eCG. Progestagen pessaries removed (PR) either at the same time or two days after treatment [348]

## 2.12 Gonadotropin releasing hormone administration

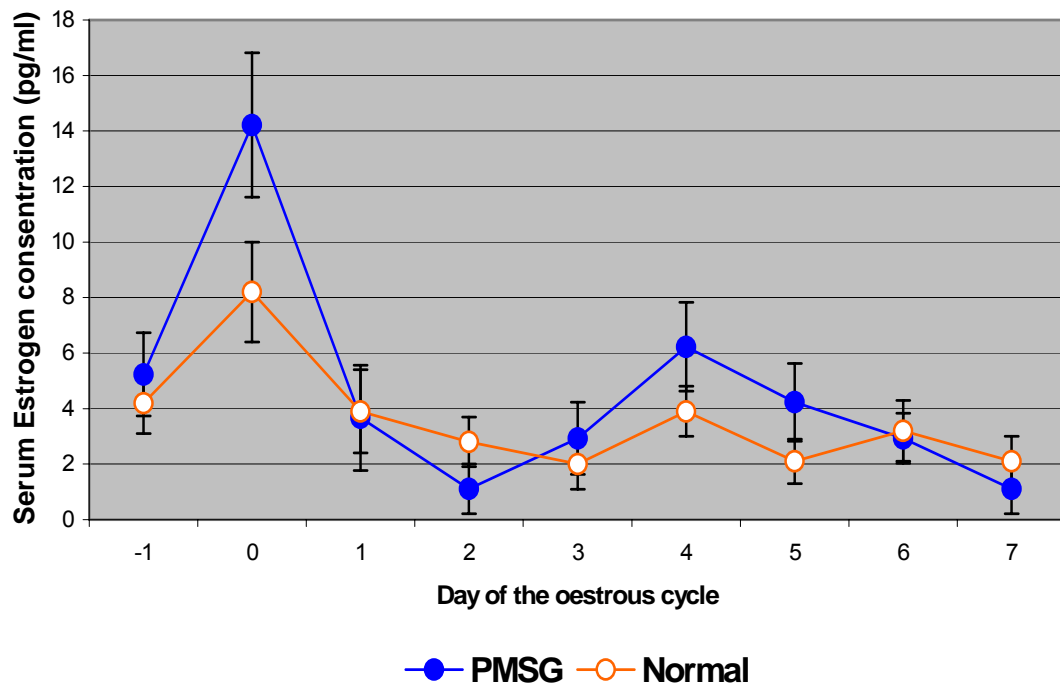
There is conflicting evidence in the literature on the influence of GnRH administration in addition to ECG with regard to the incidence of premature luteal regression. The rationale behind the use of GnRH or LH supplementation is that hyper-stimulated animals with more corpora lutea, might experience an LH shortage. This might lead to a sub-functional corpus luteum rather than a regressed corpus luteum. In the first four days after estrus there has to be adequate amounts of LH, because LH plays an important role in the completion of morphological development and establishment of intercellular channels formed by microvilli within the corpus luteum [353]. Some of the events that are mediated by LH includes the down regulation of C<sub>21</sub>- P450<sub>SCC</sub> in small luteal cells. In large luteal cells, LH induces an enhancement of cholesterol availability and increased transcription of 3 $\beta$  -HSD. This

poses the question: Does depletion of LH stores in superovulated animals lead to inadequate function?

In 1991 Jabour *et al* reported that GnRH administration 24 hours after sponge removal had no effect on the incidence of ewes with regressed corpora lutea in either autumn or spring [296] in either the breeding and non-breeding season in superovulated sheep. In contrast to the findings of Jabour *et al* (1991), Armstrong *et al* (1982) found that administration of a synthetic LH releasing hormone 38 hours after ECG administration, improved the maintenance of corpora lutea by 20 % in ferral goats [348]. The actual measurement of LH profiles in Suffolk ewes showed that there was no significant difference in LH secretion between ECG treated ewes and control animals [354]. Thus, it appears that although LH deficiency may play a role in ferral goats, it doesn't seem to be a factor in sheep.

### 2.13 Implication of estrogen

Particularly implicated in premature luteal regression, is  $E_2-17\beta$ , secreted by large follicles that are stimulated over a prolonged period after ECG administration and which often fail to ovulate and luteinise [355-358]. Reports of the  $E_2-17\beta$  profile of superovulated ewes vary dramatically. This can be attributed to the fact that the concentration of  $E_2-17\beta$  in the peripheral circulation is very low, only to be measured with extremely sensitive radioimmunoassays. Radioimmunoassays also differ with regard to specificity, and frequently this is not disclosed. These factors make it difficult to compare results between laboratories, but despite the technical problems, there seems to be consensus that  $E_2-17\beta$  levels are significantly greater in superovulated sheep ewes compared to normal cycling ewes (Figure 22).



**Figure 22** Serum  $E_2-17\beta$  profiles of ewes superovulated with 1000 IU ECG compared to normal cycling ewes not treated with ECG [48, 355].

There are also differences between species. The increased response in  $E_2-17\beta$  secretion to superovulation with ECG is more profound in ferral goats [251] (Figure 21). In this latter report  $E_2-17\beta$  levels did not follow the normal pattern seen in sheep, but stayed elevated for a prolonged period.

Administration of exogenous  $E_2-17\beta$  is known to induce luteal regression in sheep [359] and in some wild deer species [253] if administered during metestrus (day 3-5 of the estrous cycle). It was shown by Bainbridge *et al* (1996) [253] working with Red Deer (*Cervus elapus*) that the highest incidence of premature regression occurred if eCG was administered in combination with estradiol benzoate (EDB). It must be mentioned that the dose used by Bainbridge *et al* (1996) [253] produced  $E_2-17\beta$  peak levels ten times higher than those seen in eCG treated hinds.

The luteolytic effect of  $E_2-17\beta$  in sheep can be prevented by hysterectomy [359]. The role of  $E_2-17\beta$  in normal regression at the end of the cycle involving  $PGF_{2\alpha}$ , is known. A logical conclusion from the fact that  $E_2-17\beta$ -induced regression could be prevented by hysterectomy, is that  $E_2-17\beta$  induces premature luteal regression via up-regulation of rOx and the production and release of  $PGF_{2\alpha}$ .

#### **2.14 Implication of $PGF_{2\alpha}$**

Although there was always the suspicion that  $PGF_{2\alpha}$  may be involved in premature luteal regression, because of it being the signal responsible for luteal regression at the end of the natural cycle, there was no scientific evidence published until 1988. In 1988, Battye *et al* published a paper implicating the involvement of  $PGF_{2\alpha}$  in early regression in the superovulated ferral goat (*Capra hircus*)[360]. In their experiment Battye *et al* (1988) [360] used does of various ages treated with intravaginal  $P_4$  sponges for 16 days to synchronize estrus. Two days before sponge removal the goats were given 1200 IU eCG to induce superovulation. The day of sponge removal was designated as day 0. The experimental animals were divided into two groups. Group 1 received no other treatment and served as controls. Group 2 received 2.2 mg / kg body mass flunixin meglumine (Finadyne: Schering, Heriot Agvet, Melbourne) intra muscular (i.m) every 12 hours starting on day 3 after sponge removal and finishing on day 7. Flunixin meglumine is a inhibitor of prostaglandin synthesis. Like aspirin and indomethacin, it inhibits the enzyme prostaglandin-endoperoxide-synthase with a cyclooxygenase component that is responsible for the conversion of arachidonic acid to  $PGG_2$ . The same enzyme with a peroxidase component convert  $PGG_2$  to  $PGH_2$  which serves as substrate for

endoperoxide reductase to produce  $\text{PGF}_{2\alpha}$  (see section 2.4). The hormone  $\text{PGF}_{2\alpha}$  is never stored in endometrial cells and is released after production. The argument, therefore, was that if administration of flunixin meglumine prevents the occurrence of premature regression, it will prove that the phenomenon is caused by the premature release of  $\text{PGF}_{2\alpha}$  early in the cycle.

At first glance the results obtained in this study appear very convincing. All the control animals presented with premature luteal regression while all those treated with flunixin meglumine had functional corpora lutea on the 7<sup>th</sup> day after sponge removal. In their paper the authors reported that 70 % of the does had premature ovulations before sponge removal. The  $\text{P}_4$  concentration in animals with premature ovulations were significantly higher than the animals where no premature ovulations occurred (see Table 10).

**Table 10** Progesterone concentration (ng/ml) in superovulated ferral goats with and without premature ovulations on the first two days after sponge removal[360]

Day after sponge removal	Progesterone concentration (ng/ml) in does with premature ovulations	Progesterone concentration (ng/ml) in does without premature ovulations
0	0.30 ± 0.05	0.08 ± 0.04
1	1.08 ± 0.11	0.17 ± 0.05

It is known that  $\text{P}_4$  stimulates the mobilization of amphipathic phosphoacylglycerols leading to the formation of lipid droplets in the cytoplasm. The hydrolytic action of P-lase  $\text{A}_2$  will yield increased free arachidonic acid. Armstrong *et al* (1982) [251] reported increased estradiol concentrations over a prolonged period in ferral goats (see Figure 21).  $\text{E}_2\text{-}17\beta$  will activate r- $\text{E}_2\text{-}17\beta$  in the

endometrium, which will in turn lead to synthesis and up-regulation of rOx. The increased number of rOx will make the endometrium more sensitive for basal levels of Ox. In order for arachidonic acid to be turned into  $\text{PGF}_{2\alpha}$ , three enzymes are necessary namely a) prostaglandin endoperoxide synthase with cyclooxygenase component, b) prostaglandin endoperoxide synthase with peroxidase component and c) endoperoxide reductase. It is not clear how these enzymes are regulated, but it seems likely that the activated rOx via the second messenger c-AMP, plays a role in their up-regulation. Because of the similarity between prostaglandin endoperoxide synthase with the cyclooxygenase component and prostaglandin endoperoxide synthase with peroxidase component, they are possibly transcribed by the same gene. The two proteins resulting from the process known as alternative splicing, allows one piece of unprocessed mRNA to code for more than one protein. The result would be the rapid production and release of  $\text{PGF}_{2\alpha}$ . Note that this process requires two sets of protein synthesis, namely the production of the rOx, and the enzymes responsible for the conversion of arachidonic acid to  $\text{PGF}_{2\alpha}$ .

At first glance this hypothesis looks very convincing, but there are a few troublesome areas. In the study by Battye *et al* (1988) [360], the experimental groups were small. There were four animals in the control group (two had to be excluded because one animal didn't superovulate and the other animal had adhesions that prevented clear visualization of the ovaries at laparoscopy) and six animals in the group that received the flunixin meglumine. The incidence of premature corpus luteum regression in goats is in the order of 30 %, although it varies because of the influences of season, nutrition, and type and dose of

gonadotropin used. It would be more convincing if bigger experimental groups were used.

In their paper Battye *et al* (1988) [360] used the pulmonary metabolite of  $\text{PGF}_{2\alpha}$ , namely PGFM, as a indication of  $\text{PGF}_{2\alpha}$  synthesis and release, because unlike  $\text{PGF}_{2\alpha}$ , it can be easily detected in jugular venous blood. As mentioned earlier in this chapter, the usefulness of PGFM measurements has been questioned [127]. It continues to be a point of controversy in this field, [361] since it also reflects turnover of other prostaglandins, produced at other sites in the body. Battye *et al* (1988) [360] also stated that the RIA they used did not distinguish between PGFM and 13,14-dihydro-15-keto-PGF-1. But despite these objections, the study presented with a major paradox in that two out of six animals that received the flunixin meglumine showed significant peaks of PGFM without a reduction in progesterone synthesis. These animals also did not show macroscopic signs of regression when examined by laparoscopy on the 7<sup>th</sup> day after sponge removal.

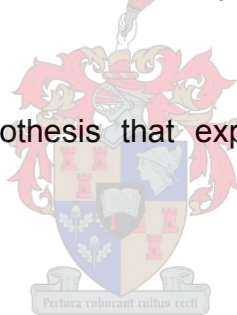
Another point of concern is the occurrence of both normal and regressed corpora lutea in the red deer reported by Bainbridge *et al* (1996)[253]. Induction of premature regression by administration of exogenous  $\text{PGF}_{2\alpha}$  always causes regression of all the corpora lutea present on the ovary. This can be compared to natural regression at the end of the cycle which always causes total regression of all the corpora lutea present on both ovaries . This raises the question: Is  $\text{PGF}_2$  really involved in the Red Deer and other animals?



There are definitive differences between species with regard to physiological mechanisms. There are adequate examples of this fact in nature, but conceivably the best example for this argument is the different  $E_2$ - $17\beta$  patterns observed between superovulated goats and sheep (see Figures 21 and 22). All the evidence for  $PGF_{2\alpha}$  involvement in premature regression is possibly limited to goat and deer species. Although the hypothesis raised in this section seems very appealing, one must refrain from making generalizations across species. More evidence is needed to unravel the mechanism of premature regression in the superovulated sheep ewe.

Therefore the aims of the current thesis were:

1. To try and unravel the mechanism behind premature luteal regression.
2. To formulate a new hypothesis that explain the mechanism behind the phenomena.



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## Chapter 3

# PGF<sub>2α</sub> Induced Regression and Premature Luteal Regression: A Comparative Study

### 3.1 Introduction

Luteal regression at the end of the natural cycle of the sheep ewe is well characterized. Knowledge on the mechanisms involved has grown tremendously from 1923 when Loeb made the observation that hysterectomy lengthens the lifespan of the corpus luteum [1]. Today it is known that the signal of uterine origin that is responsible for the initiation of luteal regression at the end of the natural cycle is PGF<sub>2α</sub> [2-38]. It is also known how PGF<sub>2α</sub> is synthesized [39], how secretion is controlled [40-71] and what pattern this hormone follows during the estrous cycle of the ewe [6, 72-90]. Maternal recognition of pregnancy is also well understood. The mechanism of how the presence of an embryo in the uterus inhibits PGF<sub>2α</sub> synthesis, and release, is clear [74-78, 91-98]. An antagonist of PGF<sub>2α</sub>, namely PGE<sub>2</sub>, has also been identified and it was shown that the embryo secretes this antagonist to prevent the actions of PGF<sub>2α</sub> [99-103]. The mechanism of PGF<sub>2α</sub> action on cellular and molecular level is still studied extensively and although a lot is known, the availability of molecular techniques will clarify the action of this hormone on luteal cells in the near future. Chapter 2 Section A gives a brief review of what is now known of natural regression in the sheep ewe.

In contrast to natural regression, literature on the phenomenon of premature luteal regression in superovulated sheep ewe is limited. Studies focussed primarily on the incidence of the phenomenon and factors influencing the incidence. Several

factors have been identified, and include seasonal influences [104, 105], nutrition [104, 105], superovulatory hormone(s) used [106], timing of administration [107], the dose of superovulatory hormones, etc. [106]. The rationale behind this approach is that the identification of a specific factor that influences the incidence could lead to the exact mechanism of the phenomenon. A difficulty experienced with this approach is that these factors are often dependent of other factors.

Studies by Ryan *et al* [104] and Jabbour *et al* [105] have shown that the incidence of premature regression is higher in autumn (breeding season) than in spring (non-breeding season). This finding was unexpected because during the non-breeding season (spring) the active suppression of the GnRH pulse generator by the  $E_2$ - $17\beta$  sensitive inhibitory neurons should be at its strongest. The question was raised whether the effect observed by these authors, was not merely a nutritional effect, because pastures will carry higher nutritional value in spring than in autumn. In their paper Jabbour *et al* [105] showed that they could reverse the effect of season by supplementation of the diet with lupin grains. This illustrated the fact that some of these influences observed are not independent. A problem with the use of lupin grains is that they alleviate  $E_2$ - $17\beta$  levels [108, 109] complicating the scenario more because  $E_2$ - $17\beta$  is also implicated in the phenomena in sheep [108, 110], goats [111], deer species [112] and cows [113]. The first aim of this study is to clarify the effect of season and nutrition.

Since it is known that  $PGF_{2\alpha}$  is the signal of uterine origin, responsible for natural luteal regression at the end of the estrous cycle of the ewe, it seems logical that this hormone could possibly be involved in premature regression. Although

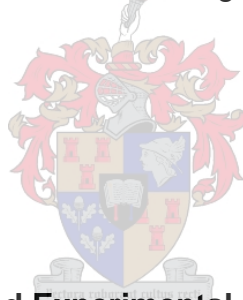
there is some convincing evidence that supports this hypothesis, there are unfortunately also some questions. To add to the confusion, the evidence for the involvement of  $\text{PGF}_{2\alpha}$  in premature regression is limited to studies in deer species [112] and goats [114]. Chapter 2 section 2.14 gives a brief summary of the evidence for and against this hypothesis.

Central to the  $\text{PGF}_{2\alpha}$  hypothesis is the role that  $\text{E}_2\text{-}17\beta$  plays. It is known that  $\text{E}_2\text{-}17\beta$  levels are much greater in superovulated goats if compared to unstimulated does [115]. Also the  $\text{E}_2\text{-}17\beta$  levels reported in superovulated ewes [110] differ from those reported in does [115]. Complicating the interpretation of  $\text{E}_2\text{-}17\beta$  data are technical difficulties in the measurement of  $\text{E}_2\text{-}17\beta$ . The concentration of  $\text{E}_2\text{-}17\beta$  in the peripheral circulation is very low ranging from 0 - 20 pg/ml in the superovulated sheep [110] and 0 - 350 pg/ml in goats [115]. Even in the  $\text{E}_2\text{-}17\beta$  pattern in untreated normally cycling ewes there is a degree of disagreement among scientists. It is generally agreed that the principal  $\text{E}_2\text{-}17\beta$  rise occurs during the two to three-day follicular phase. There is less agreement on whether or not significant increments in  $\text{E}_2\text{-}17\beta$  secretion occur at other times of the cycle. There have been several reports that  $\text{E}_2\text{-}17\beta$  secretion peaks for a second time around the 4<sup>th</sup> day of the cycle [7, 109, 116-119]. This increment, however, has not been observed in other studies [120, 121] and does not occur in all animals [116-118].

In 1996 Abbott diagnostics developed a Microparticle Enzyme Immunoassay (MEIA) for  $\text{E}_2\text{-}17\beta$  [122] that uses a solution of suspended, submicron sized latex particles to measure  $\text{E}_2\text{-}17\beta$ . The particles are coated with a capture molecule for  $\text{E}_2\text{-}17\beta$ .

17 $\beta$ . The effective surface area of microparticles increases assay kinetics and decreases assay incubation time. This results in a quicker E<sub>2</sub>-17 $\beta$  assay with higher accuracy and sensitivity. By using this technology, and by comparing PGF<sub>2 $\alpha$</sub>  induced regression, with premature luteal regression the present study finally aims to clarify the profile of E<sub>2</sub>-17 $\beta$  and critically evaluate the PGF<sub>2 $\alpha$</sub>  hypothesis.

Although the identification of factors that can influence the incidence of premature regression plays an important role in the identification of a possible mechanism for the phenomenon, it will always be limiting without functional physiological data like steroid profiles and tissue studies on microscopic and ultra structural level. This study aims to breach this gap while addressing the questions mentioned.



## **3.2 Materials & methods**

### **3.2.1 Experimental Animals and Experimental Design**

The study was conducted in spring (August – October 1996, non-breeding season) and autumn (March – May 1997, breeding season) at the Elsenburg experimental farm, near Stellenbosch in the Western Cape Province in South Africa. The farm is situated at 33°51 S, 18°50 O and is 177 metres above sea level. The average rainfall is 605 mm per annum and the average temperature 16,8 °C with wind speeds of up to 75 km per hour.



**Figure 23** Mutton Merino's grazing on Kikuyu at Elsenburg Agricultural Farm, near Stellenbosch in the Western Cape Province in South Africa

Mature Mutton Merino ewes ( $n = 50$ ) were used in the experiment. They were kept on Kikuyo (*Pennisetum clandestinum*) pastures. The ewes received oats hay (*Avena sativa L.*) and lucerne (*Medicago sativa L. sativa*) as additional feeding.

The ewes were divided in two groups ( $n = 25$ ). Group A was superovulated in autumn (March – May 1996, breeding season) and group B in spring (August – October 1997, non-breeding season). In group A, two animals had to be excluded because of retained intravaginal sponges. In group B, seven ewes were pregnant when the animals were laproscopically inseminated and had to be excluded. In



each group the ewes were subdivided in two further subgroups. Subgroup one (n = 20) was treated with a progestagen-impregnated intravaginal sponge (Repromap™ 60 mg, Upjohn,) for 12 days in combination with 1000 IU PMSG (Folligon) and 5 mg Estradiol Cypionate (ECP) (Pharmacia & Upjohn) as an intramuscular injection. The PMSG was administered at 08h00 48 hours before sponge removal and the ECP at 08h00 24 hours after sponge introduction. Subgroup two (n = 5) received a single intramuscular injection of 5 mg Lutalyze (Pharmacia & Upjohn) a  $\text{PGF}_{2\alpha}$  analogue (dinoprost promethamine) on the 6th day after sponge removal above the treatment of subgroup one. The rationale behind choosing (n=5) in subgroup two is that it was expected that 25% of the ewes would have premature regressed corpora lutea. Ideally sample size between  $\text{PGF}_{2\alpha}$  induced and premature regressed corpora lutea should be the same. The same protocol was used for Group A and Group B.

After the exclusion of the animals with retained intravaginal sponges and the ewes that were pregnant there were 23 ewes in Group A (n =18 in Subgroup one and n=5 in Subgroup two) and 18 ewes in Group B (n=16 in Subgroup one and n=2 in Subgroup two).

### **3.2.2 Macroscopic examination of ovaries**

The ovaries of all the ewes were removed 9 days after sponge removal when the ewes were slaughtered. The ovaries were transported to the laboratory and inspected within two hours of removal. Numbers of normal and regressed corpora lutea (CL) were recorded. Small and pale CL were recorded as regressed CL. The

total ovulatory response to the superovulatory treatment was given as the number of corpora lutea (normal and regressed) recorded. Old corpora albicans were not recorded.

### **3.2.3 Progesterone and estrogen analysis**

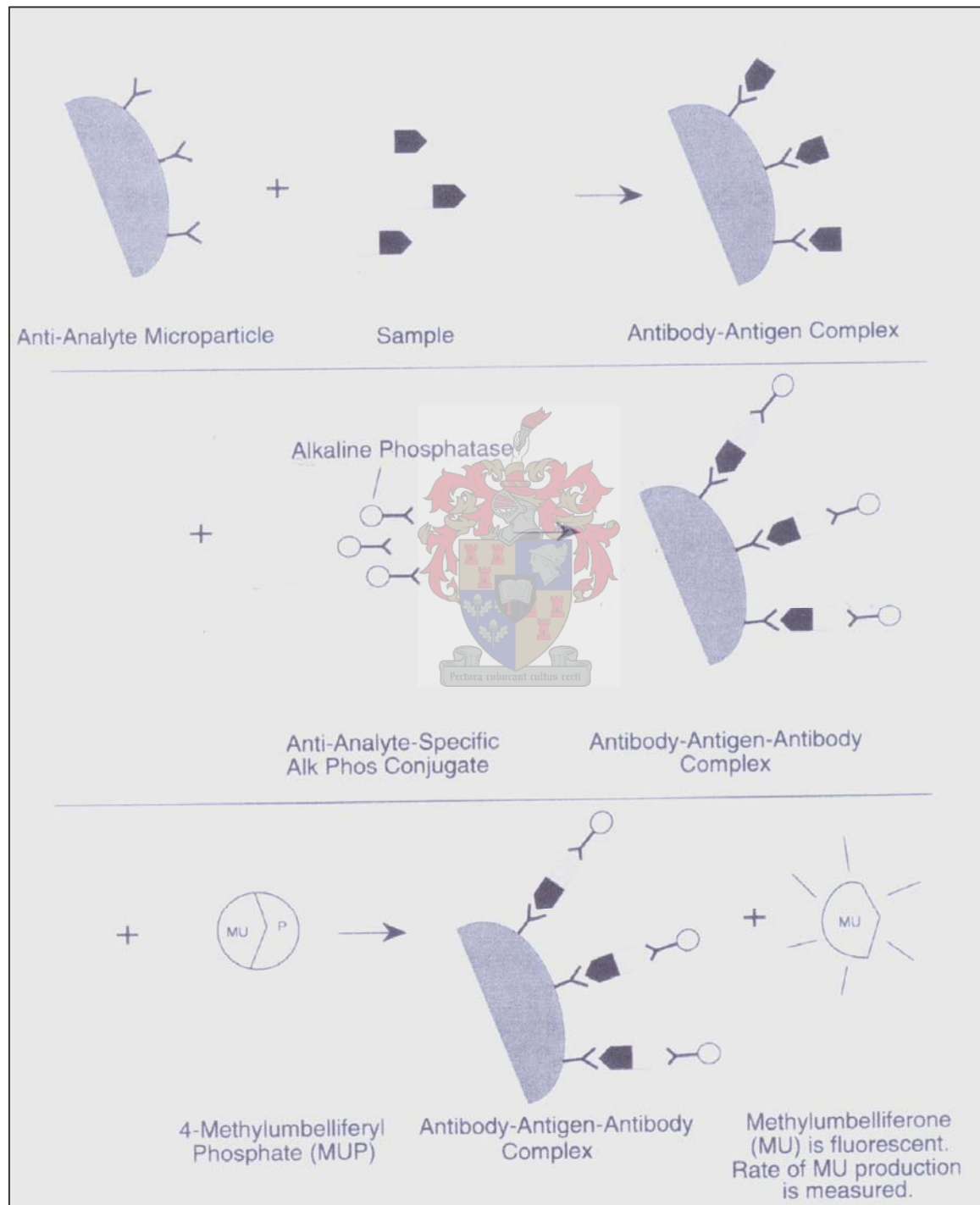
#### **3.2.3.1 Blood Sampling**

Blood samples were collected from the jugular vein by venipuncture with a 18-gauge needle in an SST (Serum Separating Tube) vacutainer (Becton Dickinson, MacMed South Africa). Samples were collected at 24 hour intervals beginning at the time of sponge removal. Serum was removed after centrifugation at 300 x g and stored at – 10 °C for later analysis for progesterone and E<sub>2</sub>-17β by Microparticle Enzyme Immunoassay (MEIA).

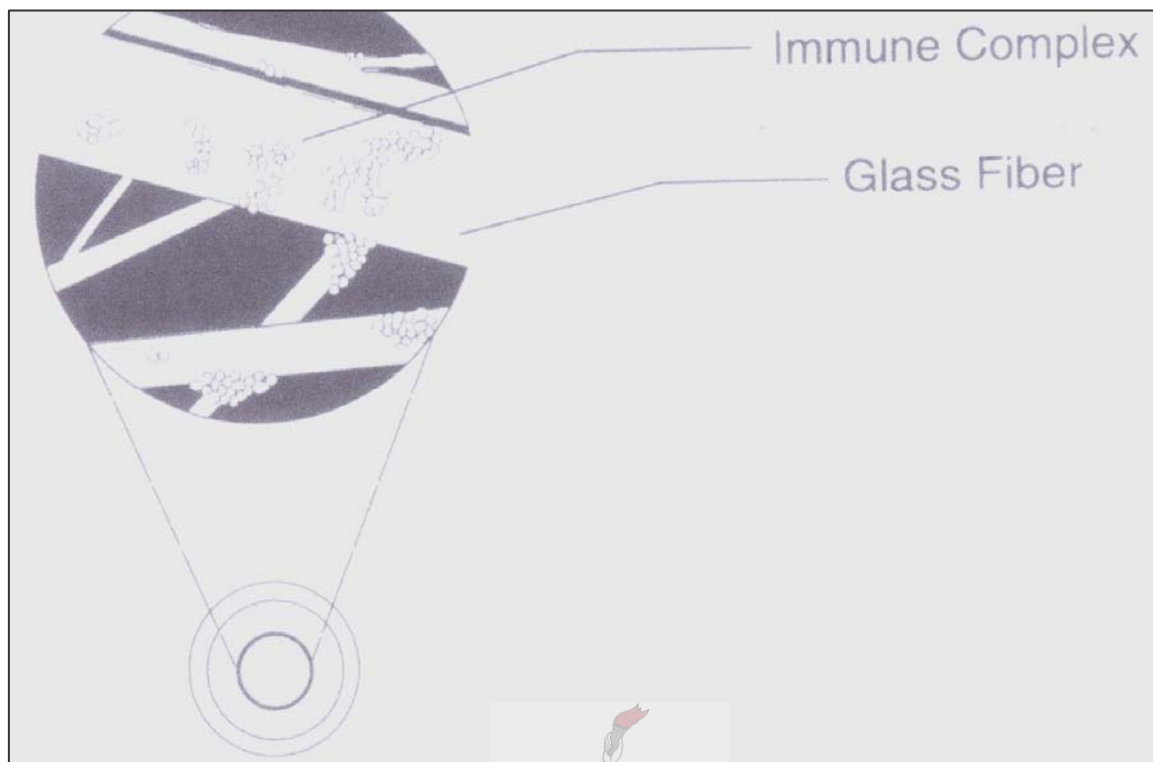
#### **3.2.3.2 Microparticle Enzyme Immunoassay**

The Microparticle Enzyme Immunoassay (MEIA) described by Buster and Abraham [122] with slight modifications to compensate for the reference range of the hormones of interest in sheep. In short the MEIA technology uses a solution of suspended, submicron sized latex particles to measure analytes. The particles are coated with a capture antibody specific for the analyte being measured. The effective surface area of microparticles increases assay kinetics and decreases assay incubation time. This permits MEIA assays to be completed in less time than other immunoassays. The concentrations of all reagents have been optimized by Abbot by titrating them out for use in diagnostic ranges [122].

The analysis was done on the Abbot AxSYM™. The reaction sequences are different for the E<sub>2</sub>-17β and P<sub>4</sub> analysis and would be dealt with separately.



**Figure 24** Typical MEIA schematic reaction sequence [122]



**Figure 25** Immune complex bound to glass fibre matrix [122]

The  $P_4$  assay uses a one step MEIA.  $P_4$  reagents and sample are pipetted in the following sequence. In the sampling centre serum, anti- $P_4$ : alkaline phosphatase conjugate and the progesterone buffer are combined in a well of the reaction vessel (RV). The  $P_4$  microparticle reagent is added to a second well of the RV. The RV is immediately transferred into the processing centre. Further pipetting is done in the processing centre by the processing probe.

In the processing centre the reaction mixture is incubated.  $P_4$  in the sample binds to the anti- $P_4$ : alkaline phosphatase conjugate. An aliquot of the reaction mixture is transferred to the well containing the  $P_4$  microparticle reagent. The  $P_4$  microparticle reagent binds to anti- $P_4$ : alkaline phosphatase conjugate not bound to  $P_4$  from sample, forming the final reaction mixture. An aliquot of the final reaction

mixture is then transferred to the matrix cell. The microparticles bind irreversibly to the glass fibre matrix. The matrix cell is washed to remove unbound materials. The substrate, 4-Methylumbelliferyl Phosphate (MUP), is added to the matrix cell and the rate of conversion to the fluorescent product Methylumbelliferone (MU) is measured by the optical system. In samples with low progesterone concentrations lower anti-P<sub>4</sub>: alkaline phosphatase conjugate will bind to the P<sub>4</sub> microparticles. This will result in a longer time to complete the reaction from MUP to MU.

The E<sub>2</sub>-17β assay uses a two step MEIA. The AxSYM E<sub>2</sub>-17β reagents and sample are pipetted in the following sequence:

In the sampling centre the serum and all the AxSYM E<sub>2</sub>-17β reagents for one test are pipetted by the sampling probe into various wells of a RV. Serum, anti- E<sub>2</sub>-17β coated microparticles, E<sub>2</sub>-17β assay buffer and line diluents are combined in a well of the RV. This is the reaction mixture. E<sub>2</sub>-17β: alkaline phosphatase conjugate is added to a second well of the RV. The RV is immediately transferred into the processing centre. Further pipetting is done in the processing centre by the processing probe.

In the processing centre the reaction mixture is incubated. E<sub>2</sub>-17β in the serum binds to the anti- E<sub>2</sub>-17β on the microparticles forming an antibody-antigen complex. After incubation, an aliquot of the reaction mixture is transferred to the matrix cell. The microparticles bind irreversibly to the glass fibre matrix. The matrix cell is washed to remove unbound materials. E<sub>2</sub>-17β: alkaline phosphatase conjugate is then dispensed onto the matrix cell and incubated. The steroid portion

of the conjugate binds to the available sites on the anti- E<sub>2</sub>-17β coated microparticles. The matrix is washed to remove unbound materials. The substrate, MUP, is added to the matrix cell and the rate of conversion to the fluorescent product MU is measured by the optical system. In samples with low estrogen concentrations lower anti- E<sub>2</sub>-17β: alkaline phosphatase conjugate will bind to the E<sub>2</sub>-17β microparticles. This will result in a longer time to complete the reaction from MUP to MU.

### 3.2.3.3 Calibration

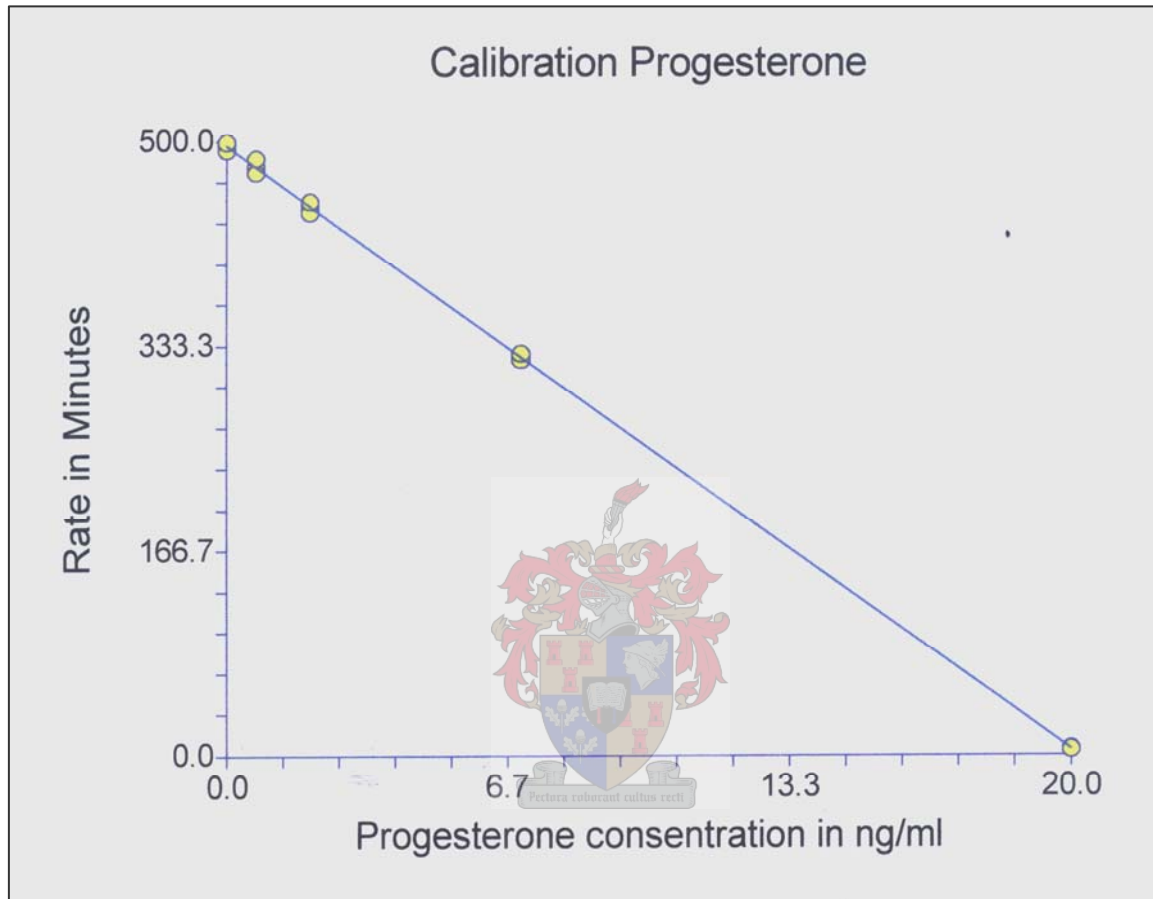
#### a) Progesterone

A standard calibration for P<sub>4</sub> on the AxSYM™ was performed. Calibrator A contains stripped human serum non-reactive for antibodies to HIV1/HIV2 (Human Immune Deficiency Virus 1 and 2), HCV (Human Cytomegalo Virus) and non-reactive for HbsAg (Hepatitis B Surface Antigen) by FDA (Food and Drug Administration) licensed tests. Callibrators B through E contain P<sub>4</sub> in TRIS buffer with chemical stabilizers and preserved in sodium azide to yield the following concentrations.

**Table 11** Progesterone calibrator concentrations

Calibrator	Progesterone	
	ng/ml	nmol/l
A	0	0
B	0.7	2.23
C	2	6.36
D	7	22.26
E	20	63.60

The reaction rate for each calibrator was determined in triplicate by a fluorescent detector. The reaction rate was plotted on the y-axis and the concentration on the x-axis. Linear regression was performed fitting a straight line using least squares.



**Figure 26** Calibration curve for progesterone analysis

Line Fitted (Regression equation) :  $y = (-24.57 X) + 496.38$

Intercept with y-axis : 496.38 seconds

Slope : -24.57

Coefficient of determination ( $R^2$ ) : 0.99

Coefficient of determination adjusted for small : 0.99

sample size ( $R^2_{\text{adjusted}}$ )

**Table 12** Calibration data for progesterone analysis

Calibrator	1	2	3	Ave	SD	Fitted Rate	Error in Fitted Rate		Span
A	496.33	492.41	499.18	495.97	3.40	496.43	-0.46	A-B	16.15
B	479.10	474.18	486.18	479.82	6.03	479.82	0.63	B-C	32.92
C	447.18	442.31	451.21	446.90	4.46	447.28	-0.38	C-D	122.21
D	324.29	322.61	327.16	324.69	2.30	324.40	0.29	D-E	319.85
E	4.79	5.11	4.63	4.84	0.24	4.92	-0.09	A-E	491.13

Before a calibration could be accepted it had to comply to the following rules as well.

- a) The Standard deviation (SD) for the reaction rate for all five calibrators had to be less than 10 seconds.
- b) The error in the fitted rate had to be in the range 1.00 to -1.00.
- c) The span between calibrators had to be in the 95% confidence interval.

The span ranges is as follows.

A-B      6.72 – 25.58      seconds

B-C      22.43 – 43.41      seconds

C-D      115.45 – 128.97      seconds

D-E      317.31 – 322.39      seconds

A-E      487.49 – 494.77      seconds

- d)  $R^2_{\text{adjusted}}$  had to be  $\geq 0.95$ .



## b) Estrogen

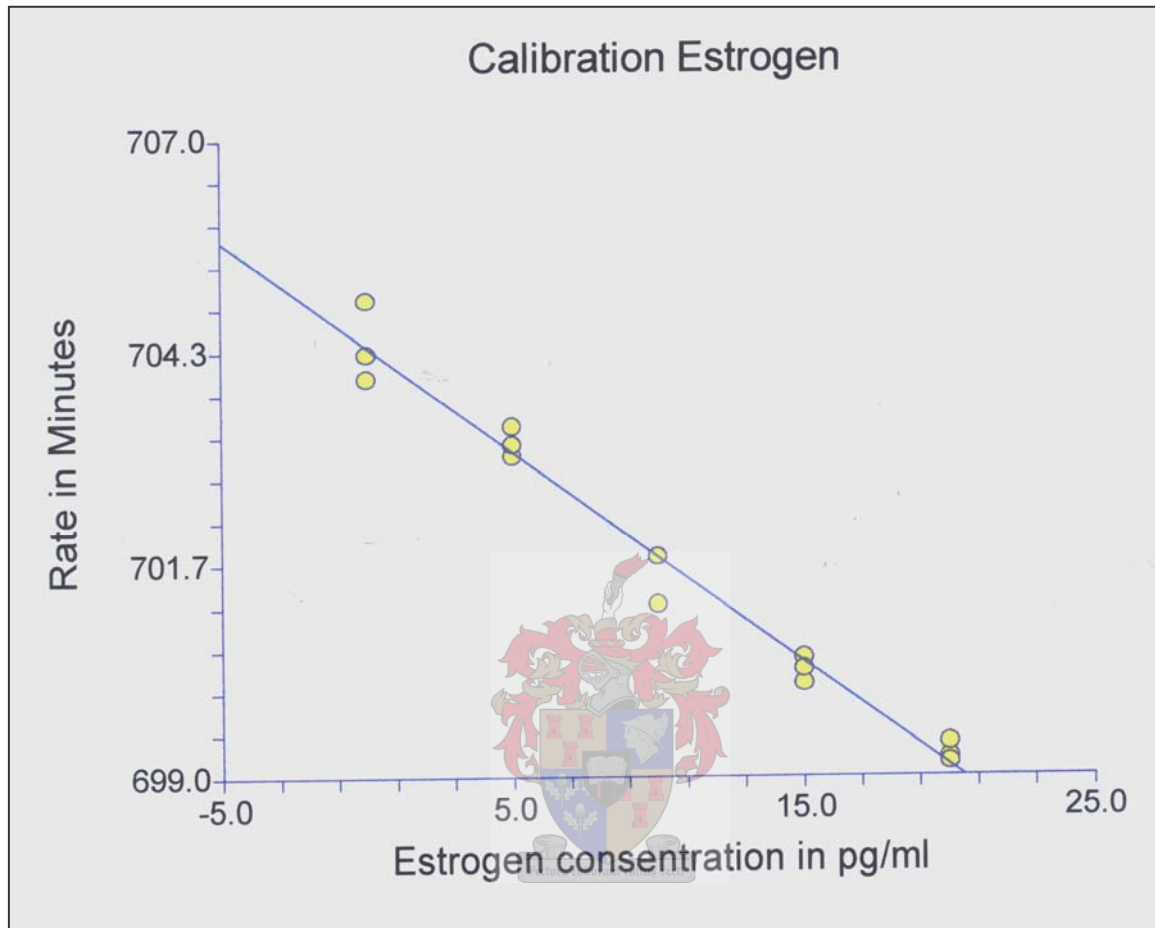
The calibration curve for E<sub>2</sub>-17β was adjusted from the standard calibration for estrogen on the AxSYM™ system. This was done because the range of measurements for humans (38 pg/ml - 300 pg/ml for normal menstruating females, and up to 1500 pg/ml for females on fertility treatment) differs from that of the sheep in this experiment (2.7 pg/ml - 16 pg/ml). The adjustment was made to make the analysis more sensitive to the range of measurements to show significant differences from day to day in estrous cycle of the sheep.

In short, five calibrators were used that contained E<sub>2</sub>-17β, TRIS buffer, a protein stabilizer (bovine) and sodium azide as preservative. The concentration of the calibrators was standardized with gas chromatography/mass spectrometry (GC/MS). The concentration of the calibrators was as follows:

**Table 13** Estrogen calibrator concentrations

Calibrator	Estradiol Concentration	
	pg/ml	pmol/l
A	0	0
B	5	18.35
C	10	36.70
D	15	55.05
E	20	73.40

The reaction rate for each calibrator was determined in triplicate by a fluorescent detector. The reaction rate was plotted on the y-axis and the concentration on the x-axis. Linear regression was performed fitting a straight line using least squares.



**Figure 27** Calibration curve for estrogen analysis

Line Fitted (Regression equation)	:	$y = (-3.753 \times 10^{-4}X) + 704.404$
Intercept with y-axis	:	704.404 seconds
Slope	:	$-3.753 \times 10^{-4}$
$R^2$	:	0.989
$R^2_{\text{adjusted}}$	:	0.982

**Table 14** Calibration data for estrogen analysis

Calibrator	1	2	3	Ave	SD	Fitted Rate	Error in Fitted Rate		Span
A	704.32	704.99	704.01	704.44	0.50	704.10	0.34	A-	1.23
B	703.04	703.41	703.18	703.21	0.19	703.09	0.12	B-	1.65
C	701.78	701.71	701.18	701.56	0.33	701.77	-0.21	C-	1.21
D	700.51	700.18	700.36	700.35	0.17	700.45	-0.10	D-	1.07
E	699.24	699.18	699.43	699.28	0.13	699.13	0.15	A- E	5.16

Before a calibration could be accepted it had to comply to the following rules as well.

- a) The SD for the reaction rate for all five calibrators had to be less than 0.65 seconds.
- b) The error in the fitted rate had to be in the range 0.50 to -0.50.
- c) The span between calibrators had to be in the 95% confidence interval. The span ranges is as follows.

A-B      0.54 – 1.92      seconds

B-C      1.13 – 2.17      seconds

C-D      0.71 – 1.71      seconds

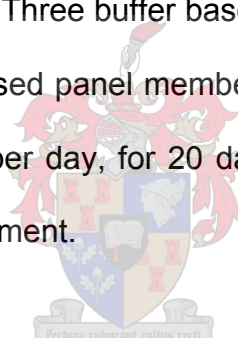
D-E	0.77 – 1.37	seconds
A-E	4.53 – 5.79	seconds

d)  $R^2_{\text{adjusted}}$  had to be  $\geq 0.95$ .

### 3.2.3.4 Specific performance characteristics

#### 3.2.3.4.1 Precision

Precision was determined as described in the National Committee for Clinical Laboratory Standards (NCCLS) protocol EP5-T2 (including an estimate of between run and between day precision). Three buffer based panel members (2,3 and 4) and one processed human serum-based panel members were assayed (1), in replicates of three, at two separate times per day, for 20 days, using a single lot of reagents and a single calibration per instrument.



#### a) Progesterone

**Table 15** Progesterone precision determined by panel member 1

Instrument	N	Mean Conc Value	Inter-assay		Intra-assay	
			SD	%CV	SD	%CV
1	80	0.60	0.19	9.66	0.06	3.91
2	80	0.54	0.13	7.06	0.06	2.86
3	80	0.56	0.13	6.96	0.03	1.14
4	80	0.54	0.16	9.58	0.06	2.85

**Table 16** Progesterone precision determined by panel member 2

Instrument	N	Mean Conc Value	Inter-assay		Intra-assay	
			SD	%CV	SD	%CV
1	80	1.05	0.16	5.10	0.06	1.72
2	80	0.99	0.16	5.42	0.09	1.94
3	80	1.00	0.19	5.63	0.05	1.83
4	80	0.97	0.13	4.22	0.16	5.01

**Table 17** Progesterone precision determined by panel member 3

Instrument	N	Mean Conc Value	Inter-assay		Intra-assay	
			SD	%CV	SD	%CV
1	80	5.40	0.57	3.32	0.06	1.62
2	80	5.58	0.51	2.84	0.38	2.23
3	80	5.55	0.54	3.03	0.25	1.44
4	80	5.66	0.54	2.92	0.19	0.91

**Table 18** Progesterone precision determined by panel member 4

Instrument	N	Mean Conc Value	Inter-assay		Intra-assay	
			SD	%CV	SD	%CV
1	80	21.67	3.21	4.62	0.98	1.41
2	80	21.08	2.77	4.11	1.14	1.70
3	80	21.33	2.73	4.00	1.84	2.70
4	80	21.90	2.54	3.61	1.81	2.53

Based on the available data the inter- and intra-assay coefficients of variation (CV) were 4.82% and 2.22% respectively.

**b) Estrogen**

**Table 19** Estrogen precision determined by panel member 1

Instrument	N	Mean Conc Value	Inter-assay		Intra-assay	
			SD	%CV	SD	%CV
1	80	5.41	5.42	10.92	3.11	6.43
2	80	4.49	4.42	8.54	1.44	2.66
3	80	5.22	5.24	10.67	2.00	3.11
4	80	4.82	4.89	9.55	2.77	5.21

**Table 20** Estrogen precision determined by panel member 2

Instrument	N	Mean Conc Value	Inter-assay		Intra-assay	
			SD	%CV	SD	%CV
1	80	24.11	6.32	7.11	3.12	3.22
2	80	25.34	5.32	5.86	3.00	3.39
3	80	23.11	5.91	6.98	2.55	2.90
4	80	24.11	5.33	5.81	3.99	4.31

**Table 21** Estrogen precision determined by panel member member 3

Instrument	N	Mean Conc Value	Inter-assay		Intra-assay	
			SD	%CV	SD	%CV
1	80	87.11	13.91	4.30	8.56	2.66
2	80	85.23	8.63	2.77	7.52	2.44
3	80	83.22	12.87	4.25	5.41	1.89
4	80	87.22	10.91	3.48	6.22	4.11

**Table 22** Estrogen precision determined by panel member 4

Instrument	N	Mean Conc Value	Inter-assay		Intra-assay	
			SD	%CV	SD	%CV
1	80	204.13	13.21	6.62	10.93	6.51
2	80	208.43	12.77	7.21	11.16	7.60
3	80	204.59	8.73	8.40	9.84	8.72
4	80	206.88	7.54	7.67	8.81	7.53

Based on the available data the inter- and intra-assay CV were 6.88% and 4.54% respectively.

### 3.2.3.4.2 Recovery

#### a) Progesterone

Known concentrations of P<sub>4</sub> were added to 3 normal human serum samples. The concentration of P<sub>4</sub> was determined using the AxSYM P<sub>4</sub> assay and the resulting percent recovery was calculated.

**Equation 1** Progesterone recovery

$$\% \text{ Recovery} = \frac{\text{Progesterone Value Observed} - \text{Endogenous Level}}{\text{Progesterone added}} \times 100$$

**Table 23** Recovery of exogenous progesterone added to biological specimens

Sample	Endogenous level ng/ml	Progesterone added ng/ml	Progesterone observed ng/ml	% Recovery
1	7.60	4.84	12.47	100.6
2	4.38	9.72	13.97	98.7
3	4.38	9.72	14.21	100.1

Based on the available data the average recovery was 99.8%

**b) Estrogen**



Known concentrations of E<sub>2</sub>-17β were added to 3 normal human serum samples. The concentration of E<sub>2</sub>-17β was determined using the AxSYM E<sub>2</sub>-17β assay and the resulting percent recovery was calculated.

**Equation 2** Estrogen recovery

$$\% \text{ Recovery} = \frac{\text{Estrogen Value Observed} - \text{Endogenous Level}}{\text{Estrogen added}} \times 100$$



**Table 24** Recovery of exogenous estrogen added to biological specimens.

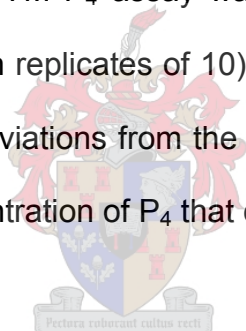
Sample	Endogenous level ng/ml	Progesterone added ng/ml	Progesterone observed ng/ml	% Recovery
1	15.99	5.00	21.12	100.6
2	5.99	15.00	20.71	98.7
3	21.00	20.00	42.11	100.5

Based on the available data the average recovery was 99.9%

### 3.2.3.4.3 Sensitivity

#### a) Progesterone

The sensitivity of the AxSYM P<sub>4</sub> assay was calculated to be better than or equal to 0.20 ng/ml (n=36 runs in replicates of 10). The sensitivity is defined as the concentration at two standard deviations from the AxSYM P<sub>4</sub> calibrator A (0 n/mol/l) and represents the lowest concentration of P<sub>4</sub> that could be distinguished from zero.



#### b) Estrogen

Based on the modified calibration, the sensitivity of the AxSYM E<sub>2</sub>-17 $\beta$  assay was calculated to be better than or equal to 1.5 pg/ml (n=40 runs in replicates of 10). The sensitivity is defined as the concentration at two standard deviations from the AxSYM E<sub>2</sub>-17 $\beta$  calibrator A (0 n/mol/l) and represents the lowest concentration of E<sub>2</sub>-17 $\beta$  that could be distinguished from zero.

### 3.2.3.4.4 Specificity

#### a) Progesterone

The specificity of the AxSYM P<sub>4</sub> assay was determined by studying cross-reactivity of the following compounds. The AxSYM P<sub>4</sub> calibrator A was spiked with 1000 ng/ml of the test compound and assayed for P<sub>4</sub>. The percent cross-reactivity of the compounds is listed for the AxSYM P<sub>4</sub> assay.

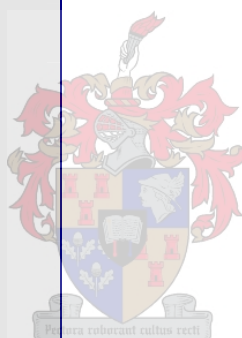
**Table 25** Cross-reactivity of progesterone related compounds in the Abbott AxSYM progesterone MEIA assay

Compound	% Cross-reactivity
Corticosterone	2.1
11-Deoxycorticosterone	1.3
17-Hydroxyprogesterone acetate	1.4
20 $\alpha$ -Dihydroxyprogesterone	0.4
20 $\beta$ -Dihydroxyprogesterone	2.2
17-Hydroxyprogesterone	0.8
5 $\alpha$ -Pregnan-3,20-dione	6.3
5 $\alpha$ -Pregnan-3 $\alpha$ -ol-20-one	1.7
5-Pregnan-3-ol-20-one	3.2
Pregnenolone	0.2
Pregnenolone-3-s	0.5
Pregnanolone	1.5

**b) Estrogen**

**Table 26** Cross-reactivity of estrogen related compounds in the modified Abbott AsXYM estrogen MEIA assay

<b>Compound</b>	<b>% Cross-reactivity</b>
Estrone	0.1
Estriol	0.1
Ethinyl Estradiol	0.1
17 $\alpha$ -Estradiol	0.1
Estradiol-3-glucuronide	0.0
Estradiol-17-glucuronide	0.0
Estradiol-3-sulfate	0.0
Estradiol-16-glucuronide	0.0
Estradiol-17-propionate	0.3
Estradiol-17-valerate	0.1
Ethisterone	0.0
Mestranol	0.0
Norethindrone	0.0
Norethynodrel	0.0
Norgestrel	0.0
Danazol	0.0
Equilenin	0.1
Equilin	0.0
DHEAS	0.0
Androstene-diol	0.0
19-Nortestosterone	0.0
DHT	0.0
Testosterone	0.0
Corticosterone	0.0
11-Deoxycorticosterone	0.0
11-Deoxycortisol	0.0



The specificity of the modified AxSYM E<sub>2</sub>-17β assay was determined by studying cross-reactivity of the above mentioned compounds. The AxSYM E<sub>2</sub>-17β calibrator A was spiked with 1000 ng/ml of the test compound and assayed for E<sub>2</sub>-17β. The percent cross-reactivity of the compounds is listed for the AxSYM E<sub>2</sub>-17β assay.

### 3.2.3.4.5 Interference

#### a) Progesterone

Interference from bilirubin, hemoglobin and triglycerides was studied in the AxSYM P<sub>4</sub> assay. A human serum specimen was spiked with the interfering substance and assayed for P<sub>4</sub>. The AxSYM P<sub>4</sub> assay demonstrated the interference stated in the following table.

**Table 27** Interference from bilirubin, hemoglobin and triglycerides in the Abbott AxSYM progesterone MEIA assay

Sample Progesterone Concentration ng/ml	Interfering Substance	Interfering Substance Concentration	% Interference
6.53	Billirubin	15 mg/dl	<5
6.25	Hemoglobin	1 g/dl	<5
34.5	Triglycerides	2000 mg/dl	<10

#### b) Estrogen

Potential interference from bilirubin, hemoglobin and triglycerides was studied in the modified AxSYM E<sub>2</sub>-17β assay. The modified AxSYM E<sub>2</sub>-17β assay demonstrated the interference stated in the following table.

**Table 28** Interference from bilirubin, hemoglobin and triglycerides in the modified AxSYM estrogen MEIA assay

Sample Estrogen Concentration pg/ml	Interfering Substance	Interfering Substance Concentration	% Interference
10.56	Billirubin	15 mg/dl	<10
5.11	Hemoglobin	1 g/dl	<10
14.77	Triglycerides	2000 mg/dl	<10

### 3.2.3.5 Quality Control

The multi-rule procedure developed by Westgard and associates [123] was utilized to verify the analytical correctness of the analytical procedure for both P<sub>4</sub> and E<sub>2</sub>-17β.



### 3.2.4 Histology

#### 3.2.4.1 Preparation and staining

The ewes were slaughtered 9 days after sponge removal and their ovaries removed. The ovaries were macroscopically inspected 2 hours after removal. Mased on the macroscopic appearance and treatment, five groups of corpora lutea were identified. Taking into account macroscopic appearance and treatment five groups of corpora lutea were identified.

- Group 1      Corpora lutea from ovaries where only normal appearing corpora lutea were present.
- Group 2      Corpora lutea from ovaries where only prematurely regressed corpora lutea were present.
- Group 3      Normal appearing corpora lutea from ovaries where regressed corpora lutea occurred with normal appearing corpora lutea (Mixed normal)
- Group 4      Regressed corpora lutea from ovaries where regressed corpora lutea occurred with normal appearing corpora lutea (Mixed regressed)
- Group 5      Corpora lutea from ovaries where regression was induced with the PGF<sub>2α</sub> analogue Dinoprost tromethamine (Lutalyse ®, Pharmacia & Upjohn).

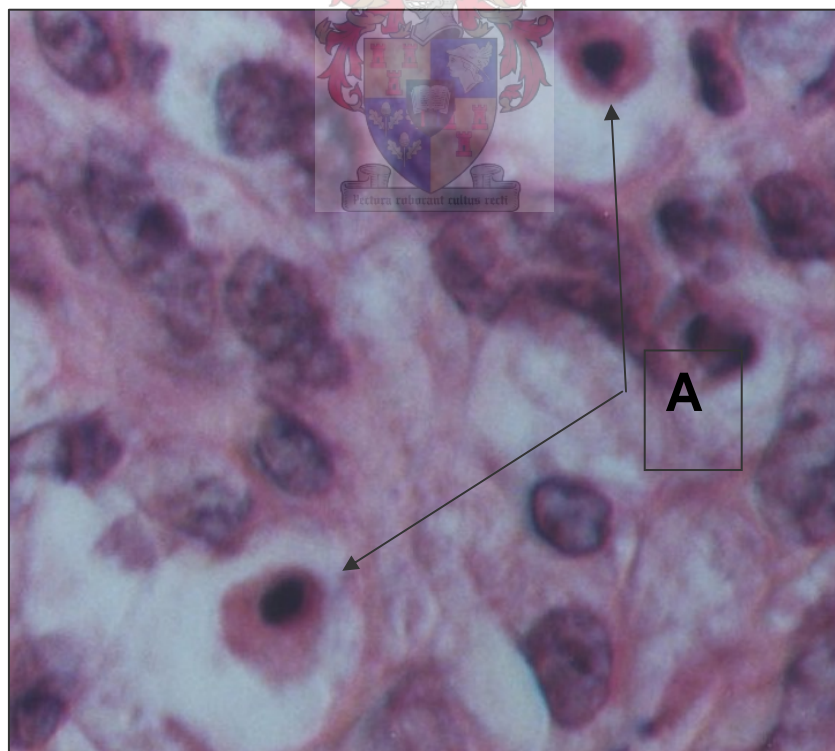


Five corpora lutea from each of these five groups were randomly selected using random numbers generated. These corpora lutea were removed from the ovary with a scalpel blade and fixed in formal-saline for 24 hours. The (formal-saline) fixed corpora lutea were then processed with a Tissue Tek II automatic tissue processing system. The tissue was dehydrated in a graded alcohol series for 12 hours 30 minutes, cleared in xylol for 3 hours 30 minutes and impregnated in paraffin wax (Histosec paraffin wax with a melting point of 56 – 58 °C) for 4 hours. The paraffin impregnated tissue was then mounted in wax blocks and air bubbles were removed with a vacuum pump. (Townson & Mercer) Tissue were sectioned with an

Optolab 820 microtome (Optolabor, Braamfontein, Johannesburg, South Africa) at 5  $\mu\text{m}$  and stained with haematoxylin and eosin for evaluation of apoptotic nuclei and with Masson's trichrome stain for connective tissue replacement.

#### 3.2.4.2 Evaluation of apoptotic nuclei

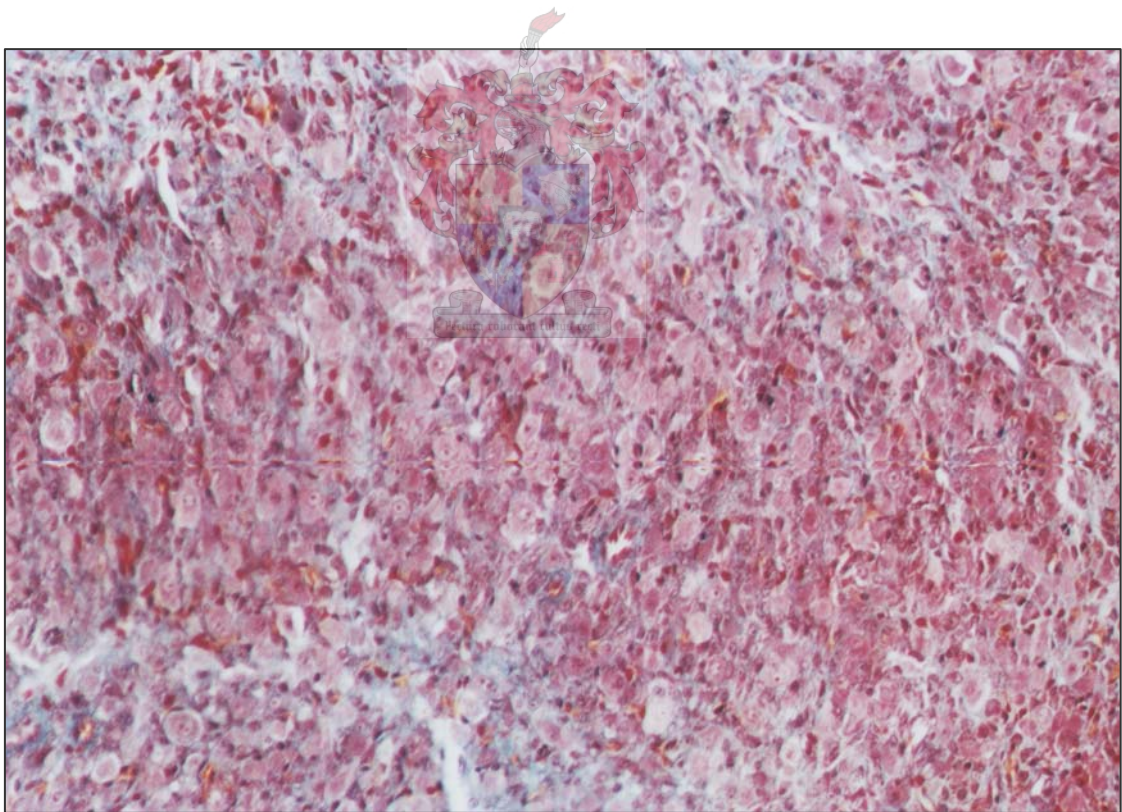
Five haematoxylin and eosin stained sections were examined by three technologists in each of the five defined groups. The technologists did not know to what group a particular slide belonged when they examined it. The number of apoptotic nuclei per field at 400 times enlargement on a light microscope (Olympus EC-2) was recorded. Ten fields per section were scored by each technologist and the average per section was calculated. These averages were then used to calculate means and SEM's for the five sections in each group.



**Figure 28** Apoptotic nuclei (A) are indicated by the arrows (H & E at 400 times enlargement)

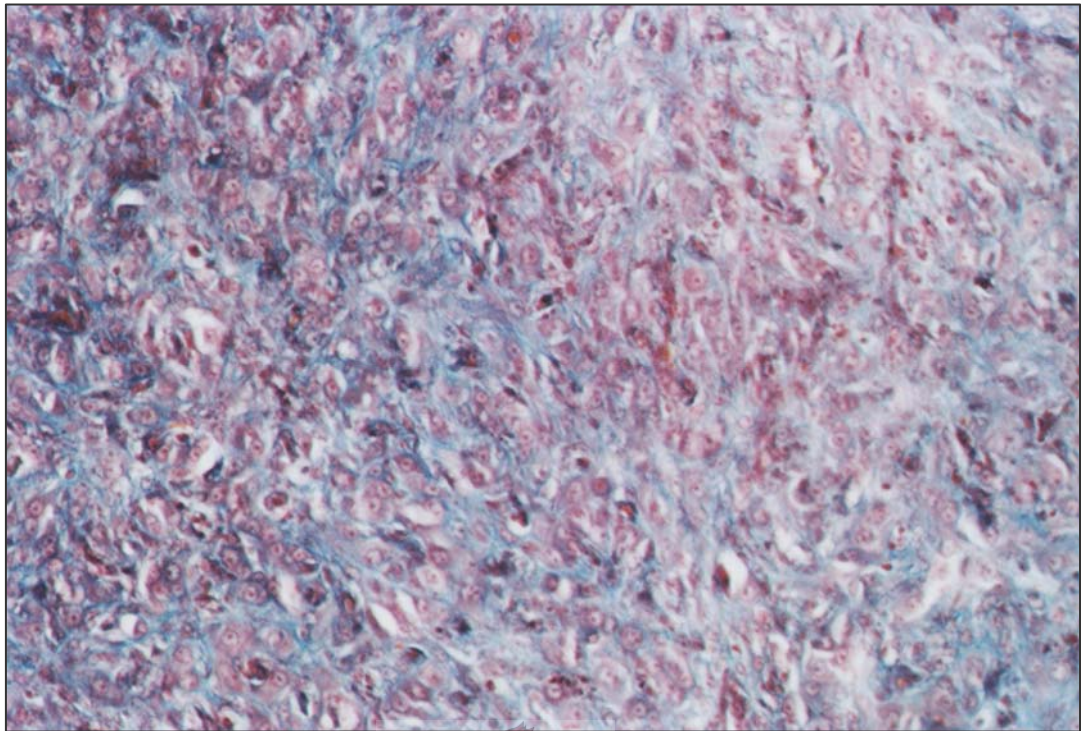
### 3.2.4.3 Evaluation for connective tissue replacement

Five sections stained with Masson's trichrome stain were examined by three technologists in each of the defined groups. The technologists did not know to what group a particular slide belonged when they examined it. Three categories of connective tissue infiltration were defined and a numerical value assigned to every category. Connective tissue stains green with Masson's trichrome stain. The defined categories are:

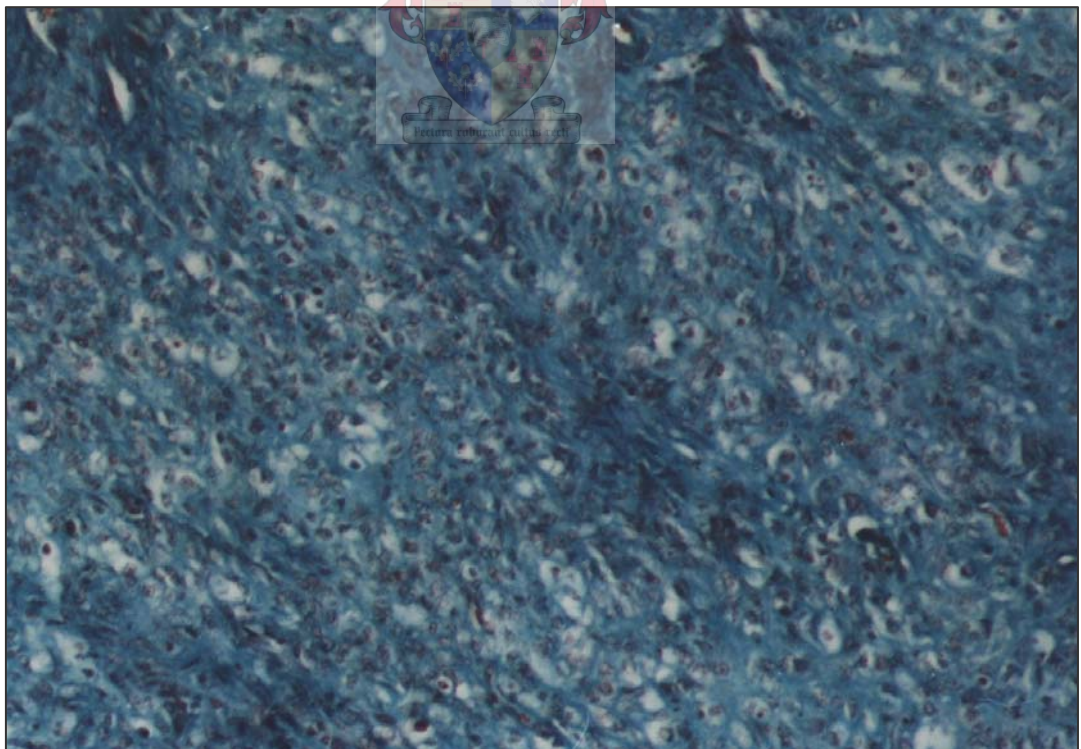


**Figure 29** Category 1: Little or no connective tissue infiltration. This amount of connective tissue is present in healthy normal functioning corpora lutea  
**Numerical value : 1**





**Figure 30** Category 2: Moderate connective tissue infiltration  
**Numerical value: 2**



**Figure 31** Category 3: Extensive connective tissue infiltration  
**Numerical value: 3**

All three technologists scored a particular slide and an average score was calculated. Using these averages, means and SEM's were calculated for the five slides in each one of the five groups.

### **3.2.5 Scanning Electron Microscopy**

Ultra structural analysis was done on a JOEL JSM – 6100 scanning electron microscope equipped with a cryotrans system.

#### **3.2.5.1 Selection**

One formalin-saline fixed corpora lutea were obtained from each of the following three groups.

Group 1 Corpora lutea with normal appearing corpora lutea.



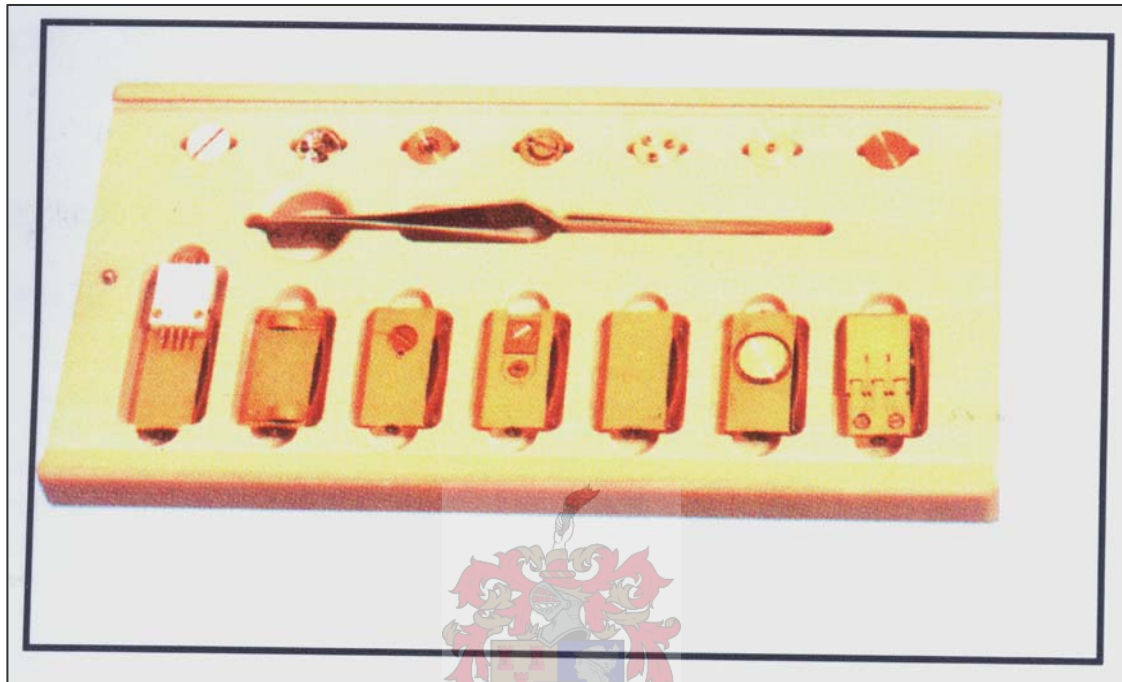
Group 2 Corpora lutea with prematurely regressed corpora lutea.

Group 3 Corpora lutea where regression was induced with the  $\text{PGF}_{2\alpha}$  analogue dinoprost tromethamine (Lutalyse ®, Pharmacia & Upjohn).

#### **3.2.5.2 Preparation**

- a) The tissue was cut with a scalpel blade into a pyramid form with a base of 5 mm x 5mm and a height of 7mm.

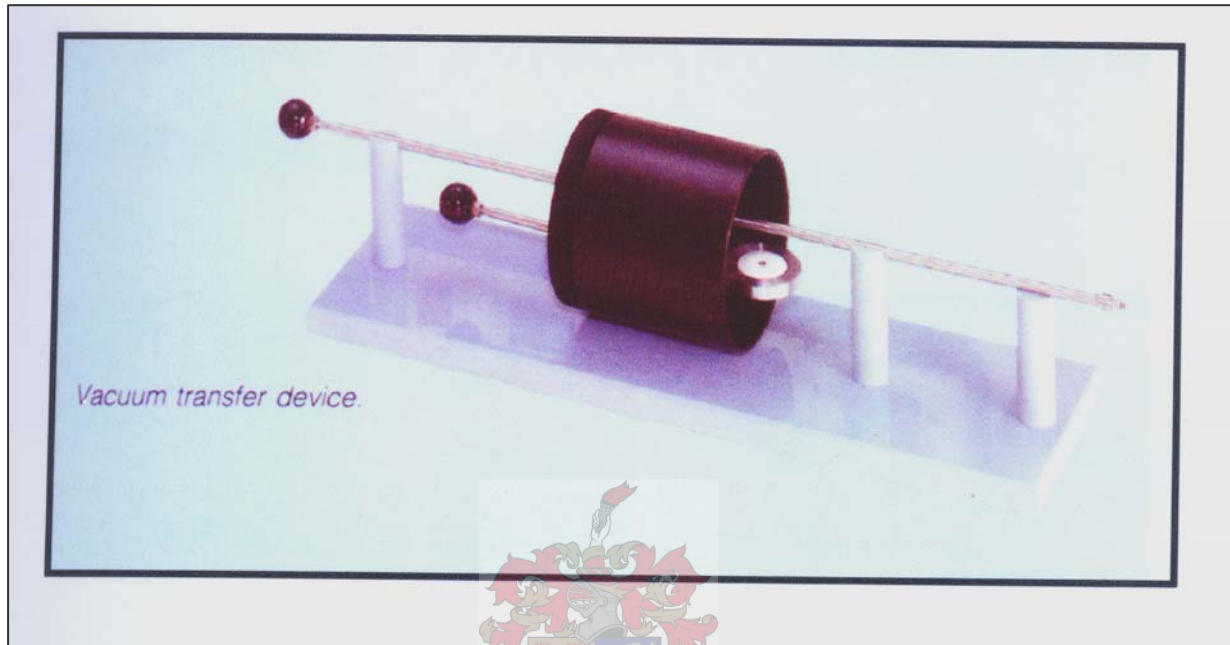
- b) The tissue was then bonded to the specimen holder at the base of the pyramid with Silver DAG in MIBK (Wirsam) followed by a 5 minute wait for the DAG to dry.



**Figure 32** A range of specimen holders for the CT 1500 cryotrans

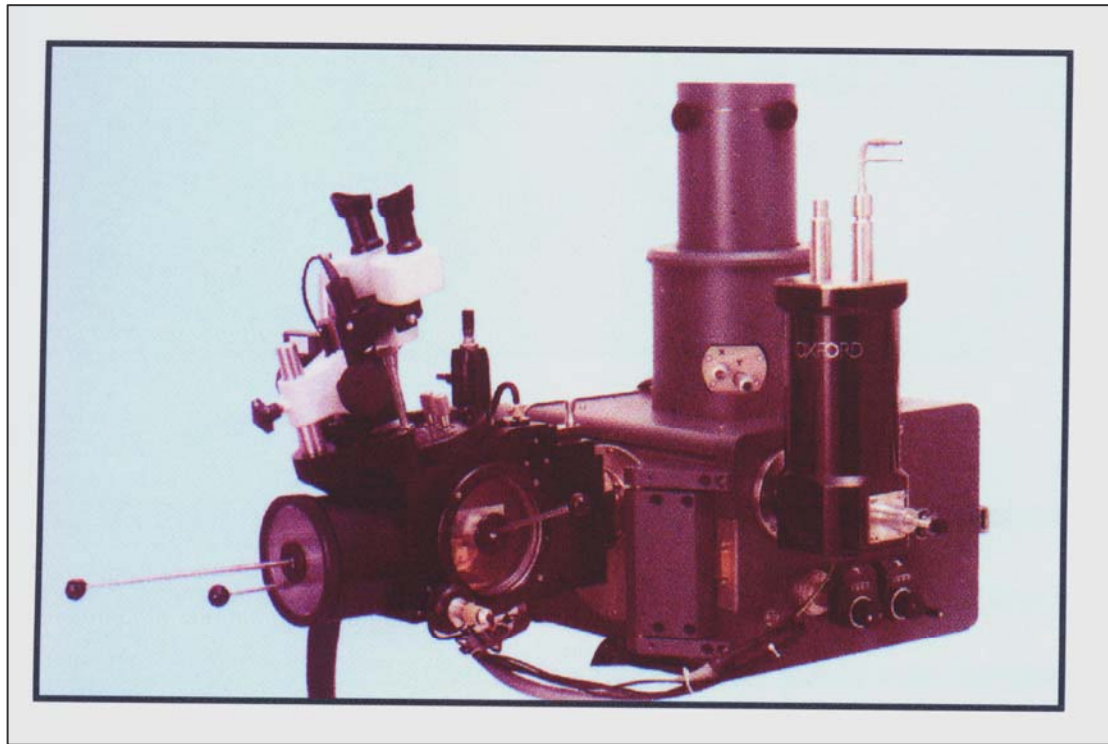
- c) The specimen holder was then connected to the vacuum transfer device.
- d) Using the vacuum transfer device, the tissue was then inserted in a slushing chamber and cooled with liquid nitrogen under a vacuum of 0.1 mBar to  $-200\text{ }^{\circ}\text{C}$ .

- e) Following the initial cooling in the slushing chamber the specimen was transferred with the vacuum transfer device to the preparation chamber while the vacuum of 0.1 mBar was kept constant.

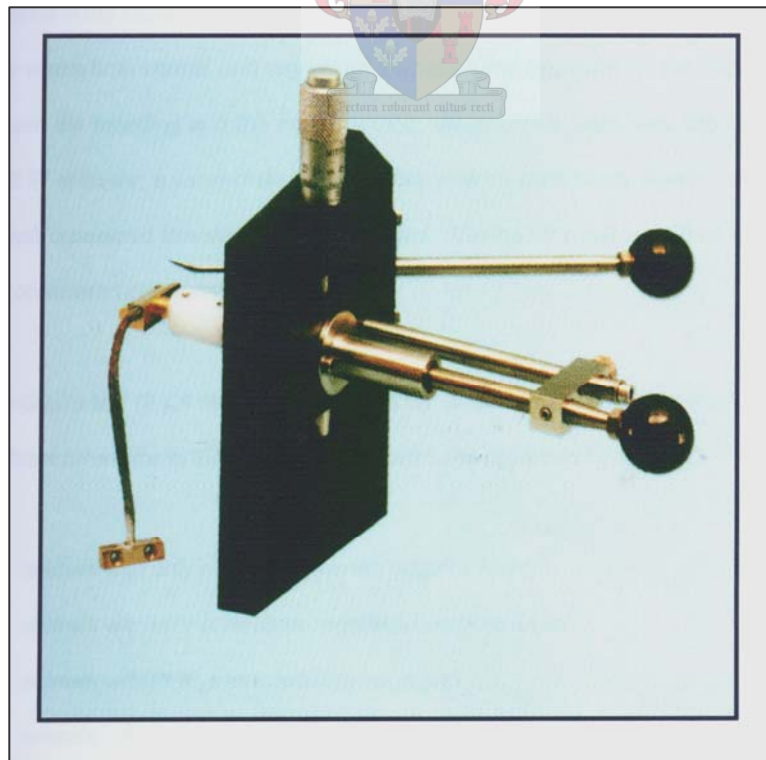


**Figure 33** The vacuum transfer device used to transfer the liquid nitrogen cooled specimen to the slushing chamber at  $-200\text{ }^{\circ}\text{C}$

- f) In the preparation chamber under a vacuum 0.1 mBar the tissue was heated to  $-70\text{ }^{\circ}\text{C}$ .
- g) Once the temperature was stable at  $-70\text{ }^{\circ}\text{C}$  the apex of the pyramid was fractured with the fracturing device.
- h) The fractured tissue was then transferred to the microscope stage with the vacuum transfer device and heated to  $-40\text{ }^{\circ}\text{C}$  to dehydrate the tissue surface.



**Figure 34** CT 1500 preparation chamber and gold stage nitrogen dewar with transfer device interfaced to an scanning electron microscope chamber



**Figure 35** Cr 15-318 fracturing and honing device

- i) Using the vacuum transfer device the dehydrated tissue was transferred to the preparation chamber where the tissue was coated with 24 ct scanning electron microscope gold.
- j) Using the vacuum transfer device the gold coated tissue was transferred to the microscope stage and the tissue was examined at low tension of 4 kV (tension would normally be 8kV).

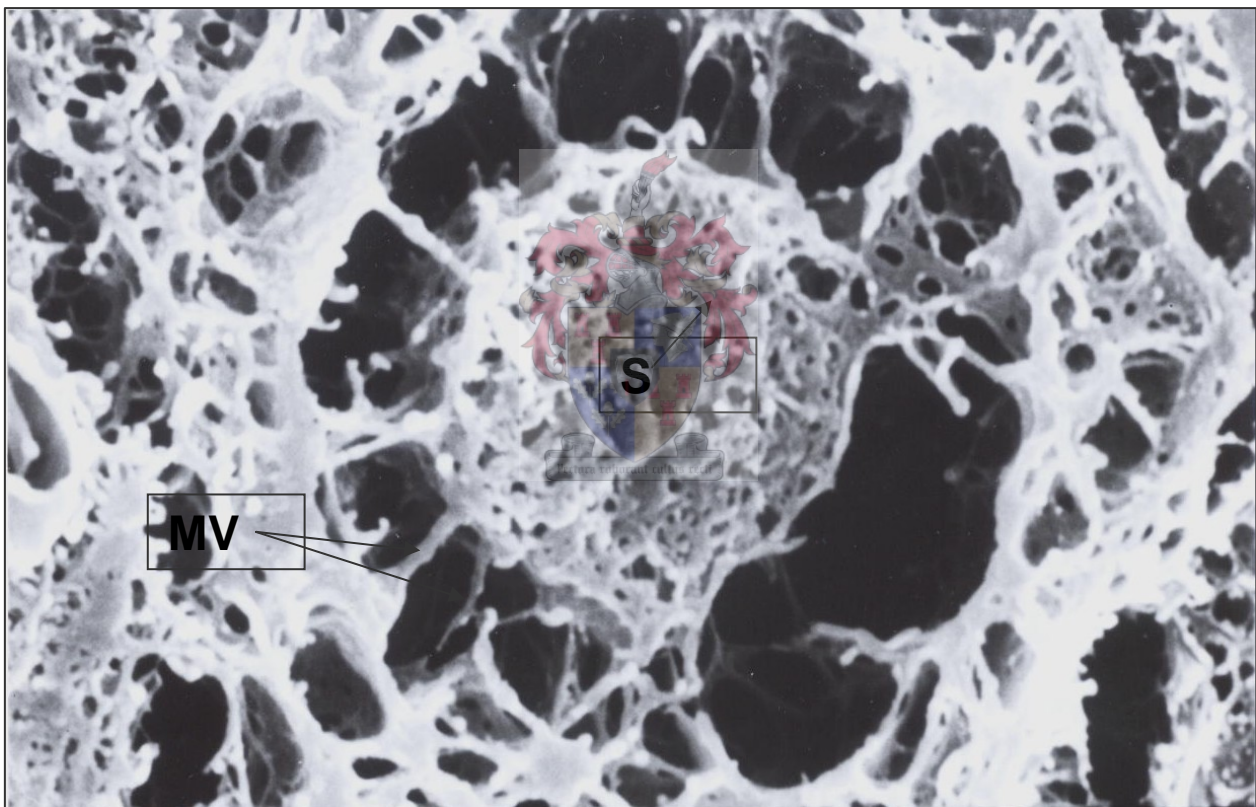
### 3.2.5.3 Evaluation of stage of apoptosis

By using a scanning electron microscope the morphological changes involving the nucleus, cytoplasm and plasma membrane [124] were studied. The progression of apoptosis follows a specific pattern with distinct stages and events that can clearly be identified by using a scanning electron microscope [125]. Characteristic scattered single cells, rather than groups of contiguous cells showing signs of apoptosis were observed. No signs of associated inflammation as seen in necrosis were observed. To be able to compare the progression of apoptosis between the three groups, three morphological stages of apoptosis were identified.

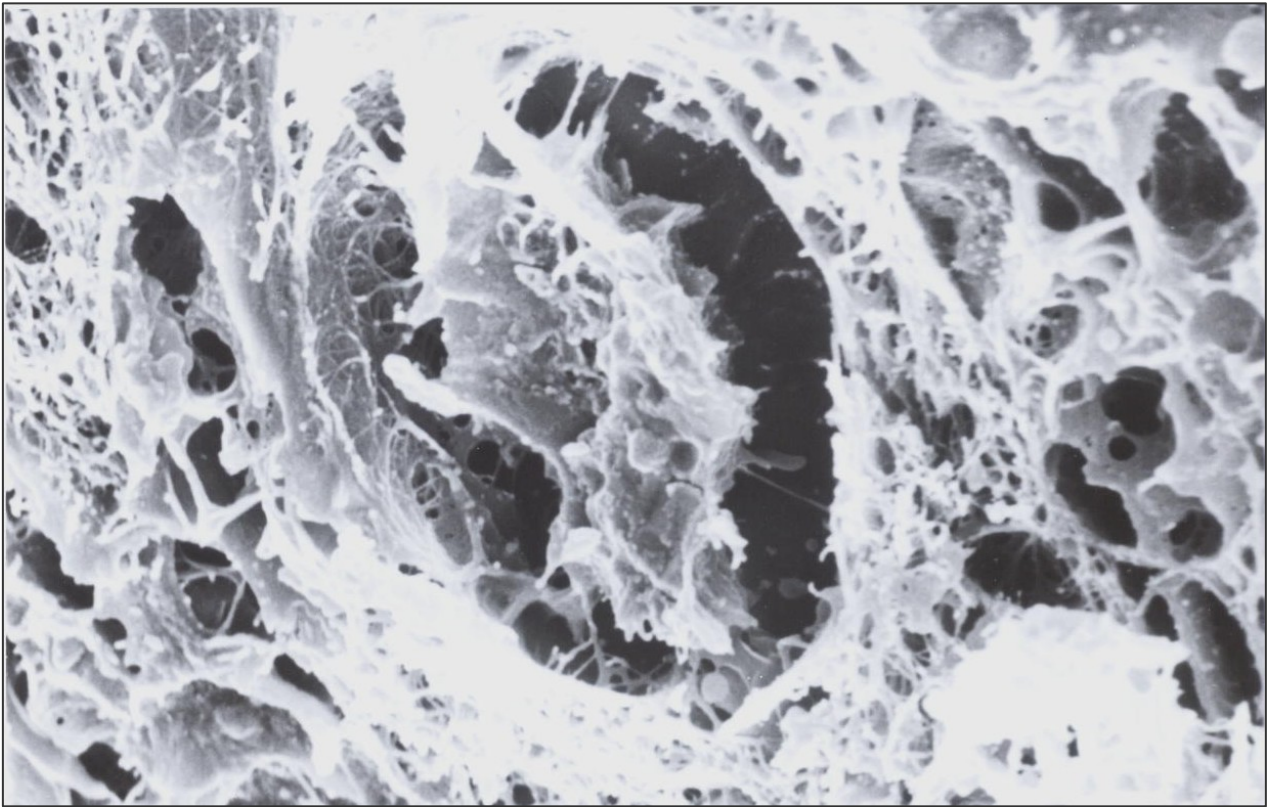
#### Stage 1

During the first stage apoptotic cells round up, loose contact with neighbours and condense or shrink. This is why the phenomenon was originally called “shrinkage necrosis” [126]. During this shrinkage they lose surface features such as microvilli and desmosomes [127] (figure 36). This stage is characterised by the loss of water. This leads to compaction of the cytoplasm, which results in an increased

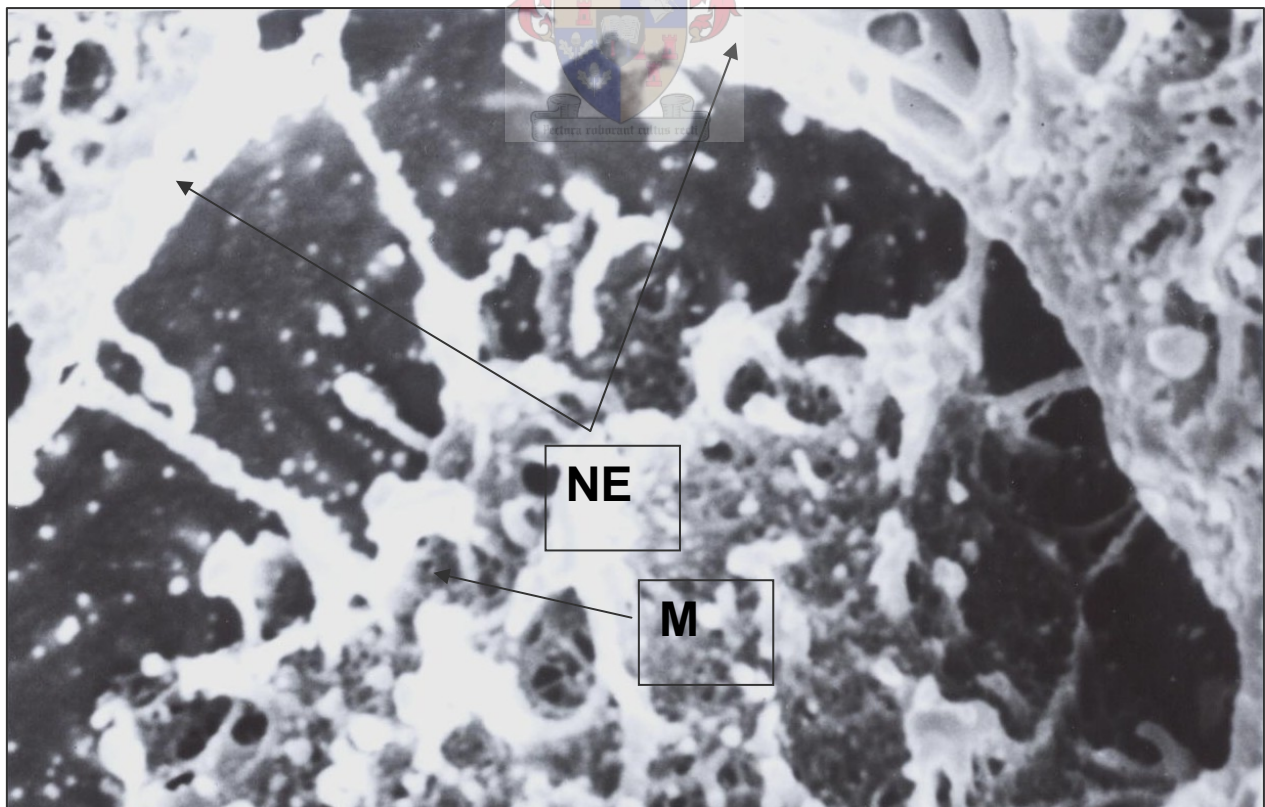
cell density. On occasion the cells may collapse and appear flattened (figure 37). On an intracellular level chromatin appears to condense and aggregates into dense compact masses along the margin of the nucleus, a process often referred to as margination of chromatin (figure 38). The endoplasmic reticulum including the outer nuclear envelope dilates. The cisternae of the reticulum swell to form vesicles and small vacuoles, many of which fuse with the plasma membrane, giving the apoptotic cell a characteristic spongy appearance [128] (figure 37).



**Figure 36** Early shrinking apoptotic cell rounding up and losing contact with neighbouring cells. During this shrinkage they lose surface features such as microvilli (MV) and desmosomes. Notice the spongy appearance (S) typical of this early stage



**Figure 37** Collapsing early shrinking apoptotic cell. During early apoptosis cells loose water, leading to increased cell density and sometimes collapsing into flattened cells as can be seen here

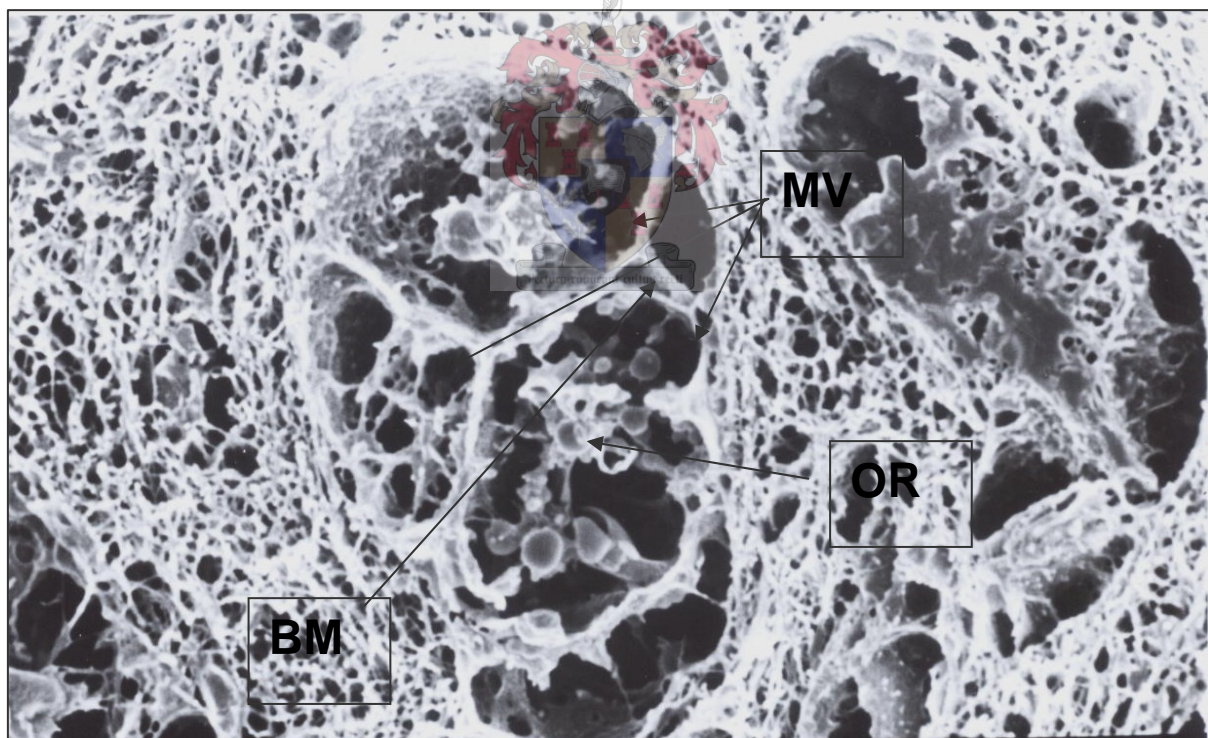


**Figure 38** Margination of chromatin (M) and dilation of the nuclear envelope (NE)

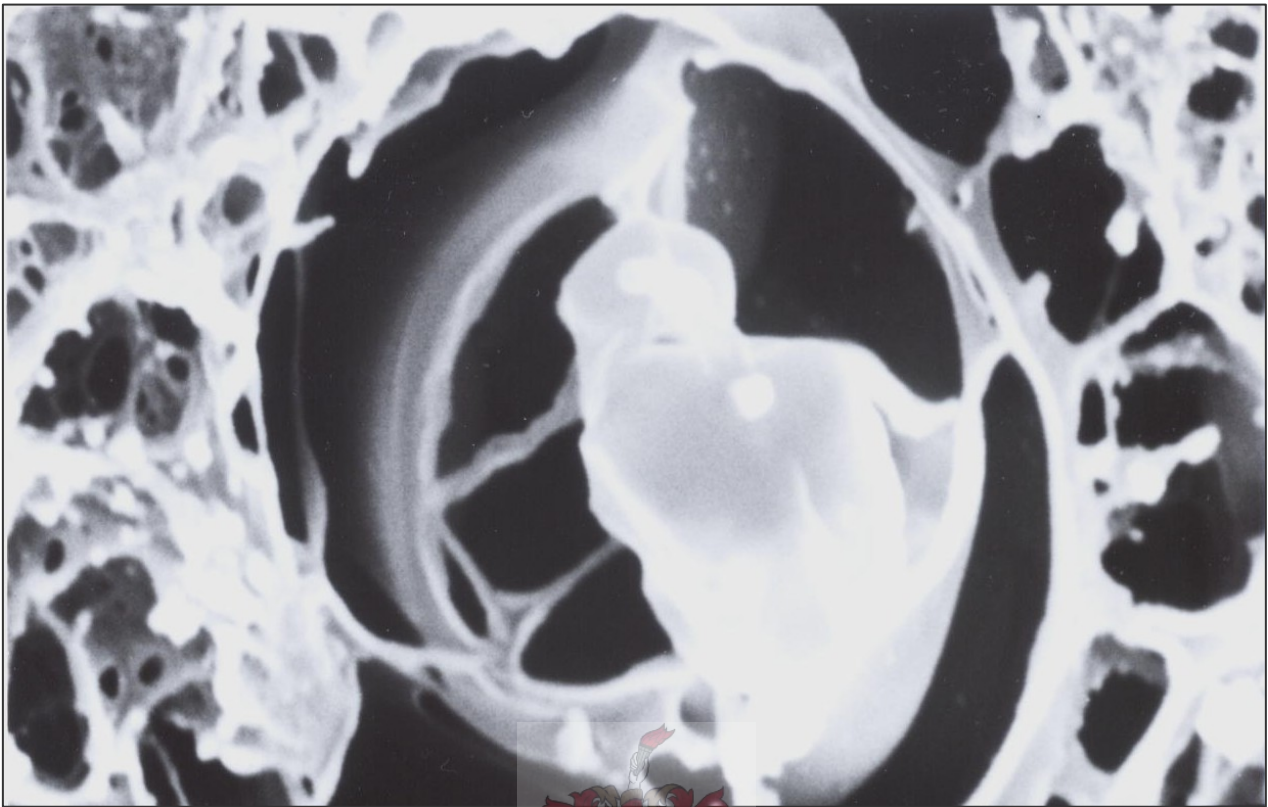


## Stage 2

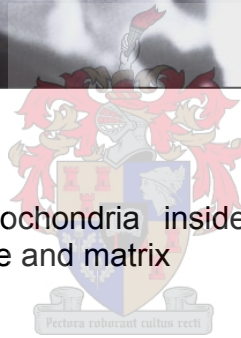
Stage two is characterised by extensive intracellular remodelling. There is a rapid loss of the cytoskeleton due to hydrolytic and degrading enzymes. These include RNAase leading to rapid lysis of ribosomal and messenger RNA, acid phosphatase, collagenase, cathepsins and proteases like the Caspases. However these enzymes are contained in large vacuoles. Membranes bud around these vacuoles leading to the formation of discrete apoptotic bodies enclosed by a cell membrane (See figure 39). These bodies sometimes enclose well preserved organelles like mitochondria which plays a vital role in controlling the process[129] (figure 40). The containment of the dangerous enzymes prevents damage to surrounding cells typical of necrosis.



**Figure 39** Stage 2 apoptotic cell displaying large membrane bound vacuoles (MV) with well-preserved organelle remnants (OR). The cell membrane buds (BM) around these vacuoles to prevent leaking of proteolytic enzymes

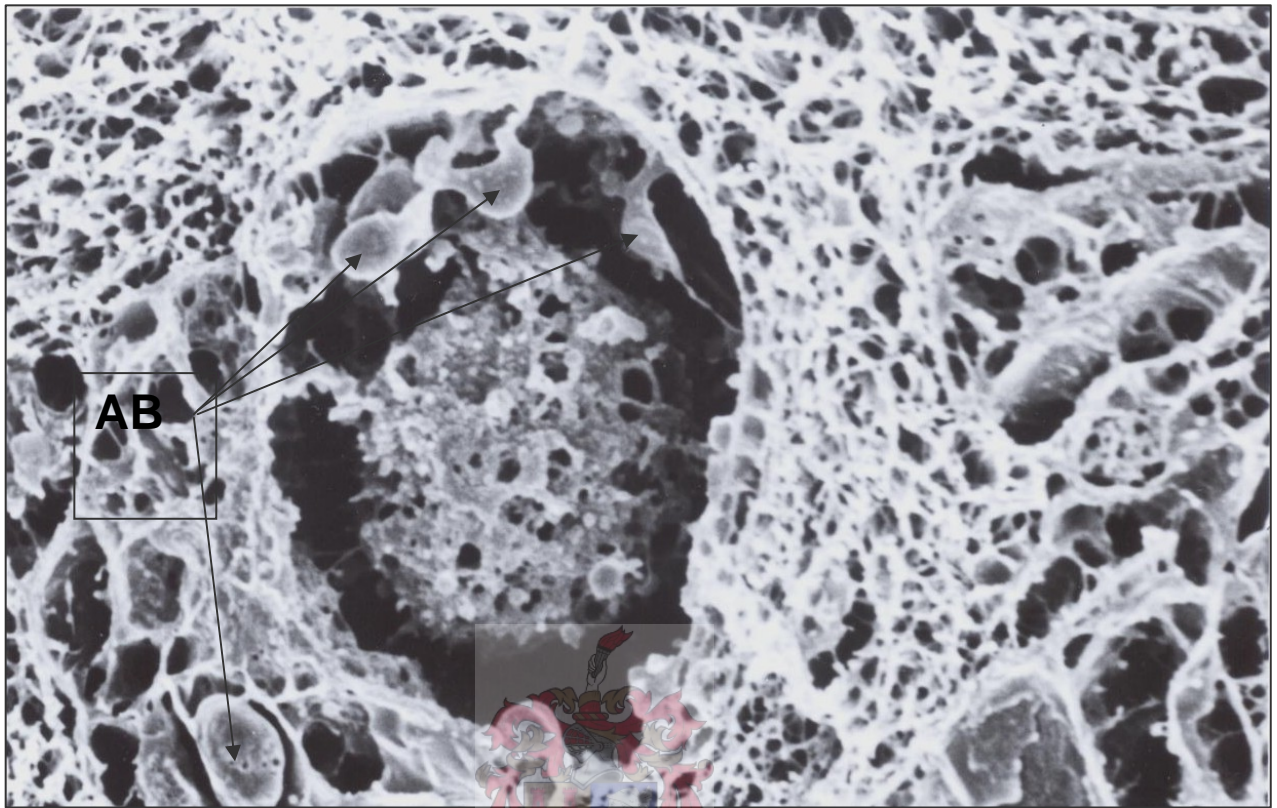


**Figure 40** Well-preserved mitochondria inside apoptotic vesicles. Note the clearly visible cristae and matrix



### Stage 3

During this stage the plasma membrane becomes active and convoluted, eventually budding or “blebbing” in such a way that the cell breaks up in a florid manner leading to a falling away (or, in Greek, apoptosis) of several membrane-bound spheres or apoptotic bodies of various sizes. Under physiological conditions these bodies remain viable and will exclude vital dyes such as trypan blue or nigrosine (figure 42).



**Figure 41** Blebbing apoptotic cell with several membrane bound apoptotic bodies of various sizes visible (AB)

In each of the three groups 100 cells were scored as either normal or stage 1-3 apoptotic.

### **3.2.6 Statistics methodology**

#### **3.2.6.3 Incidence of premature regression**

The Fisher's exact test was carried out to determine if there was any significant differences in the incidence of premature luteal regression and mixed cases between the breeding and non-breeding season. The test was performed on the NCSS 2000 software and a p-value of  $P < 0.05$  were considered significant.

#### 3.2.6.4 Ovulatory response

A Chi-Square test (4x2 matrix) was carried out to determine if there was a statistically significant difference in the amount of corpora lutea recorded between:

- a) animals with only normal appearing corpora lutea,
- b) animals with only premature regressed corpora lutea,
- c) animals with PGF<sub>2α</sub> induced regression and
- d) animals with both normal and regressed corpora lutea.

The tests were done on the NCSS 2000 software. A value of  $p < 0.05$  was considered statistically significant.

#### 3.2.6.5 Steroidogenic profiles

The progesterone, estrogen and progesterone : estrogen ratios were compared separately. The comparison was done by three-way analysis of variance (2 x 4 x 10 matrix). It was done for both the breeding and non-breeding seasons between:

- a) animals with only normal corpora lutea,
- b) animals with only premature regressed corpora lutea,
- c) animals with PGF<sub>2α</sub> induced regression and
- d) animals with both normal and regressed corpora lutea.

The analysis also extended for ten days from the day of sponge removal (day 0) to 9 days after sponge removal. The test was done on NCSS 2000 software with a P-value of  $P < 0.05$  considered statistically significant.

The correlation between the estrogen levels on the 6<sup>th</sup> day after sponge removal and the progesterone on the 7<sup>th</sup> day after sponge removal were done by means of regression analysis using the least squares method on the NCSS 2000 software. A  $R^2$  of  $\geq 0.9$  was considered an indication of a correlation between the two parameters. The analysis were carried out for linear, logarithmic, exponential and powered mathematical models.

### **3.2.6.6 Histology**

#### *3.2.6.6.1 Evaluation of the number of apoptotic nuclei*

The five group means of the number of apoptotic nuclei recorded per 100 evaluated cells were compared by one-way analysis of variance with a P-value of  $< 0.05$  considered as significant. Once significant difference among group means were established, the Tukey-Kramer adhoc test was performed to examine all possible comparisons among the five means. The analysis was done on the NCSS 2000 software.

#### *3.2.6.6.2 Evaluation of the amount of connective tissue infiltration*

The five group means of the relative connective tissue infiltration were compared by means of one-way analysis of variance with a P-value of  $< 0.05$  considered as statistically significant. Once it was established that the group means do differ significantly the Kruskal-Wallis Z adhoc test were performed to examine

all possible comparisons among the five means. This was done since the data was discrete with only three possible categories possible. The analysis was done on NCSS 2000 software.

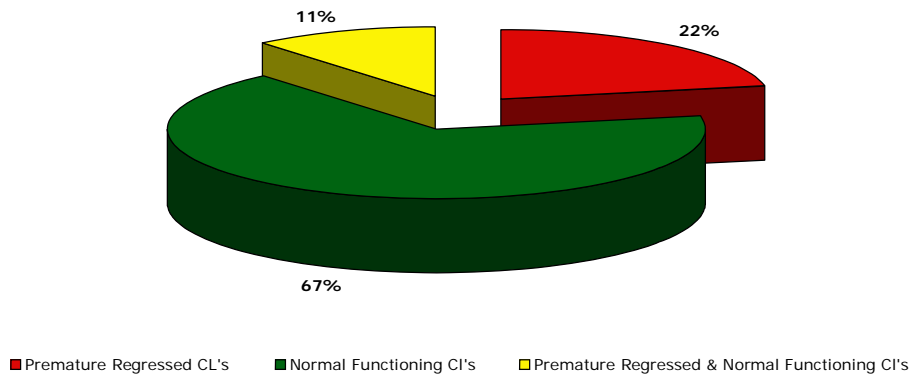
### **3.2.6.7 Scanning Electron Microscopy**

Two-way analysis of variance were performed in a 5 x 4 factorial design with  $p < 0.05$  considered statistically significant. Following the establishment of significant differences between the stage of apoptosis between the five defined groups, a Tukey-Kramer adhoc test was performed to establish all possible comparisons between the five defined groups and four defined stages of apoptosis with  $P < 0.05$  considered statistically significant. The analysis was done on the NCSS 2000 software.

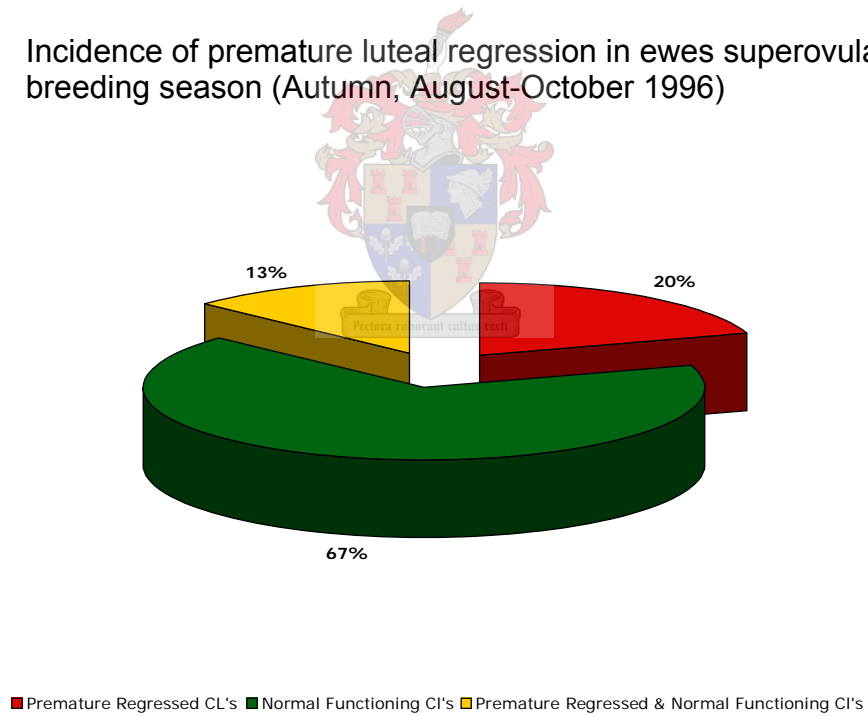


### 3.3 Results

#### 3.3.1 Occurrence of premature luteal regression



**Figure 42** Incidence of premature luteal regression in ewes superovulated in the breeding season (Autumn, August-October 1996)



**Figure 43** Incidence of premature luteal regression in ewes superovulated in the non-breeding season (Spring, March-May 1996)

There were no significant differences in the incidence of premature luteal regression ( $p>0.05$ ) and the incidence of mixed cases (both normal and regressed Cl's) between the breeding and non-breeding season. Superovulated Mutton Merino ewes received no grain supplementation in the breeding season. In both the breeding and non-breeding season about one third of the ewes showed signs of premature regression. This attributes to substantial losses in viable embryo's.

### 3.3.2 Ovulatory response

The number of corpora lutea recorded between ewes superovulated in the breeding season and the non-breeding season ( $P=0.96$ ). In the ewes with only normal appearing corpora lutea in both the breeding season and non-breeding season, there was no significant difference in the number of corpora lutea recorded compared with ewes with only premature luteal regression ( $P=0.93, P=0.98$ ), ewes with  $PGF_{2\alpha}$  induced regression ( $P=0.80, P=0.87$ ) and ewes with both normal appearing and regressed corpora lutea ( $P=0.94, P=0.91$ ). There was no significant difference in the number of corpora lutea recorded between ewes with only premature regression in the breeding season and the non-breeding season and ewes with  $PGF_{2\alpha}$  induced regression ( $P=0.65, P=0.85$ ) and ewes with both normal appearing and regressed corpora lutea ( $P=0.85, P=0.67$ ). Finally there was no significant difference in the amount of corpora lutea recorded between ewes with  $PGF_{2\alpha}$  induced regression and ewes with both normal appearing and regressed corpora lutea ( $P=0.77, P=0.79$ ). Thus, it appears that the response to superovulation with eCG was similar in the breeding and non-breeding season.



**Table 29** Ovulatory response in Mutton Merino ewes superovulated in the breeding season

Category of response	No. of ewes	Ovarian response in numbers of corpora lutea CI's (Mean $\pm$ SEM)		
		Left ovary	Right ovary	Total
Normal CI's	12	3.5 $\pm$ 0.8	4.2 $\pm$ 1.2	7.7 $\pm$ 1.1
Premature regressed CI's	4	5.1 $\pm$ 1.4	4.2 $\pm$ 1.2	9.3 $\pm$ 1.3
PGF <sub>2<math>\alpha</math></sub> induced regression	5	4.2 $\pm$ 0.9	4.0 $\pm$ 0.8	8.2 $\pm$ 0.9
<u>Mixed response</u>	2			
Normal CI's		2.4 $\pm$ 1.2	2.6 $\pm$ 2.0	5.0 $\pm$ 1.8
Premature regressed CI's		2.1 $\pm$ 1.2	2.4 $\pm$ 1.6	4.5 $\pm$ 1.4
Total CI's		4.5 $\pm$ 1.2	5.0 $\pm$ 1.8	9.5 $\pm$ 1.5

**Table 30** Ovulatory response of ewes superovulated in the non-breeding season

Category of response	No. of ewes	Ovarian response in numbers of corpora lutea CI's (Mean $\pm$ SEM)		
		Left ovary	Right ovary	Total
Normal CI's	10	4.0 $\pm$ 1.6	3.8 $\pm$ 2.4	7.8 $\pm$ 2.6
Premature regressed CI's	3	4.3 $\pm$ 0.6	6.0 $\pm$ 4.0	10.3 $\pm$ 4.0
PGF <sub>2<math>\alpha</math></sub> induced regression	3	3.6 $\pm$ 0.6	4.7 $\pm$ 3.1	8.3 $\pm$ 3.5
<u>Mixed response</u>	2			
Normal CI's		2.0 $\pm$ 1.4	2.0 $\pm$ 0.0	4.0 $\pm$ 1.4
Premature regressed CI's		1.5 $\pm$ 2.1	4.5 $\pm$ 0.7	6.0 $\pm$ 2.8
Total CI's		3.5 $\pm$ 0.7	6.5 $\pm$ 0.7	10.0 $\pm$ 1.4

### 3.3.3 Steroidogenic profiles

#### 3.3.3.1 Progesterone profiles

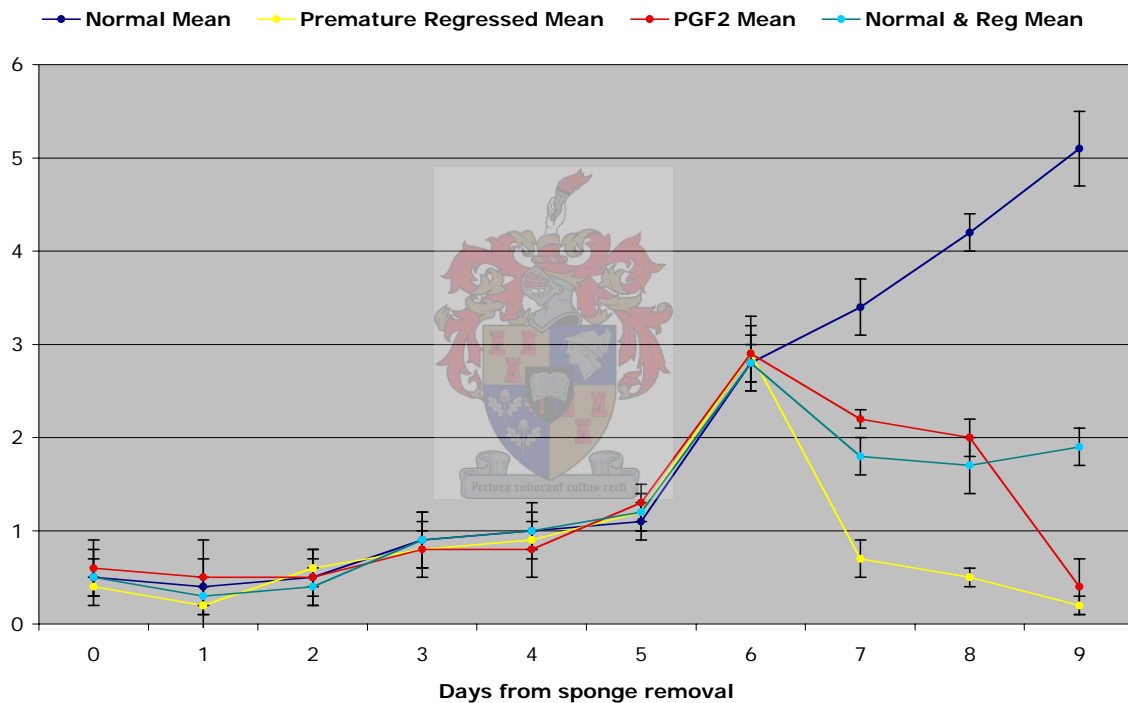
The progesterone profiles of the ewes superovulated in the breeding season were not significantly different from the progesterone profiles of the animals superovulated in the non-breeding season ( $P>0.05$ ). For the day of sponge removal up to 6 days after sponge removal there was no significant difference in progesterone profiles between:

- a) animals with only normal appearing corpora lutea,
- b) animals with only premature regressed corpora lutea,
- c) animals with  $\text{PGF}_{2\alpha}$  induced regression and
- d) animals with both normal and regressed corpora lutea.

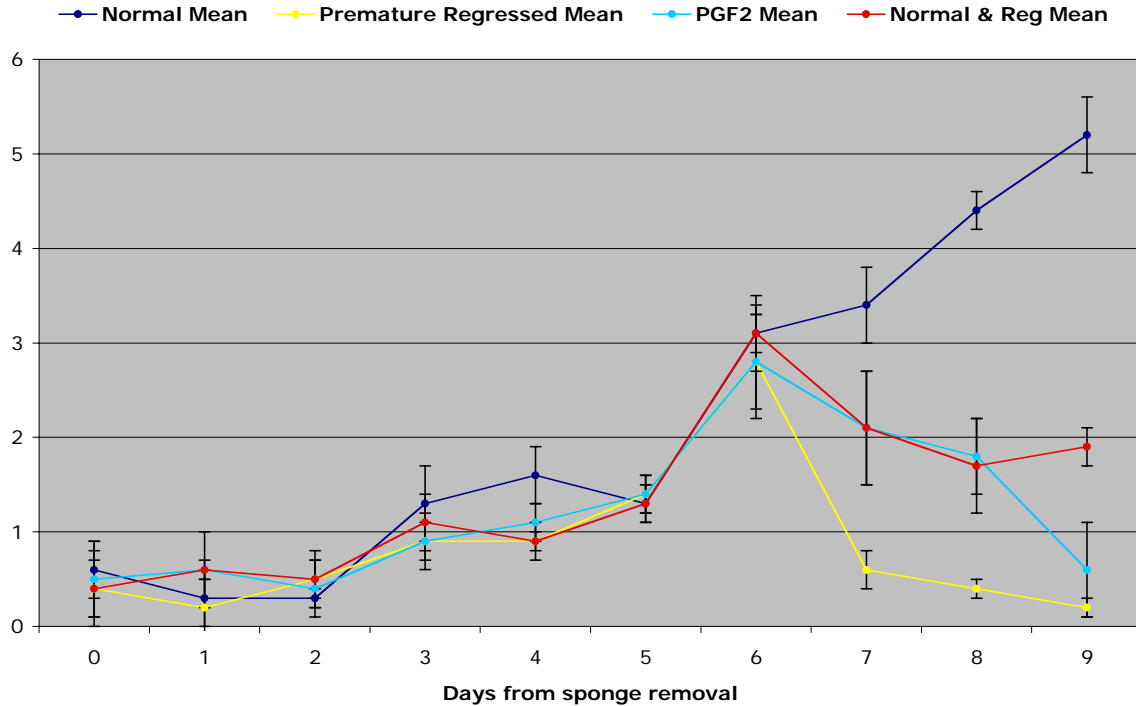


On the 7<sup>th</sup>, 8<sup>th</sup> and 9<sup>th</sup> day after the sponges were removed there were a significant difference between the four mentioned groups of animals ( $P<0.01$ ). The progesterone levels of the animals with only normal appearing corpora lutea were still increasing in the normal fashion. The progesterone levels of the other three groups all showed a decline in progesterone concentration. The decrease in progesterone concentration in the animals with only premature regressed corpora lutea was more profound than in the animals with  $\text{PGF}_{2\alpha}$  induced regression. In animals with premature regressed corpora lutea progesterone levels dropped to baseline values (1 ng/ml) within 24 hours while it took 72 hours in the ewes where regression were induced with the  $\text{PGF}_{2\alpha}$  analogue (dinoprost promethamine) until there were no significant difference between these two groups on day 9 ( $P>0.05$ ). In

the cases where regressed corpora lutea occurred with normal appearing corpora lutea there were an initial drop in progesterone after which the progesterone level was maintained at a lower concentration than the ewes with only normal appearing corpora lutea ( $P < 0.01$ ). These trends were the same in the breeding and non-breeding season (figures 44 and 45).



**Figure 44** Progesterone profiles of ewes superovulated in the breeding season



**Figure 45** Progesterone profiles of ewes superovulated in the non-breeding season

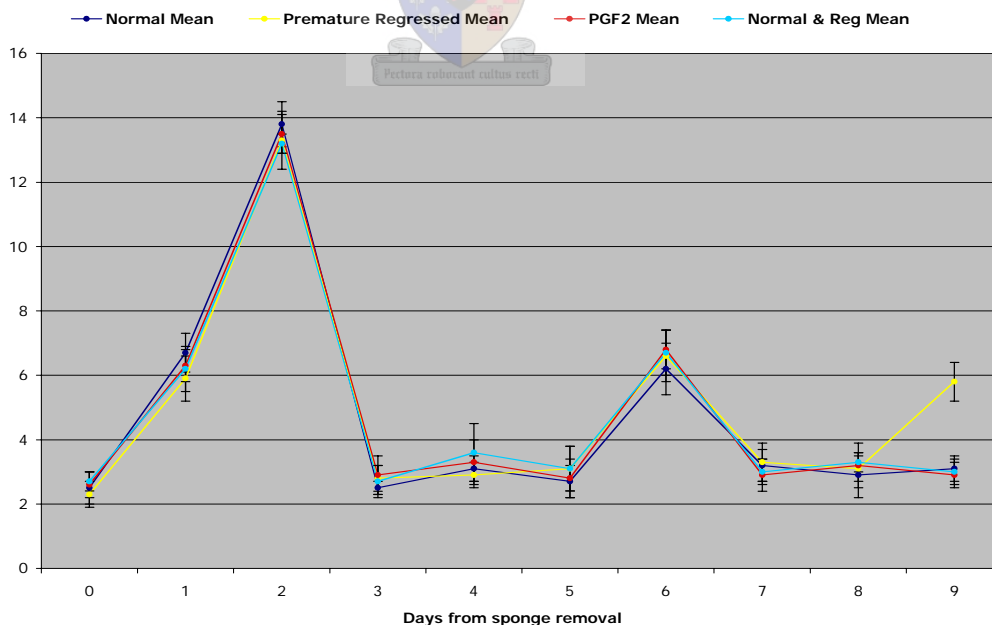


### 3.3.3.2 Estrogen profiles

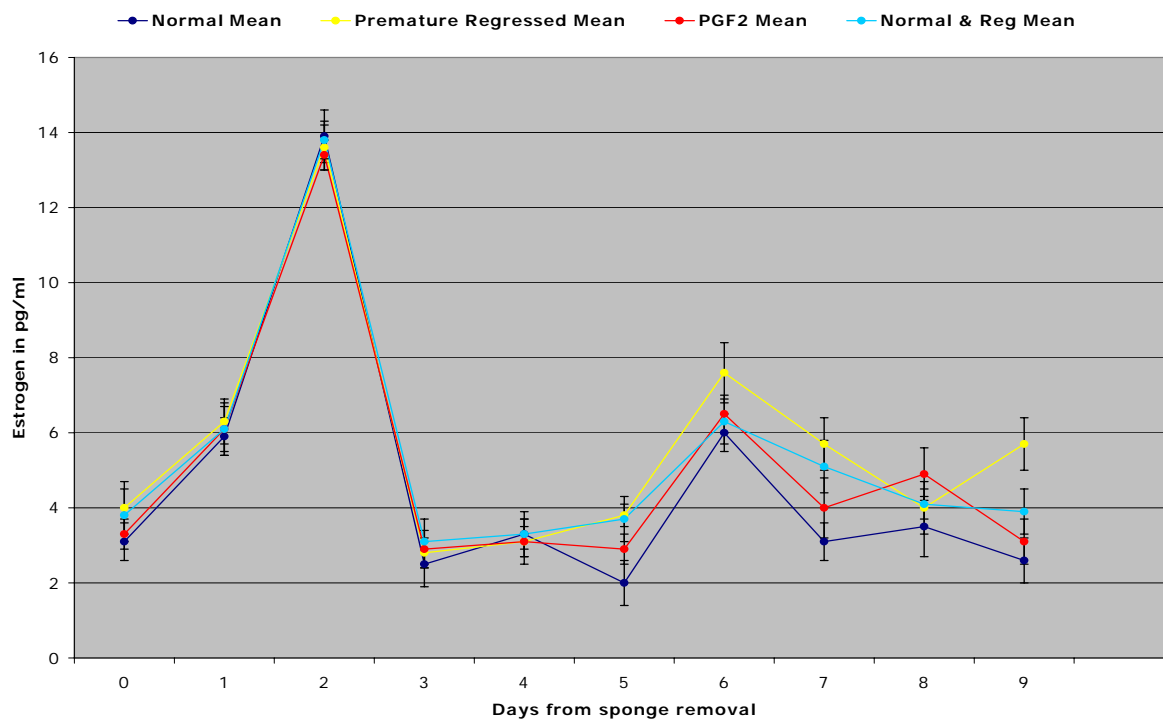
The estrogen profiles of the ewes superovulated in the breeding season were not significantly different from the  $E_2-17\beta$  profiles of the animals superovulated in the non-breeding season ( $P>0.05$ ). From the day of sponge removal up to the 8<sup>th</sup> day after sponge removal there were no significant differences in the  $E_2-17\beta$  profiles between:

- animals with only normal appearing corpora lutea,
- animals with only premature regressed corpora lutea,
- animals with  $PGF_{2\alpha}$  induced regression and
- animals with both normal and regressed corpora lutea.

On the 9<sup>th</sup> day after the sponges were removed, there was a significant difference between the animals groups ( $P < 0.01$ ). The  $E_2-17\beta$  levels of the animals with only premature regressed corpora lutea started to increase while the  $E_2-17\beta$  levels of the other groups were still at baseline levels. In all the ewes tested from all four groups in both the breeding and non-breeding season, the first principal rise peaked on the 2<sup>nd</sup> day after sponge removal ( $\pm$  day 0 of the estrous cycle, because ewes usually come into oestrus 24 to 48 hours after sponge removal). The peak  $E_2-17\beta$  levels ranged from 12.0 – 14.8 pg/ml higher than those observed in untreated ewes with peak levels ranging from 6.2 – 10.0 pg/ml. On the 3<sup>rd</sup> day after sponge removal estrogen levels dropped to baseline and remained at baseline until the 5<sup>th</sup> day after sponge removal. On the 6<sup>th</sup> day after sponge removal ( $\pm$  4<sup>th</sup> day of the estrous cycle) there was a significant additional rise in  $E_2-17\beta$  ( $P < 0.01$ ) confirming findings.



**Figure 46** Estrogen profiles of ewes superovulated in the breeding season.



**Figure 47** Estrogen profiles of ewes superovulated in the non-breeding season.

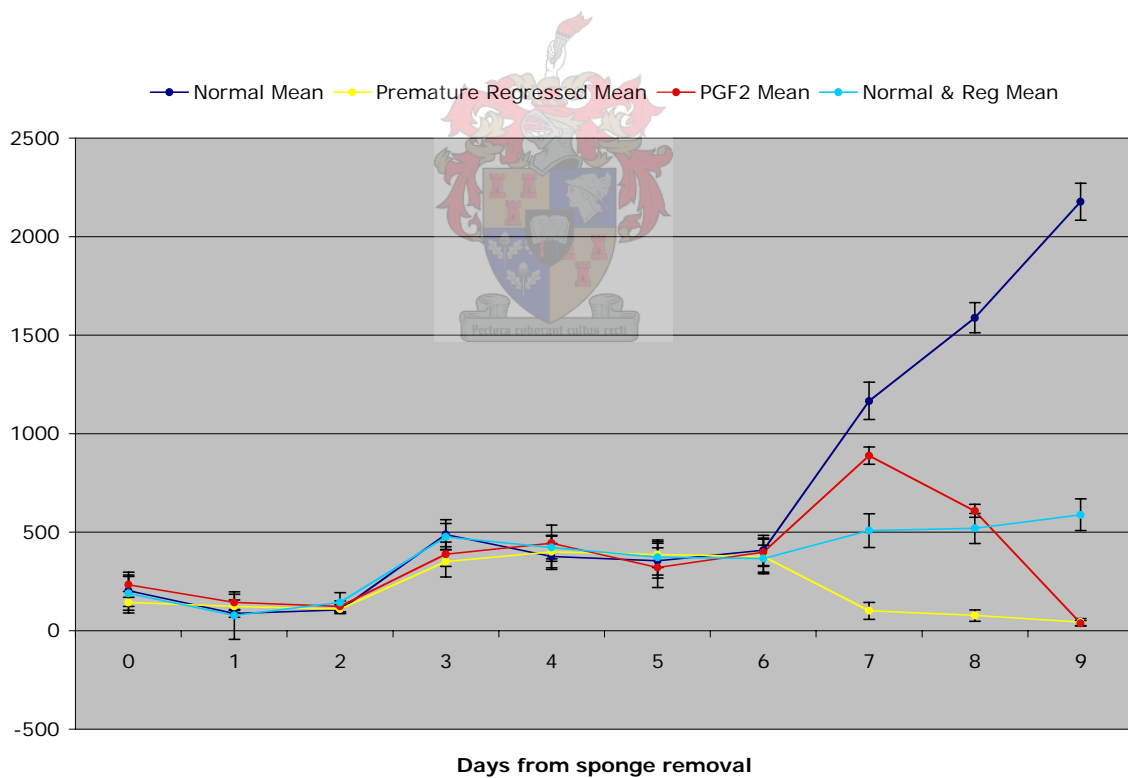
confirming findings in some other studies [7, 118-121]. Peak  $E_2-17\beta$  levels ranged from 5.4 – 8.2 pg/ml in this additional rise. On the 7<sup>th</sup> day after sponge removal  $E_2-17\beta$  returned to baseline (figures 46 and 47).

### 3.3.3.3 Progesterone:Estrogen ratio on molar base

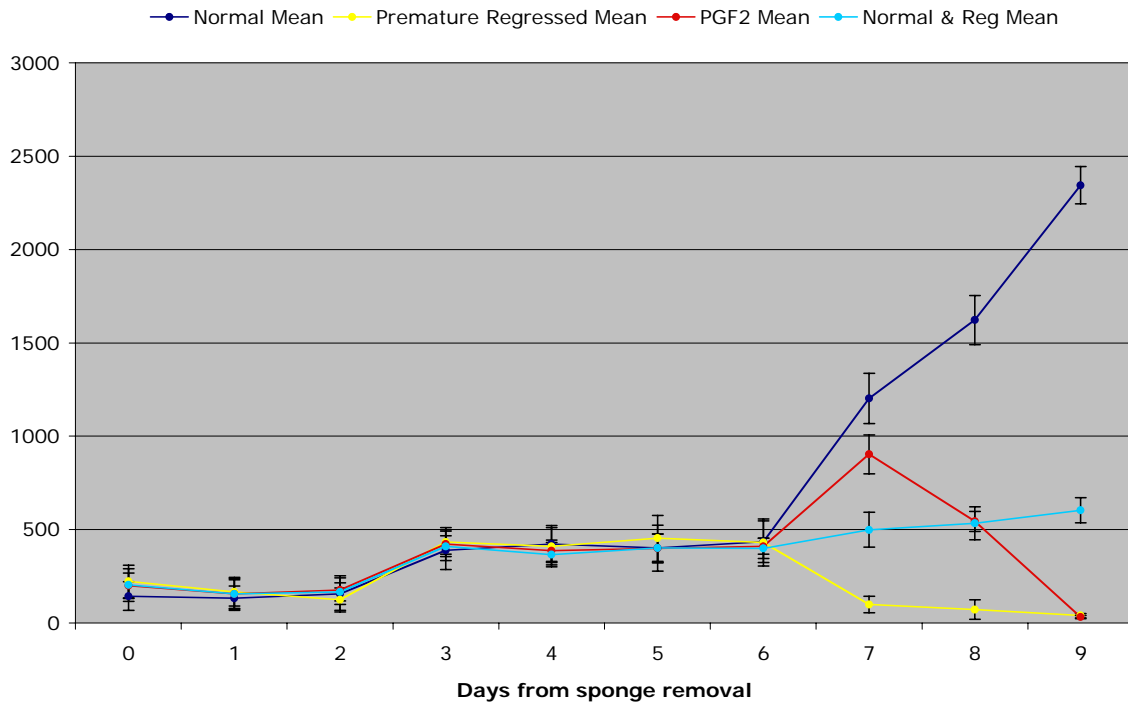
The  $P_4:E_2-17\beta$  ratio on a molar base of the ewes superovulated in the breeding season were not significantly different from the  $P_4:E_2-17\beta$  ratio's of the ewes superovulated in the non-breeding season ( $P>0.05$ ). From sponge removal up to the 6<sup>th</sup> day after sponge removal there were no significant difference in the  $P_4:E_2-17\beta$  ratio's between:

- a) animals with only normal appearing corpora lutea,
- b) animals with only premature regressed corpora lutea,
- c) animals with PGF<sub>2α</sub> induced regression and
- d) animals with both normal and regressed corpora lutea.

On days 7, 8 and 9 all the groups differed significantly from each other with respect to their P<sub>4</sub>:E<sub>2</sub>-17β ratio on a molar base except for animals with premature luteal regression and animals with PGF<sub>2α</sub> induced regression on day 9 after sponge removal where there was no significant difference on that particular day (P>0.05) (see figures 48 and 49).



**Figure 48** Profiles of the progesterone/estrogen ratio on a molar base in the breeding season



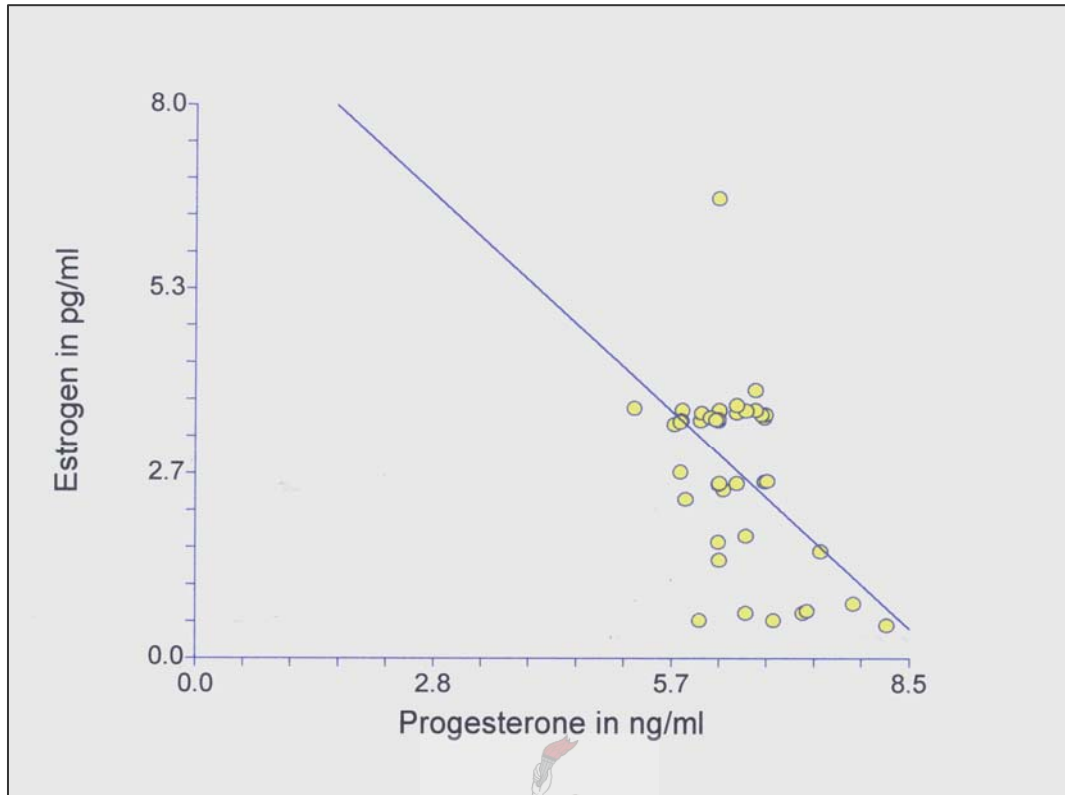
**Figure 49** Profiles of the progesterone/estrogen ratio on a molar base in the non-breeding season



**3.3.3.4** *Correlation of peak estrogen concentration on the 6<sup>th</sup> day after sponge removal and progesterone concentration on the 7<sup>th</sup> day after sponge removal*

The onset of the decline in P<sub>4</sub> levels in animals that presented signs of premature regression in both the breeding and the non-breeding season coincided with the E<sub>2</sub>-17β peak on the 6<sup>th</sup> day after sponge removal. There was, however, no correlation between the peak E<sub>2</sub>-17β level on the 6<sup>th</sup> day after sponge removal and the P<sub>4</sub> level on the 7<sup>th</sup> day after sponge removal (fig 50) (R<sup>2</sup>=0.25)





**Figure 50** Correlation of the peak estrogen concentration on the 6<sup>th</sup> day after sponge removal and the progesterone concentration on the 7<sup>th</sup> day after sponge removal in all the ewes tested in the breeding and non-breeding season

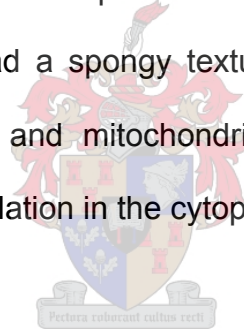
### 3.3.4 Histology

Upon histological evaluation by light microscopy on slides stained with hematoxylin and eosin and Masson's Trichrome stains, the following findings were made:

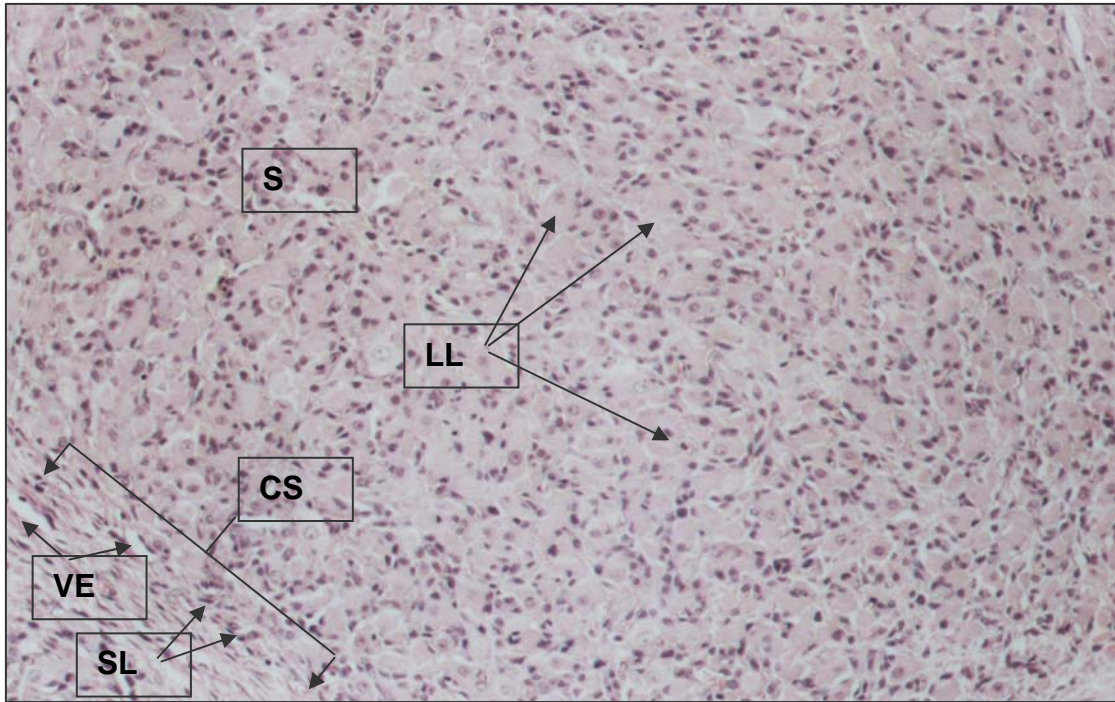
Slides from groups one and three showed no signs of apoptosis and very little connective tissue present. The corpora lutea in these slides showed all signs of advanced luteinization involving elements of theca, granulosa, vascular and reticulo-

endothelial progenitors. There was advanced vascularization with vascular components reaching the cavity of the collapsed follicle and vascular vessels carried to the internal structure of the CL by invading septa of connective tissue. Extensive capillary branching was observed.

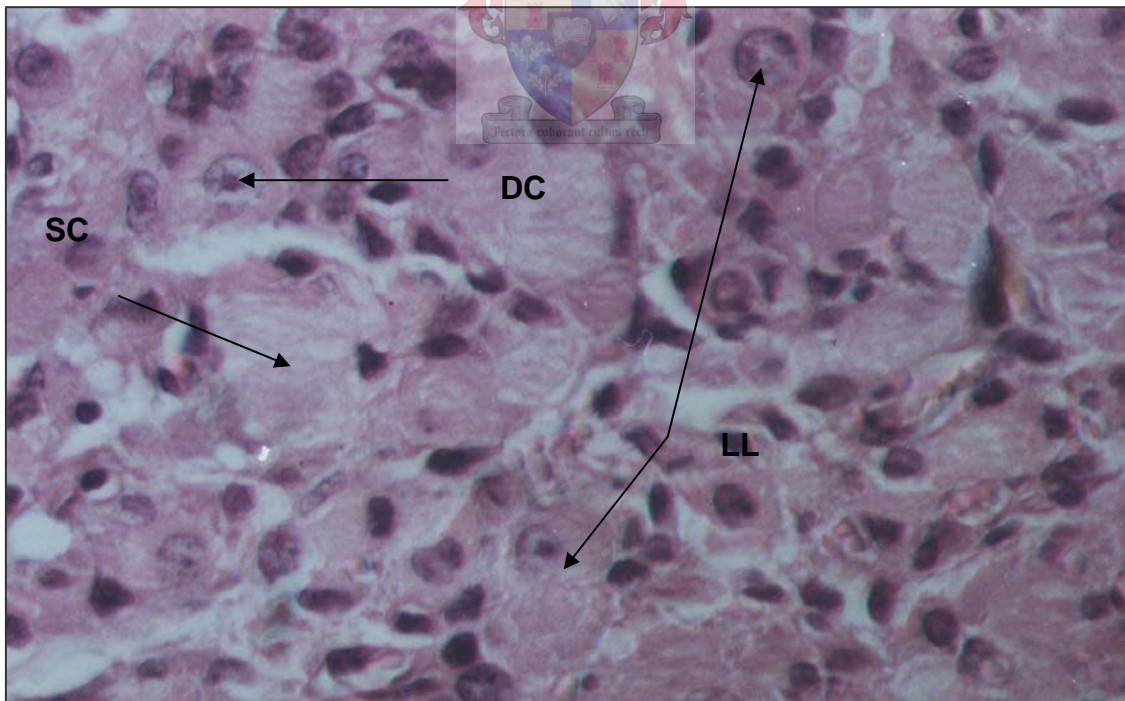
Both small (theca lutein cells) and large (granulosa lutein cells) luteal cells were present. Small lutein cells become dispersed in the septa as single entities or in small groups. These cells show moderate enlargement and assume an epithelioid shape. Granulosa cells underwent massive hypertrophy as they assumed a typical polyhedral shape. The cells were pale staining with a lot of cytoplasm. The chromatin in these cells were dispersed a sign of actively transcribing cells. The pale staining cytoplasm had a spongy texture typical of cells with abundant smooth endoplasmic reticulum and mitochondria. Especially on the Masson's Trichrome stain the lipid accumulation in the cytoplasm was pronounced.



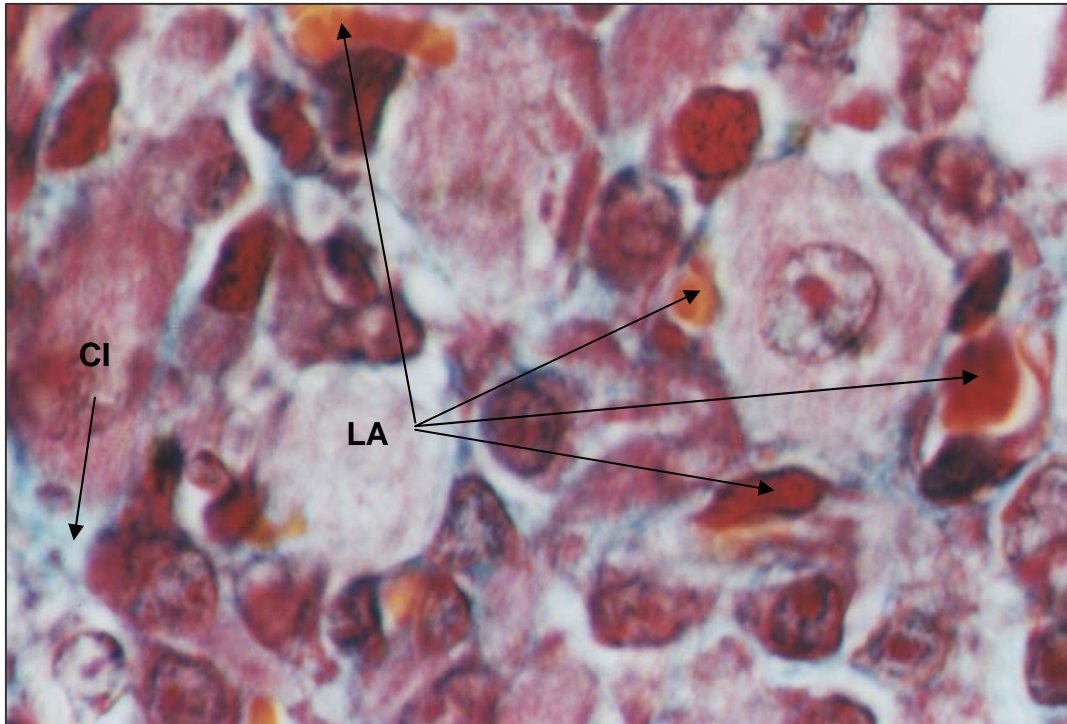
Slides from groups two, four and five showed all the signs of apoptotic luteal regression. Discrete well-preserved apoptotic bodies were present. Convoluted budding cells were rarely seen. The apoptotic bodies were roughly round or oval in shape and varied in shape. Apoptotic bodies comprised of single dense chromatin masses surrounded by barely detectable cytoplasmic rims and not numerous nuclear fragments as seen in thymocytes [128]. The apoptotic cells were mostly seen as scattered solitary bodies. There was extensive connective tissue infiltration on the Masson's Trichrome stained slides as well as no visible lipid accumulation as seen in slides of group one and three.



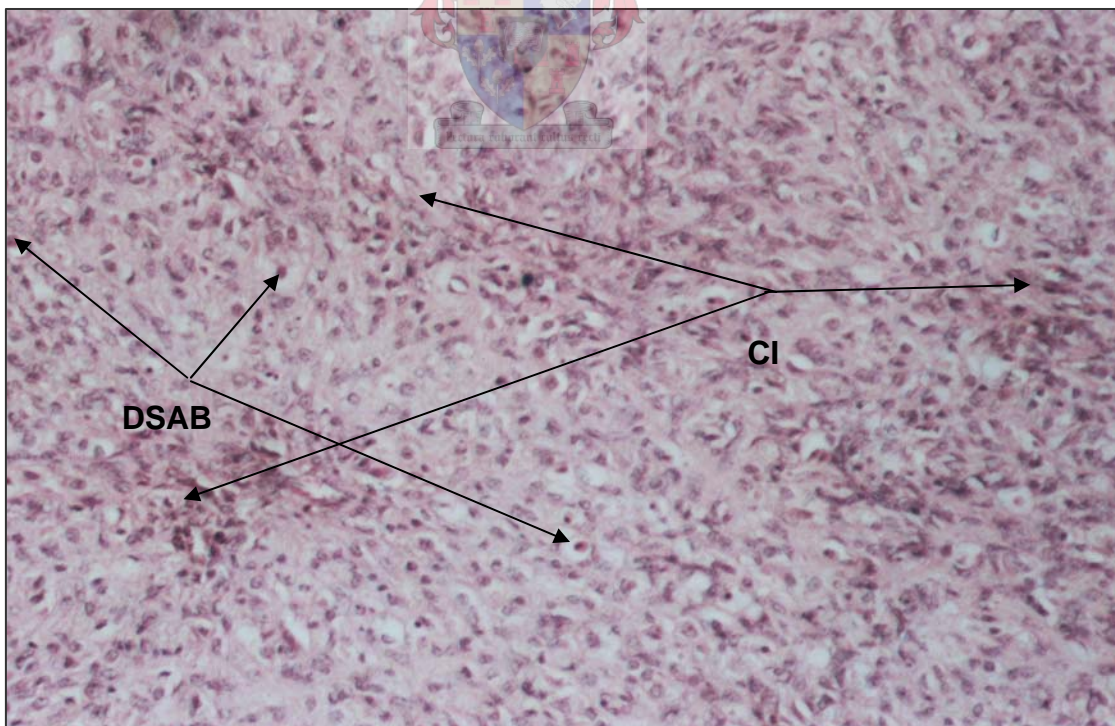
**Figure 51** H & E stained section of a normal corpus luteum as seen on x 100 magnification. Note the clearly visible connective tissue septa (CS) with groups of epithelioid small lutein cells (SL) and vascular elements (VE). The stroma (S) has numerous pale staining enlarged polyhedral large lutein cells (LL).



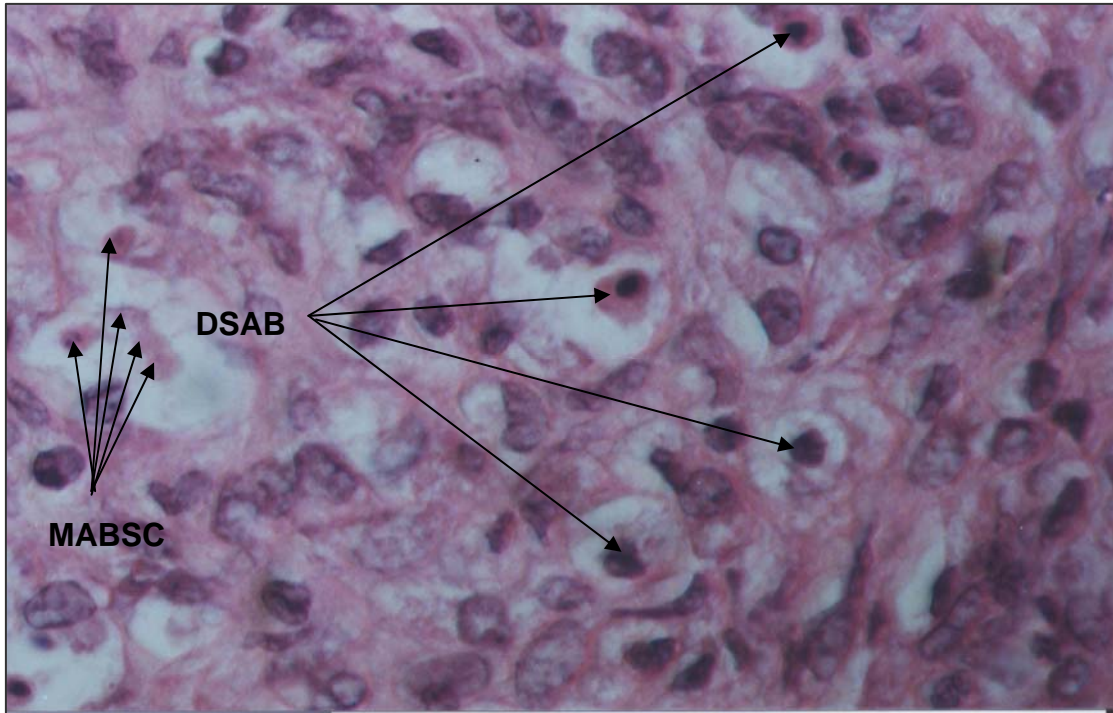
**Figure 52** H & E stained normal corpus luteum seen at x 400 magnification. Note the pale staining polyhedral large lutein cells (LL) with dispersed chromatin (DC) and spongy cytoplasm (SC)



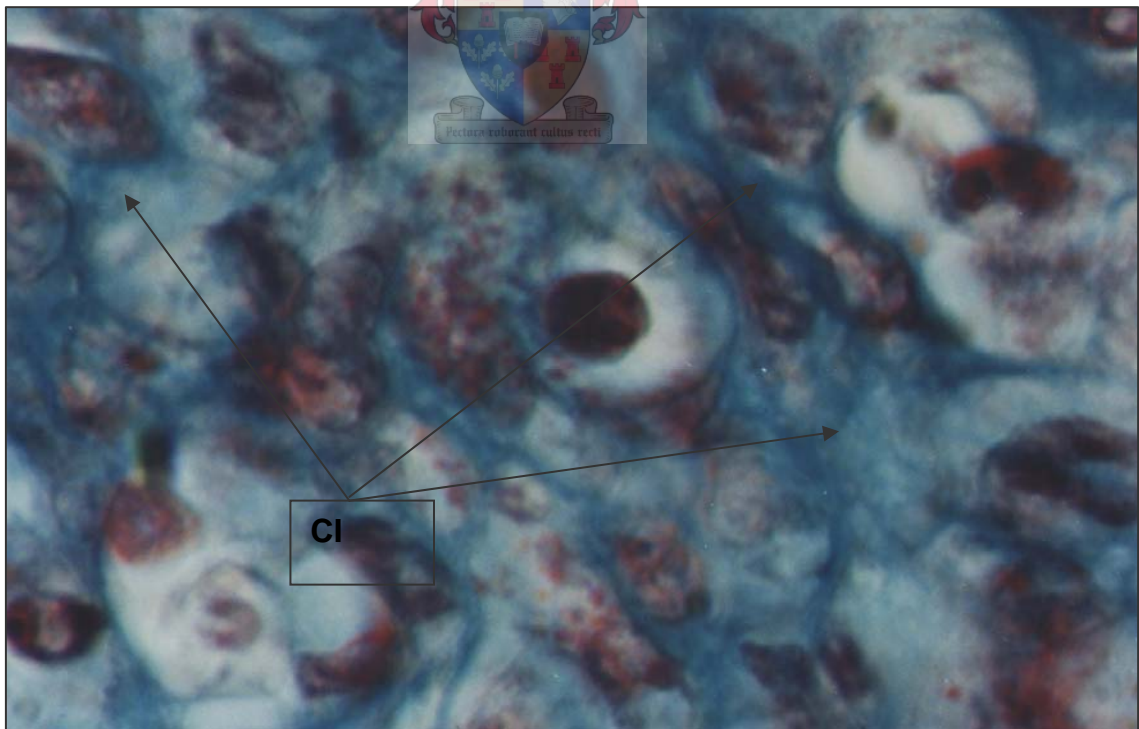
**Figure 53** Masson's Trichrome stained normal corpus luteum at x 800 magnification. There is very little connective tissue infiltration (CI) staining green with Masson's Trichrome stain. Clearly visible is the lipid accumulation (LA) staining orange



**Figure 54** H & E stained section of a regressed corpus luteum as seen on x 100 magnification. Note the clusters of connective tissue infiltration (CI) and discrete singular apoptotic bodies (DSAB)



**Figure 55** H & E stained section of a regressed corpus luteum as seen on x 400 magnification. Note the scattered discrete singular apoptotic bodies (DSAB) roughly round or oval in shape with rims of cytoplasm. On occasion, multiple apoptotic bodies with various amounts of nuclear material can be seen in a singular cell (MABSC)

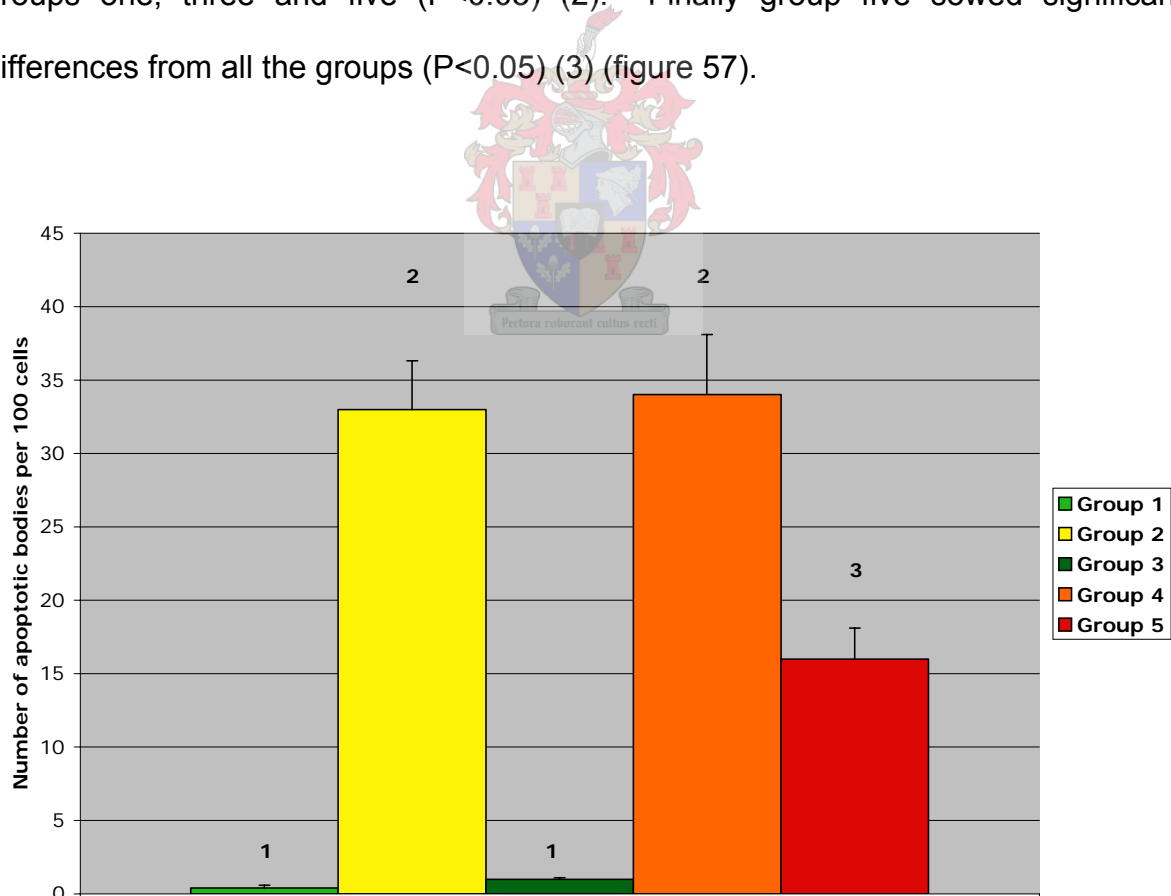


**Figure 56** Masson's Trichrome stained regressed corpus luteum at x 800 magnification. There is extensive connective tissue infiltration (CI) staining green with Masson's Trichrome stain

An attempt was made to compare the extent of luteal regression in the five groups by comparing the amount of apoptotic bodies per 100 cells and the amount of connective tissue infiltration on a categorical scale by assigned numerical values.

### 3.3.4.1 Evaluation of apoptotic nuclei

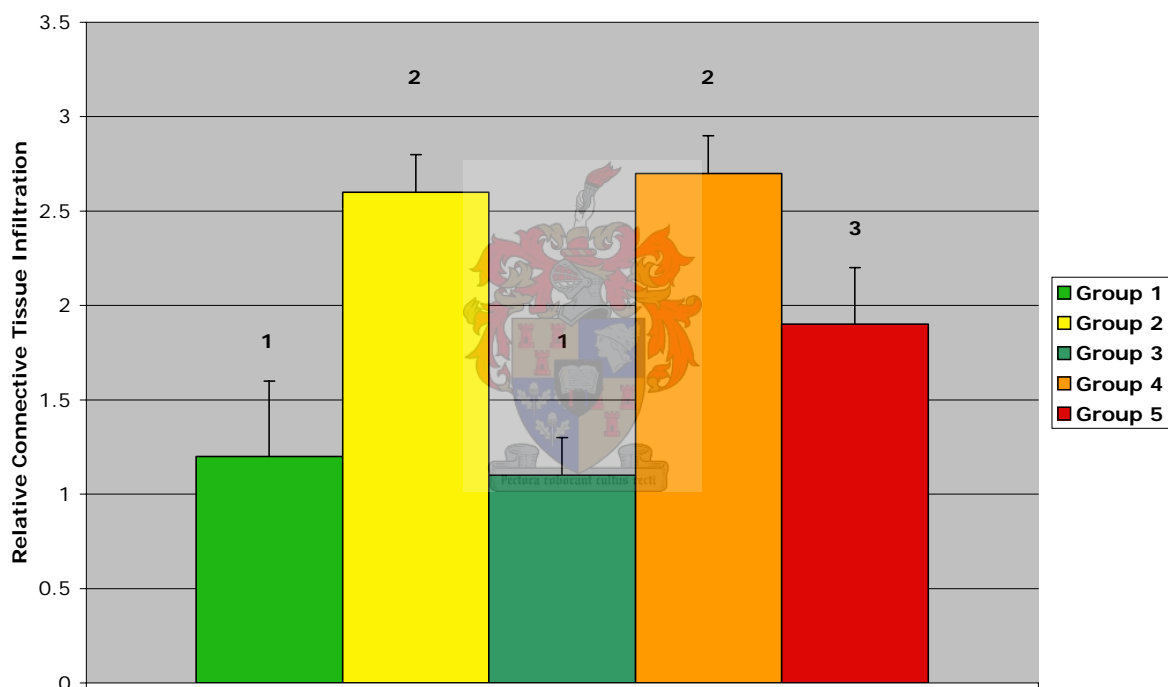
One-way analysis of variance revealed that the group means were statistically significantly different ( $P < 0.05$ ). The Tukey-Kramer adhoc test showed that groups one and three were not statistically significantly different ( $P > 0.05$ ) although they differed statistically from groups two, four and five ( $P < 0.05$ ) (1). Groups two and four did not differ statistically significantly ( $P > 0.05$ ) but they did differ significantly from groups one, three and five ( $P < 0.05$ ) (2). Finally group five showed significant differences from all the groups ( $P < 0.05$ ) (3) (figure 57).



**Figure 57** Number of apoptotic nuclei recorded per 100 cells between the five defined groups represented as means + SEM

### 3.3.4.2 Evaluation of connective tissue replacement

One-way analysis of variance revealed that the group means were significantly different ( $P < 0.05$ ). The Kruskal-Wallis Z adhoc test showed that groups one and three were not significantly different ( $P > 0.05$ ) although they differed from groups two, four and five ( $P < 0.05$ ) (1). Groups two and four did not differ significantly ( $P > 0.05$ ) but they did differ statistically significantly from groups one, three and five ( $P < 0.05$ ) (2). Finally group five differed statistically significantly from all the groups  $P < 0.05$  (3) (figure 58).

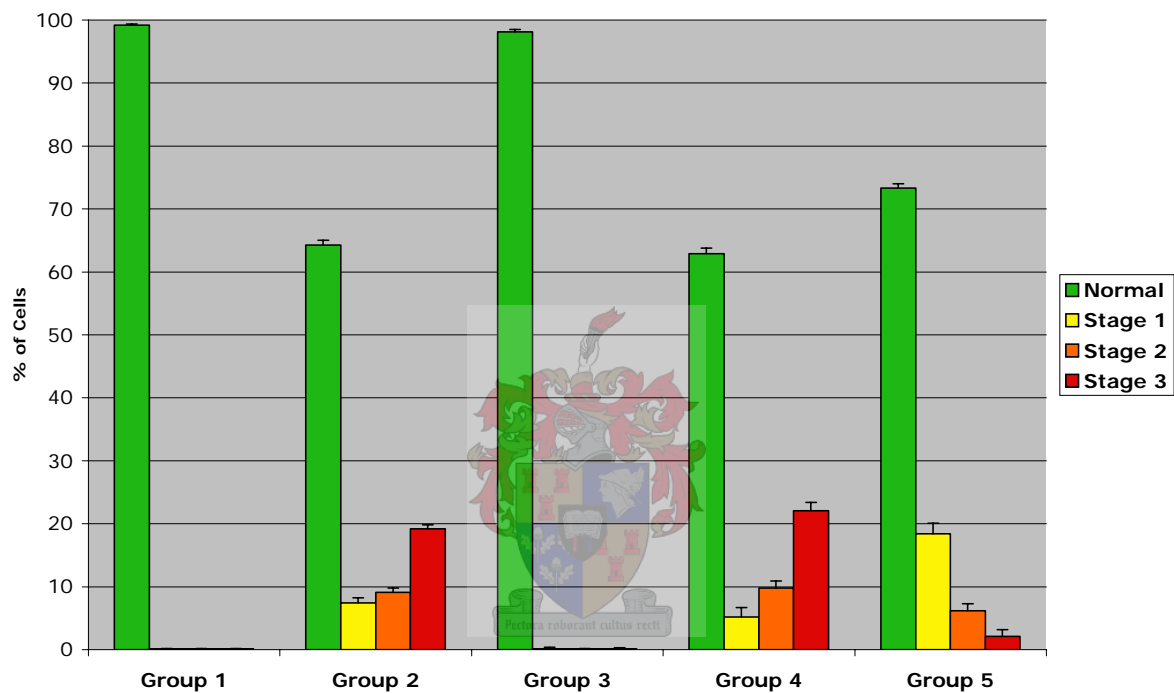


**Figure 58** Relative connective tissue infiltration recorded between the five defined groups represented as group means + SEM

### 3.3.5 Scanning Electron Microscopy

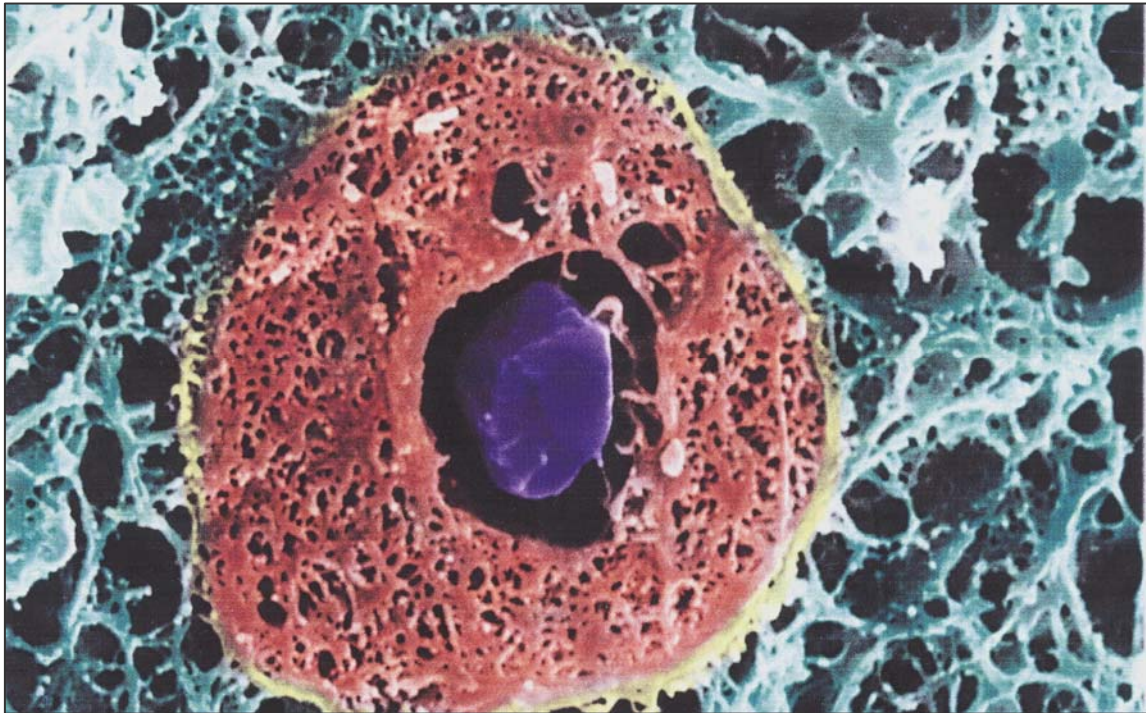
Scanning electron microscopy was used to establish the stage of apoptosis as described in section 3.2.5.2 in each of the five defined groups. Two-way analysis of variance revealed that there were significant differences in the stage of apoptosis between the five defined groups. The Tukey-Kramer adhoc test showed that groups

one and three were not significantly different ( $P>0.05$ ) although they differed from groups two, four and five ( $P<0.05$ ). Groups two and four did not differ significantly ( $P>0.05$ ) but they did differ statistically significantly from groups one, three and five ( $P<0.05$ ). Finally group five differed significantly from all the groups ( $P<0.05$ ) (figure 59).

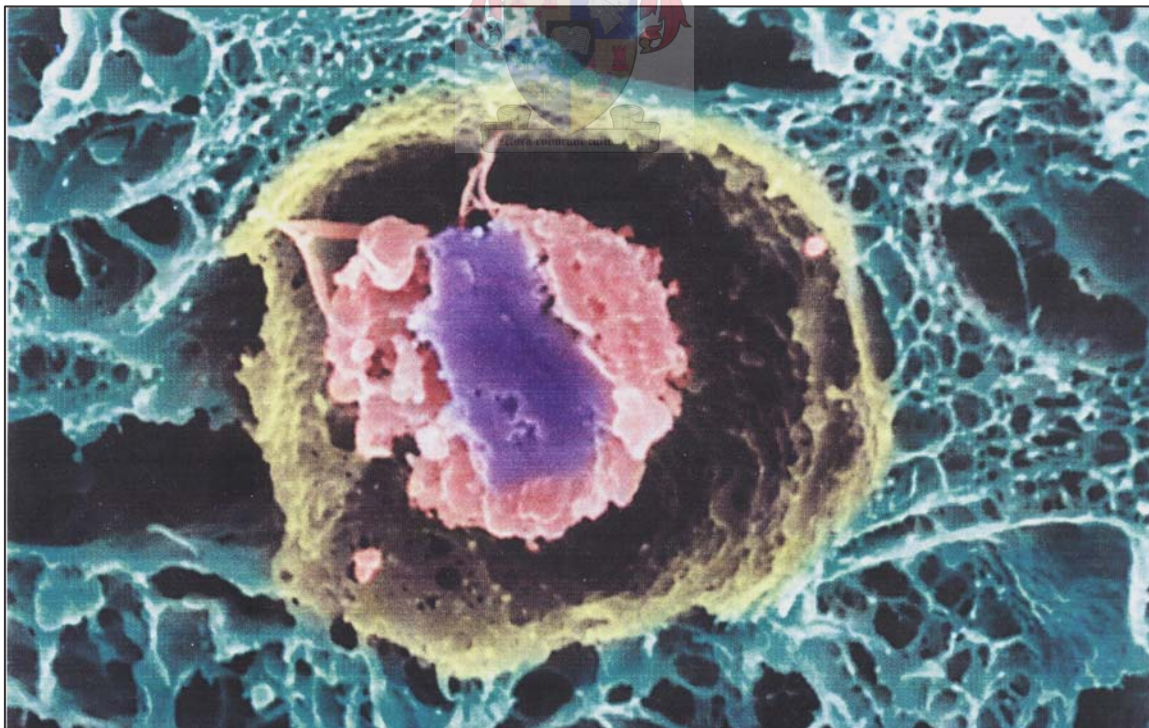


**Figure 59** Evaluation of the stage of apoptosis between the five defined groups represented as % of cells plus Scanning electron microscope





**Figure 60** Normal large lutein cell freeze fractured and magnification x 10 000 with a scanning electron microscope. Note the extensive development of the cyto-skeleton typical of steroid producing cells



**Figure 61** Apoptotic lutein cell freeze fractured and magnification x 10 000 with a SEM. Note that the cytoskeleton is almost completely broken down by the action of proteases like caspases.

### 3.4 Discussion

In the current study, season had no effect on the occurrence of premature luteal regression. The occurrence of both only premature regressed corpora lutea and mixed cases, where premature regressed corpora lutea occurred with normal appearing corpora lutea, were similar between the breeding and non-breeding seasons (see section 3.3.1). This finding differs from the observations made by Ryan *et al* [104] and Jabbour *et al* [105] in that the incidence of the phenomena is higher in the breeding season than in the non-breeding season.

It is known that during the non-breeding season (spring) with longer day lengths MT secretion decrease leading to up-regulation of  $rE_2-17\beta$  in  $E_2-17\beta$  sensitive neurons that inhibit the GnRH pulse generator. In the breeding season with short days increased MT secretion down-regulates  $rE_2-17\beta$  in  $E_2-17\beta$  sensitive neurons and the inhibition of the GnRH pulse generator does not take place. The theory is that in the non-breeding season, because of the inhibition of the GnRH pulse generator, the preovulatory LH surge is prevented, thus ovulation does not take place and the animals goes into anestrus (see section 2.9 for a review) [130-167]. It is known that the early corpus luteum needs LH to become functional [168]. The theca and granulosa cells need to differentiate into small and large lutein cells, respectively and extensive vascularisation needs to take place to make this possible. The differentiation of the granulosa and theca cells is in part LH dependant.

In granulosa cells and theca cells LH causes massive synthesis of  $P_4$  by alterations in cholesterol metabolism and steroidogenic enzymes (see section

2.6.1.2 for a review) [169-172]. A concurrent morphological change occurs in the mitochondria, where some of the steroidogenic enzymes are located. Mitochondria tend to exhibit an increasing complexity of their cristae, often with lammelliform-villiform cristae, after exposure to LH [173-178]. A clustering of mitochondria and other organelles such as lysosomes in the perinuclear region has been observed, which presumably facilitates movement of substrates for steroidogenesis [179, 180]. An increase in the amount of lipid inclusions has been described as well [173-178]. A number of plasma membrane receptors are modulated in response to LH. Prolactin,  $\beta$ -adrenergic and lipoprotein receptor is increased, whereas the LH receptor itself can be down-regulated [181-183]. The smooth endoplasmic reticulum (SER) develops and expand to the most conspicuous organelle in luteal cells in response to LH. It has been shown to occur in various forms, including tubular and vesicular networks. In the SER the enzymes  $3\beta$ -HSD,  $17\beta$ -HSD and Aromatase-P450 are modulated (see section 2.6.1.2 for a review). The effect of LH on the nucleus and proliferative activity is particular interesting. Before exposure to LH, granulosa cells exhibit a high nuclear-cytoplasmic ratio and proliferative activity[184, 185]. After exposure to LH, the mitotic index falls, and a precipitous increase in the cytoplasmic volume occurs [184-187]. The nuclear heterochromatin is transformed to a lighter staining euchromatin pattern [187, 188]. Finally there is rearrangement of cytoskeletal elements with microvilli forming and gap junctions decreasing by as much as 85% upon LH exposure [189-193].

The effect of season on premature luteal regression is interesting since it poses the question if the phenomenon is in essence really regression, or merely inadequate luteal support. It is known that ovulation rate greatly increase if the

release of endogenous gonadotropins is increased, or if exogenous gonadotropins are administered. Increased ovulation rate implies an increase in the amount of corpora lutea formed. Does this also imply increasing amounts of LH required for the early corpora lutea to differentiate into functional structures? The seasonal influences in the Mutton Merino is not as pronounced as in some other breeds of sheep, with oestrous cycles occurring throughout most of the year [135, 136]. The fact that the ewes ovulated in the non-breeding season and that there was no significant difference in the amount of corpora lutea recorded between the breeding and the non breeding seasons, implies that there is enough LH during the pre-ovulatory LH surge to induce ovulation in the developing follicles. It is possible that the seasonal inhibition of the GnRH pulse generator in the Mutton Merino is not enough to prevent the pre-ovulatory LH surge but leads to insufficient LH support after ovulation especially in the superovulated animal with more than usual corpora lutea to support early in the luteal phase. In the current study there was on average more corpora lutea recorded in animals with premature regressed corpora lutea (only premature regression and mixed responses) than in animals with normal functioning corpora lutea. This was observed in both the breeding and non-breeding season, but these differences were not statistically significant (see section 3.3.2). Were there too many structures for the extent of LH support in the animals where the phenomenon occurred.

Although this question cannot be directly answered because LH concentration was not measured, there is enough indirect clues to suggest the contrary. The first argument against the theory is that the incidence of premature luteal regression should be the highest in the non-breeding season when the active suppression of

the GnRH pulse generator by E<sub>2</sub>-17β sensitive inhibitory neurons should be at its strongest. The finding by Ryan *et al* [104] and Jabbour *et al* [105] that the incidence of premature regression increase in the breeding season and the observation in this study that there is no difference in the incidence between the breeding and non-breeding season, does not support this theory. Secondly, this study shows that in both the breeding and non-breeding season P<sub>4</sub> levels increase for the first four days in animals where the phenomena did occur.

The fact that there are conflicting reports on the incidence of the phenomenon in the breeding and non-breeding season raises the question if the seasonal influences observed is not really nutritional influences. It is known that usually pastures carry higher nutritional value in the non-breeding season than in the breeding season [134, 135]. It has long been recognized that nutrition has a profound effect on reproductive performance in female farm animals, although the underlying mechanisms remain poorly understood [135, 136, 194, 195]. An increase in the amount of protein digested post-ruminally [196] or increased availability of energy-yielding substrates, like carbohydrates [197], may be responsible for the higher fecundity observed. One proposed theory is that higher protein intake increases the availability of essential amino acids, the building blocks of protein hormones [198, 199]. For this theory to be valid, there has to be a deficiency in essential amino acids. In at least one study, it was proven that increased protein intake does not increase the amplitude of the LH surge [105]. A more popular theory is that increasing the intake of carbohydrates leads to increasing circulating insulin and IGF-1 [194, 195]. It is known that due to their structural similarity, insulin will bind to RIGF-1, but the receptor's affinity for insulin is not as high as for IGF-1 [200-

202] . The beneficial role of insulin and IGF-1 on follicular growth synergising with pituitary gonadotropins is well known [202-207]. These two metabolic hormones have also been shown to increase the number of ovulations in superovulated heifers [208]. Although an increase of progesterone production per cell basis, thus eliminating the effect of proliferation have been reported [202-205, 207] the effect of these hormones on luteal function remains poorly understood [209]

Perhaps the most compelling evidence that the seasonal influences observed by Ryan *et al* [104] and Jabbour *et al* [105] is in fact nutritional influences possibly acting by increasing insulin and IGF-1 is that Jabbour *et al* reported that they can reverse the effect by nutritional supplementation. It is known that under normal circumstances, the inhibitory effect of photoperiod will override the stimulatory effects of increased nutritional state in most breeds of sheep [198]. The current study added a couple of interesting insights since no seasonal effects were seen and nutritional supplementation of oats hay (*Avena sativa L.*) and lucerne (*Medicago sativa L. sativa*) did not reduce the incidence of the phenomenon. These findings support the idea that seasonal influences are merely nutritional influences, but which nutrients, and why those nutrients, need to be established. More research on the effect of nutrition on the incidence of premature luteal regression is needed.

By using the more sensitive MEIA technology, a second peak was observed in E<sub>2</sub>-17β around the 4<sup>th</sup> day of the cycle confirming previous reports [73, 109, 117-119]. This second increment was observed in both the breeding and non-breeding seasons and occurred in all the animals unlike reported by some authors [73, 117, 118]. The superovulated ewes had higher than normal E<sub>2</sub>-17β peaks around estrus

(12.0-14.8 pg/ml vs 6.2-10.0 pg/ml). The second E<sub>2</sub>-17β peak ranged between 5.4-8.2 pg/ml. An interesting observation was that the second E<sub>2</sub>-17β peak preceded the decline in P<sub>4</sub> in animals with signs of premature luteal regression. It is known that E<sub>2</sub>-17β increase the recombinant oxytocin, mediating the production and release of PGF<sub>2α</sub> by the uterus upon the binding of Ox. By increasing the amount of rOx the uterus becomes more sensitive to basal levels of Ox (see section 2.5). In this study, however, the second peak was observed in both animals with signs of premature luteal regression and animals with normal functioning corpora lutea. This raised the question, if these high E<sub>2</sub>-17β levels leads to the early release of PGF<sub>2α</sub>, why did this not happen in all the animals? The possibility that a certain threshold needs to be reached was considered. We investigated a potential correlation between the peak E<sub>2</sub>-17β level on the 6<sup>th</sup> day after sponge removal (4<sup>th</sup> day of the cycle) and the P<sub>4</sub> level on the 7<sup>th</sup> day, but failed to find any correlation, thus concluding that there is no threshold level of E<sub>2</sub>-17β that will initiate the early release of PGF<sub>2α</sub>. In an interesting study by McCracken [89] using the autotransplanted uterus in ovariectomized ewes, it was shown that E<sub>2</sub>-17β exposure can lead to PGF<sub>2α</sub> release after about 6 hours and that increasing peaks will occur every 6 hours (It takes approximately 6 hours for the oxytocin receptors to be manufactured) in the absence of P<sub>4</sub> exposure. However, in P<sub>4</sub> primed animals this effect will be prevented if animals were primed for 2 or 6 days while the effect will be enhanced after 10 days of P<sub>4</sub> exposure.

In the current experiment, animals were exposed to P<sub>4</sub> for 4 days. According to the mentioned experiments by McCracken [89], using the autotransplanted uterus as a model the E<sub>2</sub>-17β induced release of PGF<sub>2α</sub> should not take place. An

interesting observation was that in the animals where  $\text{PGF}_{2\alpha}$  was administered, the decline in  $\text{P}_4$  was slower than in animals displaying signs of premature luteal regression (see figures 45 & 46). Upon histological examination it was shown that there was significantly more apoptotic nuclei in premature regressed corpora lutea (see section 3.3.4.1) and connective tissue replacement (see section 3.3.4.2) than in corpora lutea where regression was induced by  $\text{PGF}_{2\alpha}$ . Scanning electron microscopy also demonstrated that premature regressed corpora lutea were in later stages of apoptosis than in  $\text{PGF}_{2\alpha}$  induced regression (see section 3.3.5). In several species the newly formed corpus luteum shows considerable resistance to the luteolytic effect of  $\text{PGF}_{2\alpha}$ , including sheep[210], horse[211], cow[212, 213], rat[214] and pig[215]. In the sheep, horse, cow and rat the corpus luteum is resistant to the luteolytic effects of  $\text{PGF}_{2\alpha}$  for the first 4 days after its formation while in the pig the corpus luteum is resistant to  $\text{PGF}_{2\alpha}$  for up to 12 – 14 days.

The effects of  $\text{PGF}_{2\alpha}$  on both functional and structural luteolysis have been described (see sections 2.6.1 & 2.6.2). The enzyme  $20\alpha$ -hydroxysteroid dehydrogenase ( $20\alpha$ -HSD) is considered a marker of functional luteolysis[216]. The predominantly cytosolic enzyme is NADP-dependent and catalyzes the reduction of progesterone to the inactive  $20\alpha$ -hydroxyprogesterone ( $20\alpha$ OHP). It was shown that the early corpus luteum of eCG treated immature rats,  $\text{PGF}_{2\alpha}$  can't up-regulate the expression of this enzyme in the first 4 days [217]. It was also shown that the block in the LH-induced c-AMP increase does not take place in the early corpus luteum of the rat [214]. In agreement with the mentioned studies slow functional and structural



regression in corpora lutea where regression was induced by  $\text{PGF}_{2\alpha}$  was shown. This was not the case in premature regressed corpora lutea.

Another interesting observation was that in premature luteal regression, normal appearing corpora lutea sometimes occurred with regressed corpora lutea on the same ovary. This was also observed by Bainbridge *et al* [218] in the superovulated red deer (*Cervus elaphus*). The occurrence of these mixed responses was never observed in animals where regression was induced by  $\text{PGF}_{2\alpha}$ . We confirmed that these regressed corpora lutea were from the same cycle and not merely corpora albicans from previous cycles by doing a histological examination. The regressed corpora lutea were in the same stage of regression than the corpora lutea of animals only displaying premature regressed corpora lutea. In the animals where there was mixed responses, there were also multiple corpora lutea in the same stage of regression. The animals we used in the experiment were only superovulated once. Thus premature luteal regression does not necessarily influence all the corpora lutea present on the ovary at the time. It is known that 2% [86] of the  $\text{PGF}_{2\alpha}$  produced in the uterus is transferred from the uterine vein to the ovarian artery by a counter current transfer mechanism (see figure 1). The branches of the ovarian artery will reach all the corpora lutea upon the completion of vascularization following ovulation and luteinization. The only possible explanations to the occurrence of mixed responses is a) that Prostaglandin  $\text{F}_{2\alpha}$  receptor ( $\text{r-PGF}_{2\alpha}$ ) is not present in all the corpora lutea, b) that vascularization is not complete in these corpora lutea or c) that  $\text{PGF}_{2\alpha}$  is not the stimulus that initiates premature luteal regression. If vascularization is incomplete the corpora lutea will be devoid of LH, Prolactin and other systemic trophic factors,  $\text{O}_2$  and nutrients. Therefore this option

is unlikely. The fact that the administration of  $\text{PGF}_{2\alpha}$  cause regression of all corpora lutea in ewes under the same experimental conditions, although at a slower rate than in premature luteal regression, suggests the presence of  $\text{r-PGF}_{2\alpha}$ . This poses an interesting question: Is  $\text{PGF}_{2\alpha}$  responsible for premature luteal regression?

The concept that  $\text{PGF}_{2\alpha}$  is the only signal indicating luteal regression in the normal estrous cycle, was challenged by Sugimoto *et al* [219] in perhaps the most groundbreaking paper on luteal function published in the 1990's. They created knockout mice with homozygous null mutations for  $\text{R-PGF}_{2\alpha}$ . Very surprisingly the mice had normal estrous cycles with luteal regression occurring in cycles where animals did not become pregnant. Ovulation and implantation were also normal in the  $\text{R-PGF}_{2\alpha}$  knockout mice. But in pregnant mice the animals did not give birth, because the timely regression of corpora lutea of pregnancy had been prevented, and this in turn prevented the up-regulation of  $\text{rOx}$ , thus preventing myometrial contractility. Ovariectomy released this inhibition of  $\text{rOx}$  and allowed myometrial contractility to resume. Thus, in mice at least,  $\text{PGF}_{2\alpha}$  is not the only signal initiating luteal regression. It is, therefore, possible that premature luteal regression in the superovulated Mutton Merino can be caused by a signal other than  $\text{PGF}_{2\alpha}$ .

### 3.5 Conclusions

The following conclusions were made:

1. Premature luteal regression is not merely inadequate luteal support, but indeed early luteal regression, since seasonal influences could merely be nutritional influences, and a definitive increase in  $\text{P}_4$  were recorded in animals exhibiting the phenomena.

2. Nutritional influences could play a role, but the type and quality of nutrients and mechanism involved, is still unclear.
3.  $\text{PGF}_{2\alpha}$ -induced regression differs from premature luteal regression in that:
  - a) The progression of functional and structural regression in  $\text{PGF}_{2\alpha}$  - induced regression is slower than in premature luteal regression.
  - b) Regressed corpora lutea do not occur with normal functioning corpora lutea.
4. There is a distinct second  $\text{E}_2$ - $17\beta$  peak preceding the decline in  $\text{P}_4$  in animals that exhibits signs of premature luteal regression.
5. A threshold initiating premature luteal regression was not established.



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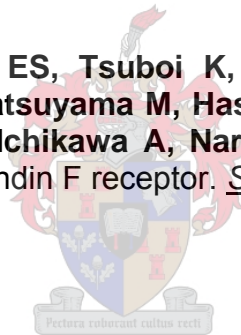


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## Chapter 4

# A Potential Role for Estrogen Receptors in Premature Luteal Regression

### 4.1 Introduction

Administration of exogenous  $E_2-17\beta$  in metestrus (day 3-5 of the estrous cycle) causes luteal regression in sheep [1] and some wild deer species [2]. At the end of the normal estrous cycle, it is known that  $E_2-17\beta$ , upon binding to its nuclear receptor, leads to transcription of the gene encoding the ROx, which in turn results in up-regulation in the membrane receptor making endometrial cells more responsive to basal levels of Ox. If Ox binds to its membrane receptor, cyclase is activated and intracellular c-AMP increases, resulting in activation of Phospholipase  $A_2$ , Prostaglandin endoperoxide synthase with cyclooxygenase and peroxidase components and endoperoxide reductase represent the machinery necessary for the production of  $PGF_{2\alpha}$ .  $PGF_{2\alpha}$  is never stored and is released within minutes upon production.

In the corpus luteum,  $PGF_{2\alpha}$  cause a influx of  $Ca^{++}$  into the cytoplasm [3, 4] leading to activation of phosphokinase C [5] and decreasing progesterone syntheses [6]. Other effects of  $PGF_{2\alpha}$  exposure includes uncoupling of the LH receptor from adenylate cyclase [7] thus decreasing the LH induced increase in c-AMP levels. This is associated with increased production of progesterone seen

in luteinized granulosa cells by modulating metabolic and steroidogenic enzymes [8]. Finally a rapid decrease in luteal blood flow [9, 10] leading to increased formation in oxygen-derived free radicals [11] have been shown to induce apoptosis in a variety of cell types [12, 13]. It is generally accepted that these events initiates luteal regression at the end of the estrous cycle in the sheep if a viable embryo doesn't implant in the uterus. Therefore it seems logic that the luteolytic action of  $E_2-17\beta$  in metestrus is caused by the described mechanism.

It is known that induction of superovulating in sheep by administration of eCG, also increases circulation levels of  $E_2-17\beta$  [14]. One theory to explain the phenomenon of premature luteal regression is that increased  $E_2-17\beta$  levels leads to the early release of  $PGF_{2\alpha}$ . But resistance to the luteolytic actions of  $PGF_{2\alpha}$  in the early corpora have been described in several species[15-19],including the sheep [20]. In a previous study (see Chapter 3) we showed that premature luteal regression differs from  $PGF_{2\alpha}$  -induced regression in that both functional and structural regression in  $PGF_{2\alpha}$  induced regression in the early corpus luteum is slower than premature luteal regression. Secondly, it was found that in premature luteal regression, regressed corpora lutea sometimes occurs with normal functioning corpora lutea while this was never the case in  $PGF_{2\alpha}$  induced regression. Sugimoto *et al* [21] showed in r- $PGF_{2\alpha}$  knockout mice, that mice had normal estrous cycles with luteal regression occurring if a viable embryo didn't implant despite the absence of r- $PGF_{2\alpha}$ . This challenged the notion that  $PGF_{2\alpha}$  is the only signal to induce luteal regression. In the mentioned study (see chapter

3) it was observed that there is a definitive second peak in  $E_2$ -17 $\beta$  peaking on the 4<sup>th</sup> day of the estrous cycle preceding the decline in progesterone observed in premature luteal regression. There was, however, no correlation between the  $E_2$ -17 $\beta$  level on the 4<sup>th</sup> day of the cycle and the progesterone outcome the following day, dismissing the notion that there is a threshold of  $E_2$ -17 $\beta$  that needs to be reached before premature luteal regression will occur.

A common misconception is that only hormone levels follows a pattern, increasing and decreasing during the estrous cycle and that cells will always be receptive to their actions. In fact, hormone receptors are highly dynamic and are up- and down-regulated by various stimuli.  $E_2$ -17 $\beta$  acts via specific nuclear receptors that in turn regulate transcription of hormone-responsive target genes. In the case of  $E_2$ -17 $\beta$ , two receptors have been identified, namely estrogen receptor alpha ( $ER\alpha$ ) and estrogen receptor beta ( $ER\beta$ ). In the sheep ovary  $ER\beta$  is the predominant, or the only estrogen receptor expressed in granulosa cells [22]. The present study investigate the possibility that  $E_2$ -17 $\beta$  rather than  $PGF_{2\alpha}$ , initiates premature luteal regression by acting directly on luteal cells via the  $ER\beta$  and that the presence of  $ER\beta$  will determine the fate of the early corpus luteum on the 4<sup>th</sup> day of the cycle when the  $E_2$ -17 $\beta$  peaks for a second time.

## **4.2 Materials & methods**

### **4.2.1 Experimental animals and experimental design**

Refer to section 3.2.1 and 3.2.2

### **4.2.2 Progesterone & estrogen analysis**

#### **4.2.2.1 Blood collection**

Refer to section 3.2.3.1

#### **4.2.2.2 Progesterone**

The concentration of progesterone in serum were measured by MEIA on a Abbot AxSYM™ described by Buster and Abraham[23]. The sensitivity of the assay was 0.20 ng/ml. The inter- and intra-assay coefficients of variation (C.V.) were 4.82 % and 2.22 % respectively and the recovery was 99.8%.

#### **4.2.2.3 Estrogen**

The concentration of estrogen in serum were measured by MEIA on a Abbot AxSYM™ described by Buster and Abraham[23]. The sensitivity of the assay was 1.5 pg/ml. The inter- and intra-assay coefficients of variation (C.V.) were 6.88% and 4.54% respectively and the recovery was 99.9%.

See section 3.2.3 for more detailed information.



### 4.2.3 Immunohistochemistry

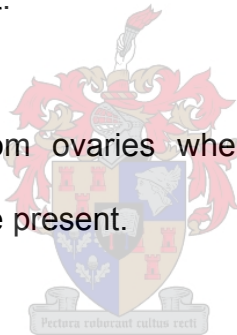
The ovaries of all the ewes were removed and macroscopically inspected 9 days after sponge removal when the ewes were slaughtered. Taking into consideration macroscopic appearance and treatment, five groups were identified.

Group 1 Corpora lutea from ovaries where only normal appearing corpora lutea were present.

Group 2 Corpora lutea from ovaries where only prematurely regressed corpora lutea were present.

Group 3 Normal appearing corpora lutea from ovaries where regressed corpora lutea occurred with normal appearing corpora lutea (Mixed normal)

Group 4 Regressed corpora lutea from ovaries where regressed corpora lutea occurred with normal appearing corpora lutea (Mixed regressed)



Group 5      Corpora lutea from ovaries where regression was induced with the PGF<sub>2α</sub> analogue Dinoprost tromethamine (Lutalyse ®, Pharmacia & Upjohn).

Six corpora lutea from each of the five defined groups were randomly selected using random numbers generated. The corpora lutea were removed from the ovary with a scalpel blade and fixed in formal-saline for 24 hours. The formal-saline fixed tissue were processed for histology (see section 3.2.4.1) A standard immunohistochemical technique (avidin-biotin-peroxidase) was used to visualize ERβ in the sheep corpora lutea as described previously[22]. Briefly a polyclonal rabbit anti-rat ERβ antibody (PA1-310; Affinity Bioreagents, Golden, CO), raised against a C-terminal synthetic peptide which corresponds to the C-terminal amino acid residues 530-549, was used for detection of oERβ. Antigen retrieval was achieved by pre-treatment in a microwave oven at high power, in 0.01 M sodium citrate buffer (pH 6.0) for 10 min, and then allowed to cool for a further 20 minutes. After washing in buffer (0.1 M PBS, pH 7.4) non-specific endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide (Merck) in methanol for 10 minutes at room temperature. After a 10 minute wash in buffer, samples were exposed to a 30 minute non-immunoblock using diluted normal goat serum (Santa Cruz Biotechnology, Santa Cruz, CA) in TBS containing 5% (w:v) BSA in a humidified chamber at room temperature. The samples were then incubated with the primary antibody. The ERβ antibody was diluted 1:200 in TBS with 5% BSA and incubated at 4°C overnight. Negative

controls were obtained by replacing the primary antibody with non-immunoserum of equivalent concentration. In addition, the ER $\beta$  antibody was preabsorbed with neutralizing synthetic ER $\beta$  peptide (PEP-007, Affinity Bioreagents) overnight to demonstrate antigen specificity. After primary antibody binding, the samples were incubated for 30 minutes at room temperature with a biotinylated goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology) diluted in normal goat serum. Thereafter, samples were incubated for 45 minutes at room temperature with a horseradish peroxidase-avidin-biotin complex (BD Biosciences Pharmingen). The site of the bound enzyme was visualized by the application of 3,3'-diaminobenzidine in H<sub>2</sub>O<sub>2</sub> (DAB kit BD Biosciences Pharmingen), a chromogen that produces a brown, insoluble precipitate when incubated with enzyme. The samples were counterstained with hematoxylin and dehydrated before they were mounted with Pertex. For each slide, 100 cells were scored as either positive or negative. Each slide was scored by three independent technologists and the mean score for each slide was calculated. Six slides in each of five groups were scored.

#### **4.2.4 Statistical analysis**

##### **4.2.4.1 Progesterone & estrogen analysis**

The progesterone and estrogen levels were compared separately. The comparison was done by three-way analysis of variance (2 x 4 x 10 matrix). It was done for both the breeding and non-breeding seasons between:

- a) animals with only normal corpora lutea,
- b) animals with only premature regressed corpora lutea,
- c) animals with PGF<sub>2α</sub> induced regression and
- d) animals with both normal and regressed corpora lutea.

The analysis also extended for 10 days from the day of sponge removal (day 0) to 9 days after sponge removal. The test was done on NCSS 2000 software with a P value of ( $P < 0.05$ ) considered significant.

#### **4.2.4.2 Immunohistochemistry**

The five group means of the percentage ER $\beta$  positive cells recorded per 100 evaluated cells were compared by means of one-way analysis of variance with a p-value of  $< 0.05$  considered as statistically significant. Once it was established that the group means do differ significantly the Tukey-Kramer adhoc test were performed to examine all possible comparisons among the five means. The analysis was done on NCSS 2000 software.

### **4.3 Results**

#### **4.3.1 Steroidogenic Profiles**

##### *4.3.1.1 Progesterone profiles*

The progesterone profiles of the ewes superovulated in the breeding season were not significantly different from the progesterone profiles of the animals

superovulated in the non-breeding season ( $P>0.05$ ). From the day of sponge removal up to 6 days after sponge removal, there was no significant difference in progesterone profiles between:

- a) animals with only normal appearing corpora lutea,
- b) animals with only premature regressed corpora lutea,
- c) animals with  $\text{PGF}_{2\alpha}$  induced regression and
- d) animals with both normal and regressed corpora lutea.

On the 7<sup>th</sup>, 8<sup>th</sup> and 9<sup>th</sup> day after the sponges were removed, there was a significant difference between the four mentioned groups of animals ( $p<0.01$ ). The progesterone levels of the animals with only normal appearing corpora lutea were still increasing in the normal fashion. The progesterone levels of the other three groups all showed a decline in progesterone concentration. The decrease in progesterone concentration in the animals with only premature regressed corpora lutea was more profound than in the animals with  $\text{PGF}_{2\alpha}$  induced regression. In animals with premature regressed corpora lutea progesterone levels dropped to baseline (1 ng/ml) within 24 hours while it took 72 hours in the ewes where regression was induced with the  $\text{PGF}_{2\alpha}$  analogue (dinoprost promethamine) to do so ( $P>0.05$ ). In the cases where regressed corpora lutea occurred with normal appearing corpora lutea, there were an initial drop in progesterone after which the progesterone level was maintained at a lower concentration than the ewes with only normal appearing corpora lutea ( $p<0.01$ ).

These trends were the same in the breeding and non-breeding season (see figures 63-70).

#### 4.3.1.2 *Estrogen profiles*

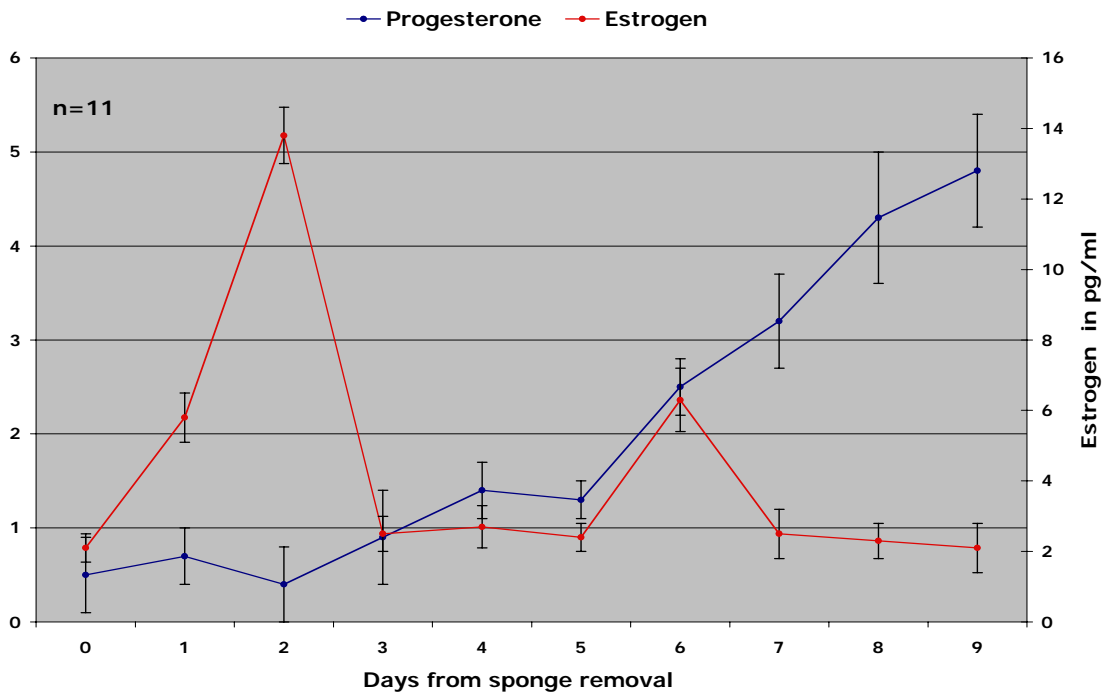
The estrogen profiles of the ewes superovulated in the breeding season were not significantly different from the estrogen profiles of the animals superovulated in the non-breeding season ( $p>0.05$ ). From the day of sponge removal up to the 8<sup>th</sup> day after sponge removal there were no significant differences in the estrogen profiles between:

- a) animals with only normal appearing corpora lutea,
- b) animals with only premature regressed corpora lutea,
- c) animals with PGF<sub>2 $\alpha$</sub>  induced regression and
- d) animals with both normal and regressed corpora lutea.

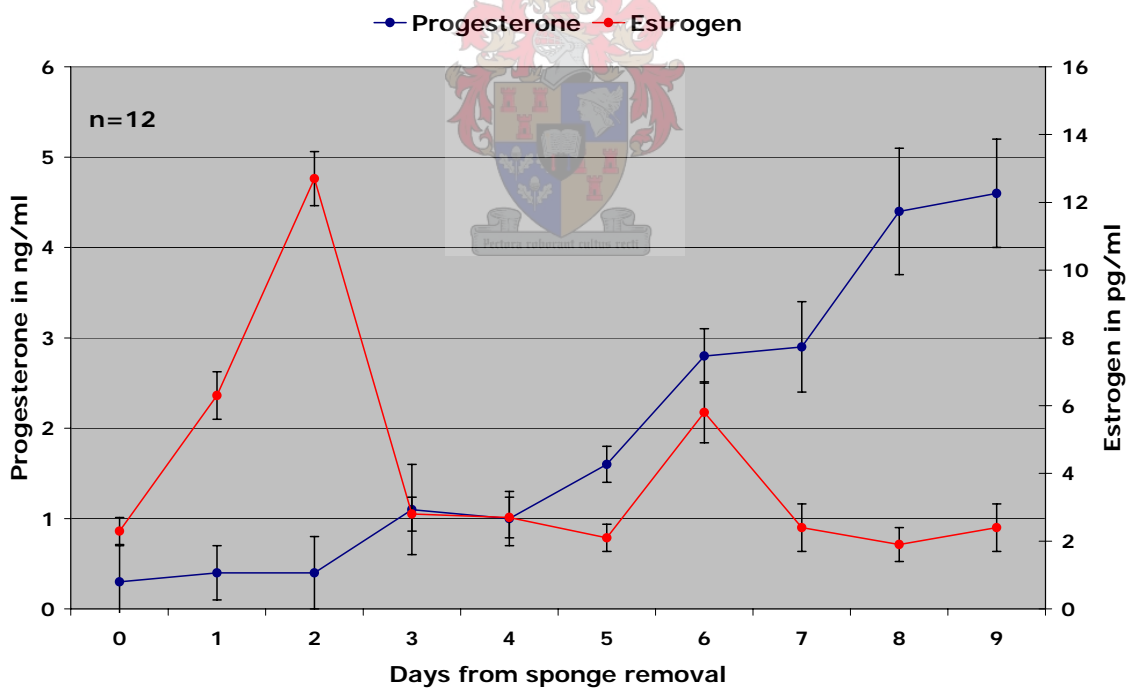
On the 9<sup>th</sup> day after the sponges were removed there were a significant difference between the mentioned groups of animals ( $P<0.01$ ). The estrogen levels of the animals with only premature regressed corpora lutea started to rise while the estrogen levels of the other groups were still at baseline. In all the ewes tested from all four groups in both the breeding and non-breeding season the first principal rise peaked on the 2<sup>nd</sup> day after sponge removal ( $\pm$ day 0 of the oestrous cycle, because ewes usually come into oestrous 24 to 48 hours after sponge removal). The peak estrogen levels ranged from 12.0 – 14.8 pg/ml higher than

those observed in untreated ewes with peak levels ranging from 6.2 – 10.0 pg/ml. On the 3<sup>rd</sup> day after sponge removal estrogen levels dropped to baseline and remained at baseline until the 5<sup>th</sup> day after sponge removal. On the 6<sup>th</sup> day after sponge removal ( $\pm$  4<sup>th</sup> day of the oestrous cycle) there was a significant additional rise in estrogen ( $P < 0.01$ ) confirming findings in some other studies[24-28]. Peak estrogen levels ranged from 5.4 – 8.2 pg/ml in this additional rise. On the 7<sup>th</sup> day after sponge removal, estradiol returned to baseline (figures 62-69).



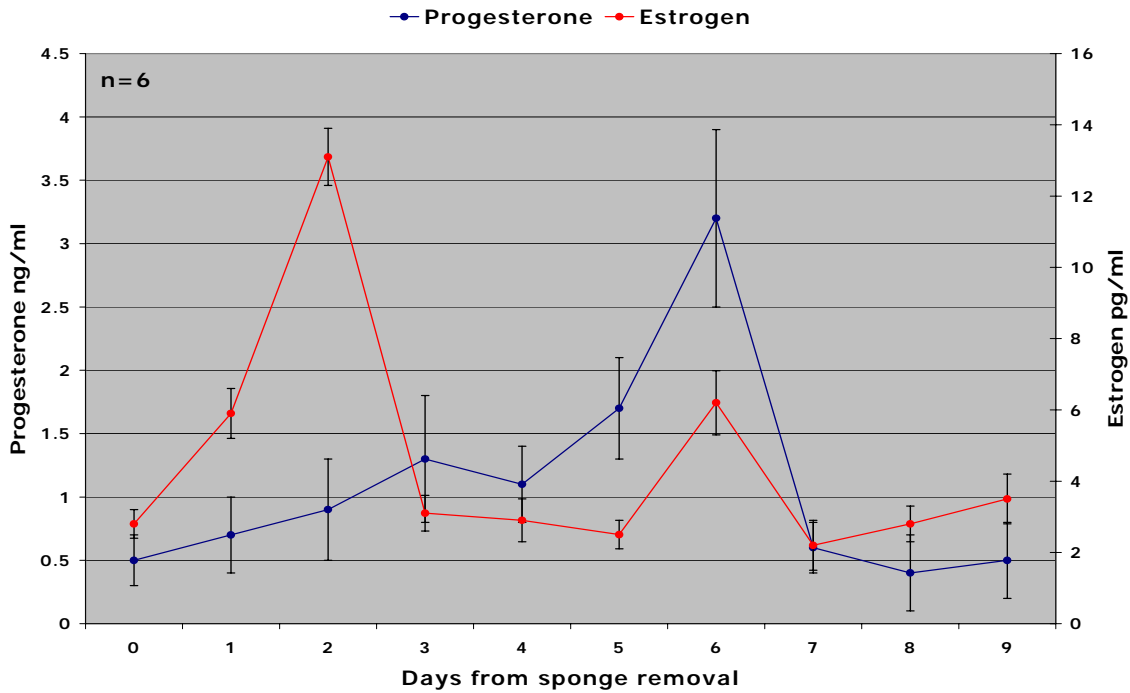


**Figure 62** Progesterone and estrogen profiles of superovulated Mutton Merino ewes with normal appearing corpora lutea. The ewes were superovulated in the breeding season (March – May 1996)

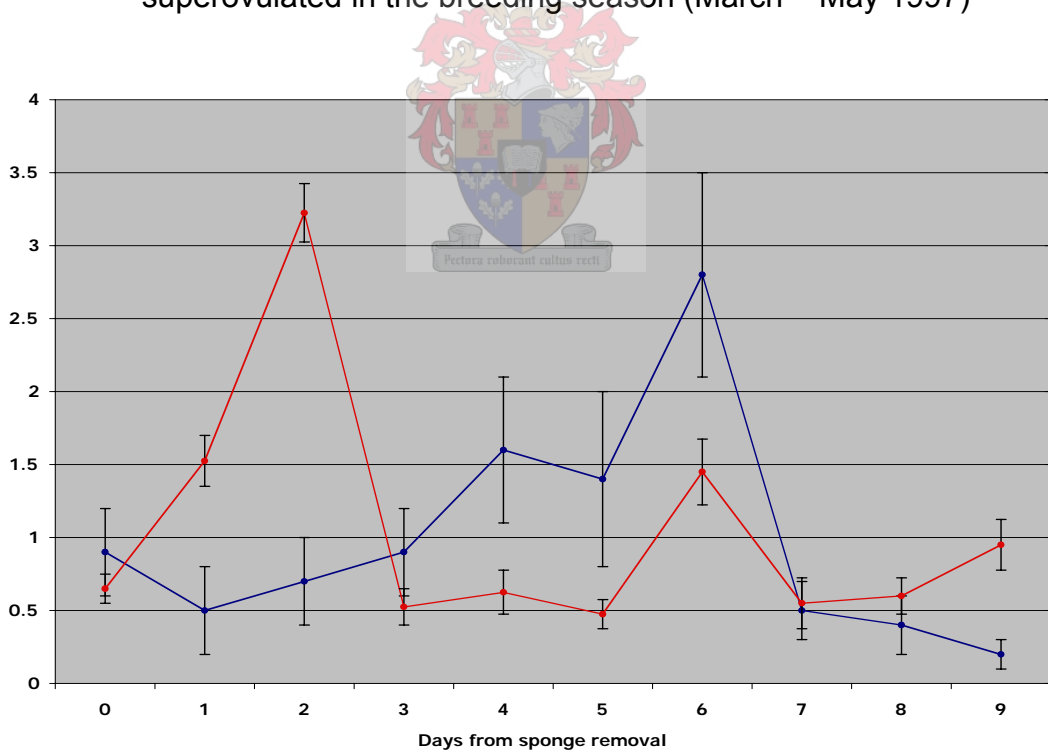


**Figure 63** Progesterone and estrogen profiles of superovulated Mutton Merino ewes with normal appearing corpora lutea. The ewes were superovulated in the non-breeding season (August - October 1997)

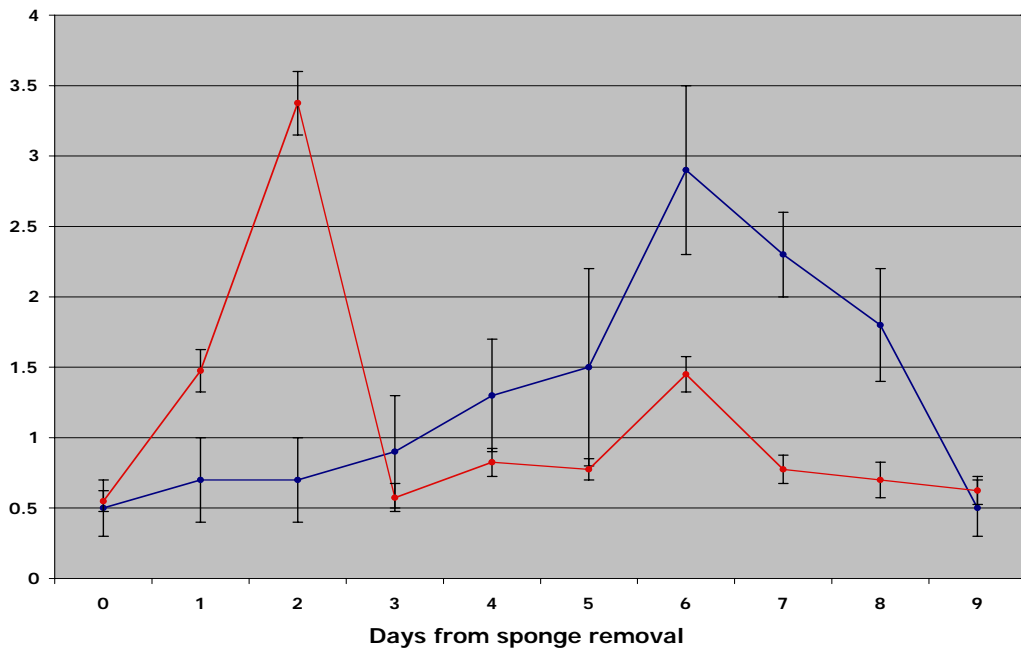




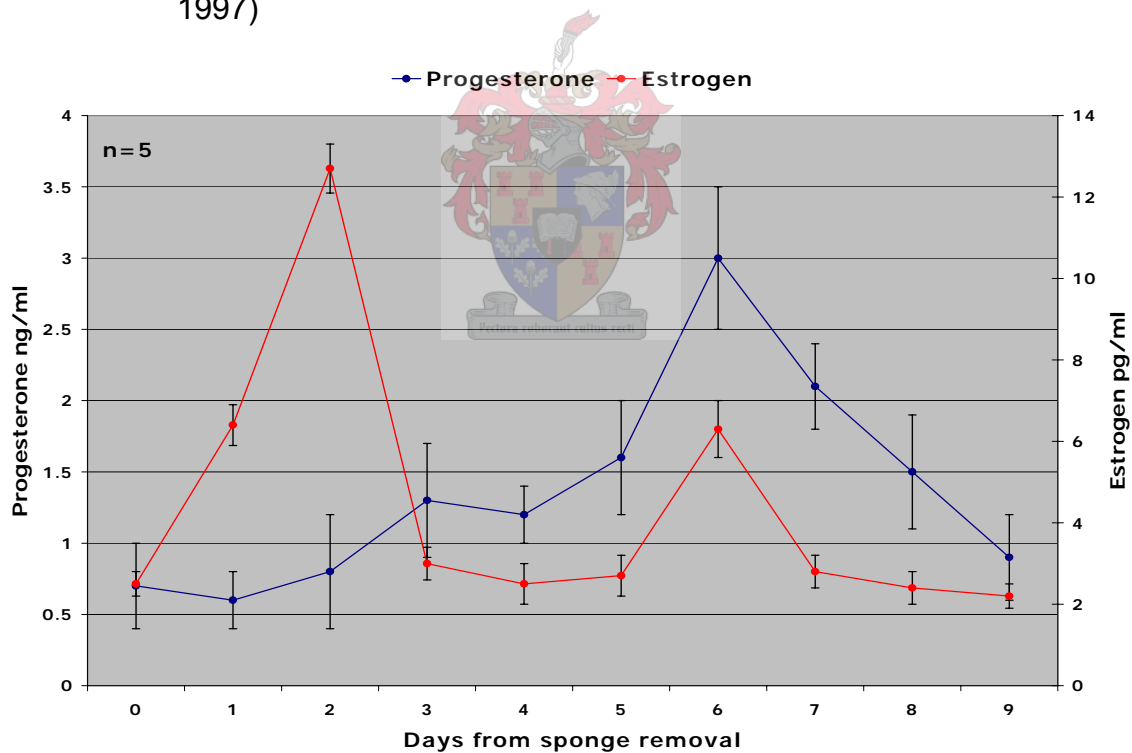
**Figure 64** Progesterone and estrogen profiles of superovulated Mutton Merino ewes with premature regressed corpora lutea. The ewes were superovulated in the breeding season (March – May 1997)



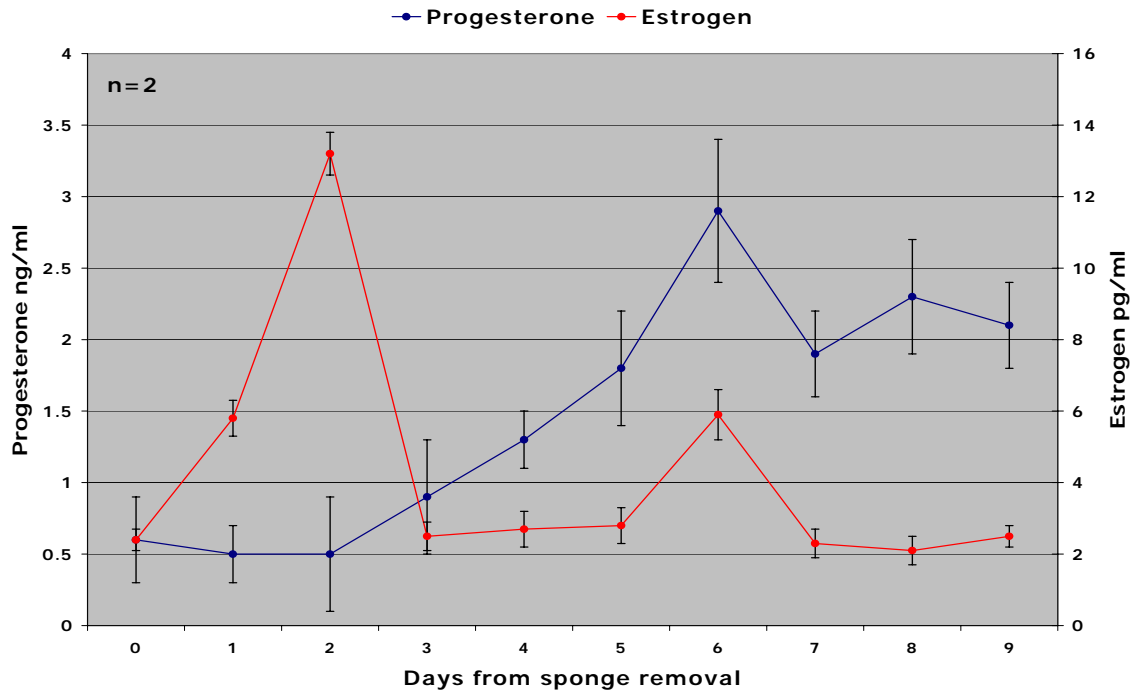
**Figure 65** Progesterone and estrogen profiles of superovulated Mutton Merino ewes with premature regressed corpora lutea. The ewes were superovulated in the non-breeding season (August - October 1997)



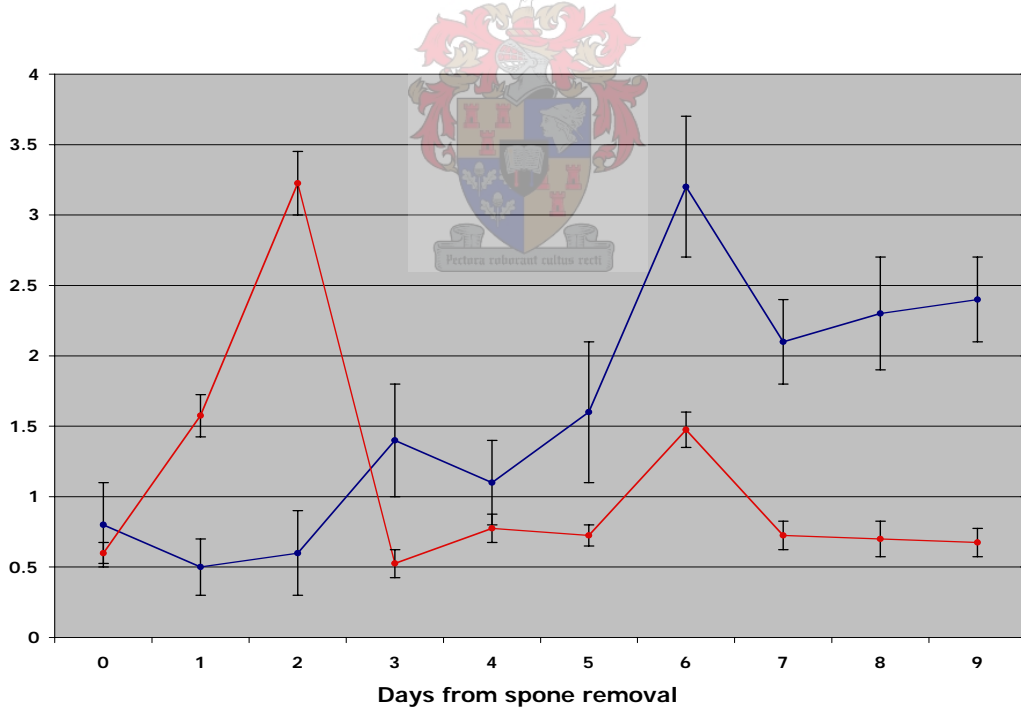
**Figure 66** Progesterone and estrogen profiles of superovulated Mutton Merino ewes with  $\text{PGF}_{2\alpha}$  induced regression of the corpora lutea. The ewes were superovulated in the breeding season (March – May 1997)



**Figure 67** Progesterone and estrogen profiles of superovulated Mutton Merino ewes with  $\text{PGF}_{2\alpha}$  induced regression of the corpora lutea. The ewes were superovulated in the non-breeding season (August – October 1997)



**Figure 68** Progesterone and estrogen profiles of superovulated Mutton Merino ewes with mixed responses. The ewes were superovulated in the breeding season (March – May 1997)



**Figure 69** Progesterone and estrogen profiles of superovulated Mutton Merino ewes with mixed responses. The ewes were superovulated in the non-breeding season (August - October 1997)

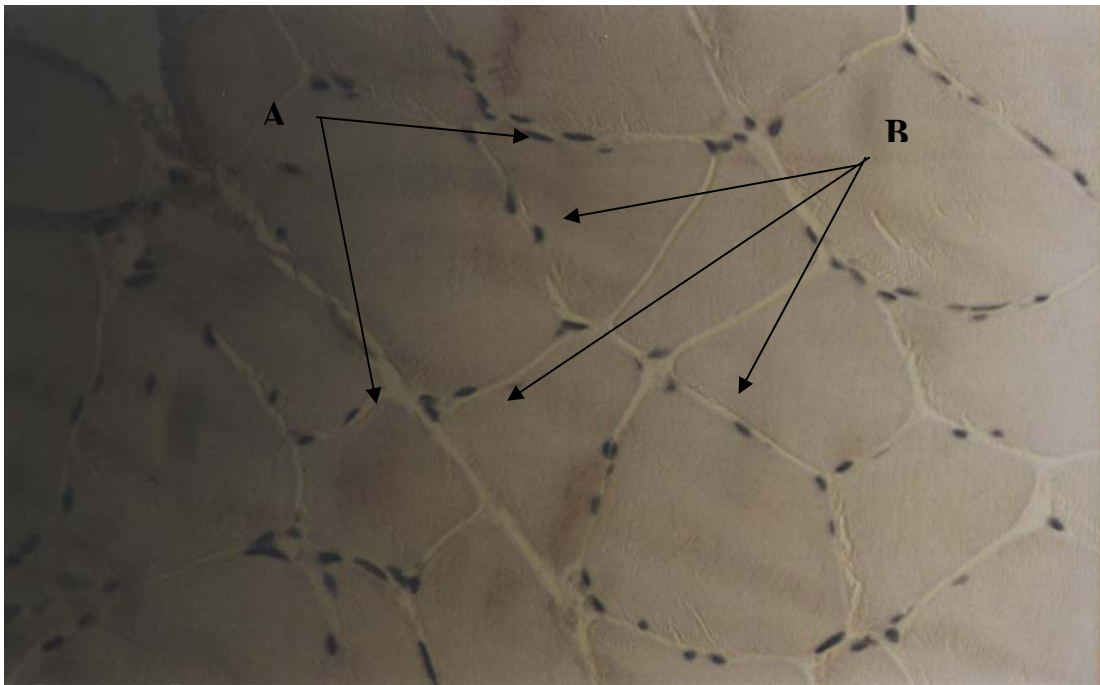
### 4.3.2 Immunohistochemistry

The polyclonal rabbit antibody directed against a 19 amino acid C-terminal synthetic peptide of the rat ER $\beta$  (**CSSTEDSKNKESSQNLQSQ**) with 9 residues (47%) identical to the equivalent ovine sequence (RNLA**EDSESKEGSQKPQAQ**) proved to be effective in localising the full length 527 amino acid oER $\beta$  (the red letters indicate common residues). The full-length rat ER $\beta$  is 87.3% homologous with the oER $\beta$  while the region homology varies between 83% and 99% (table 31) [22].

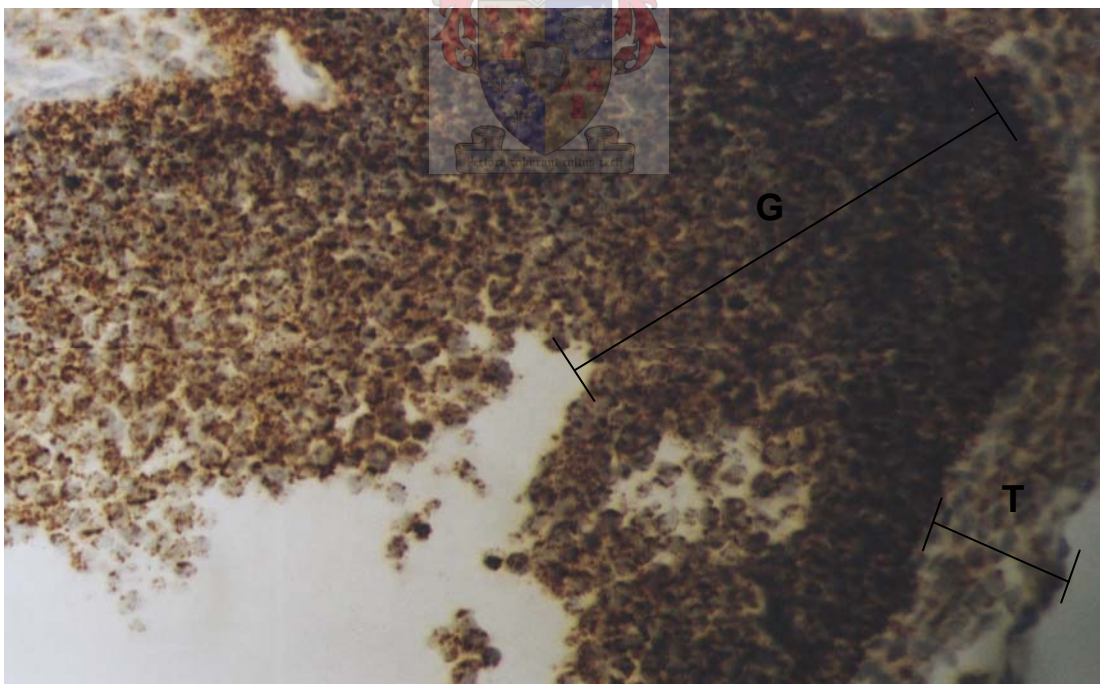
**Table 31** Percent homology between the rER $\beta$  and the oER $\beta$  per region [22]

	A/B (1-145)	C (146-211)	D (212-245)	E/F (246-527)
oER $\beta$	100	100	100	100
rER $\beta$	83	99	88	87

Skeletal muscle from the *musculus biceps brachii* in Mutton Merino sheep stained negative (figure 70) and in the antral follicle, granulosa cells stained strong positive and theca interna weak, positive (figure 71) as described by Cárdenas *et al* [22].

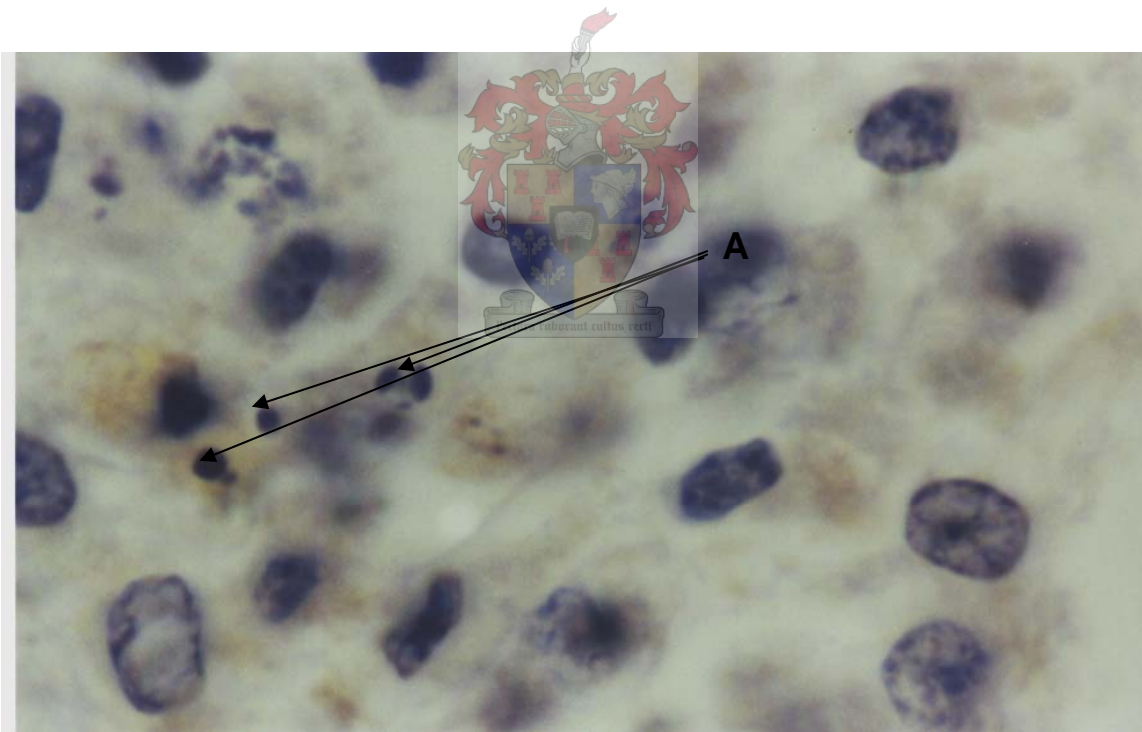


**Figure 70** A Cross section of skeletal muscle cells from the *Musculus Biceps Brachii* of the Mutton Merino stained immunohistochemically and counterstained with hematoxylin. LM x 800 magnification As can be seen there is no brown insoluble precipitate confirming the absence of ER $\beta$  in the skeletal muscle cell. A Note the multiple elongated peripheral nuclei typical of skeletal muscle. B Cross section of muscles cells. Skeletal muscle served as a negative control

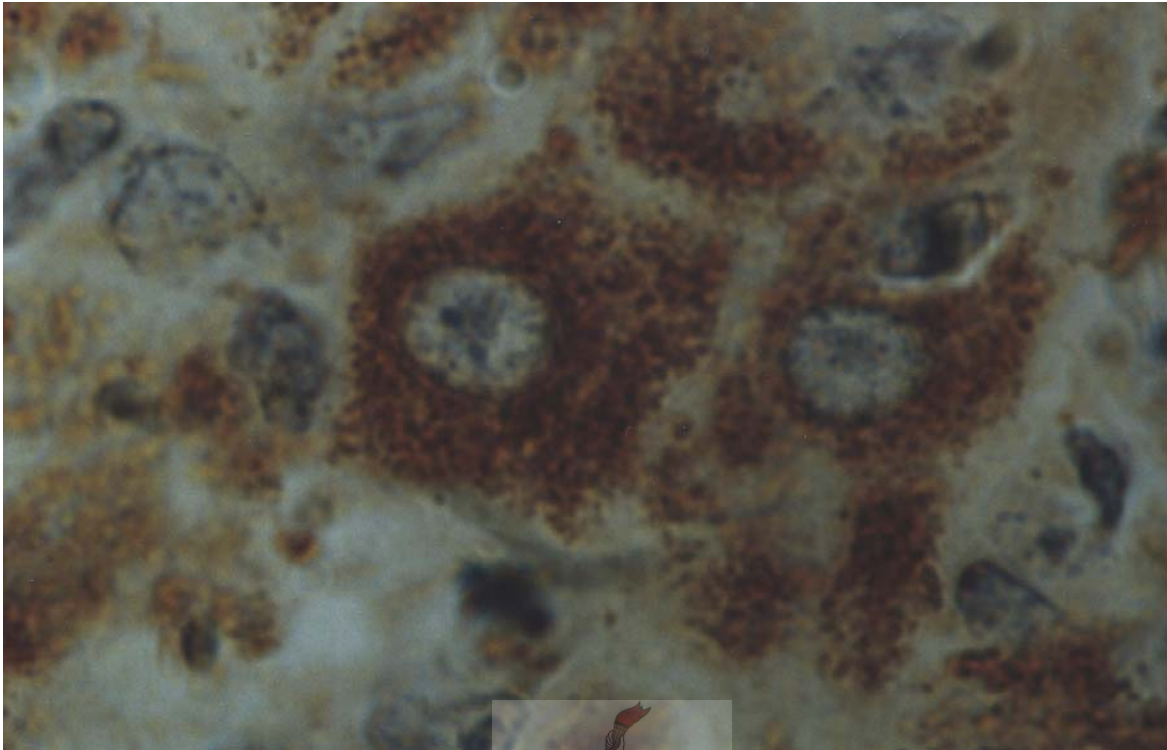


**Figure 71** A Cross section from an antral follicle in the mutton merino. LM x 800 magnification The granulosa cells G stain strong positive while the theca interna T stain weak positive. The antral follicle served as a positive control

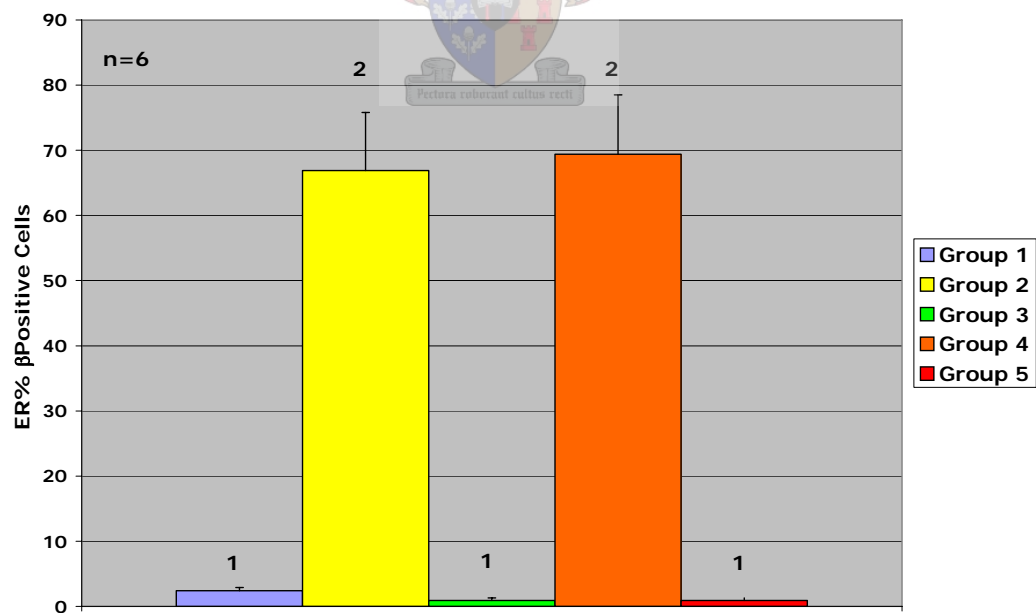
A section from a premature regressed corpus luteum stained by using a non specific polyclonal rabbit antibody stained negative proving that there is no non-specific binding (figure 72) while a section from a normal corpus luteum pre-adsorbed with the control peptide stained positive (figure 75) showing antigen specificity. Note that only the cytoplasm stained with the pre-adsorbed section. Appel *et al* [29] reported that on average, 6 amino acids constitute antigenic determinants in peptides. There is evidence that the 9 common residues between the amino acid sequence of the rat ER $\beta$  and the oER $\beta$  account for the antigenic determinants.



**Figure 72** Premature regressed corpus luteum stained by a non-specific polyclonal rabbit antibody staining negative proving there is no non-specific binding present. LM x 800 magnification Note apoptotic nuclei A present.



**Figure 73** Normal corpus luteum pre-adsorbed with the control peptide. LM x 800 magnification



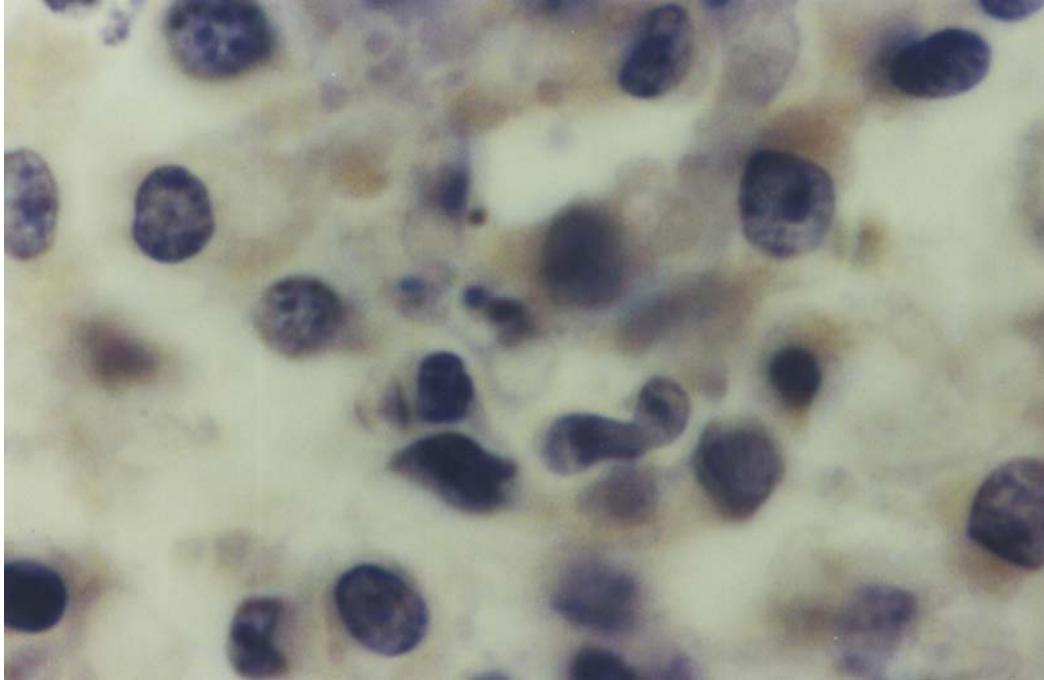
**Figure 74** Number of ER $\beta$  positive cells recorded per 100 cells between the five defined groups represented as means + SEM

One-way analysis of variance revealed that the group means were significantly different ( $P < 0.05$ ). The Tukey-Kramer adhoc test showed that groups 1,3 and 5 were not s different ( $P > 0.05$ ) although they differed from groups 2 and 4 ( $P < 0.05$ ). Groups 2 and 4 were not different ( $P > 0.05$ ) but the differed statistically from groups 1,3 and 5 ( $P < 0.05$ ) (figure 74). We can thus conclude that animals with only normal corpora lutea had insignificant amounts of oER $\beta$  on the 9<sup>th</sup> day after sponge removal (roughly 7<sup>th</sup> day of the estrous cycle) (see figure 75).

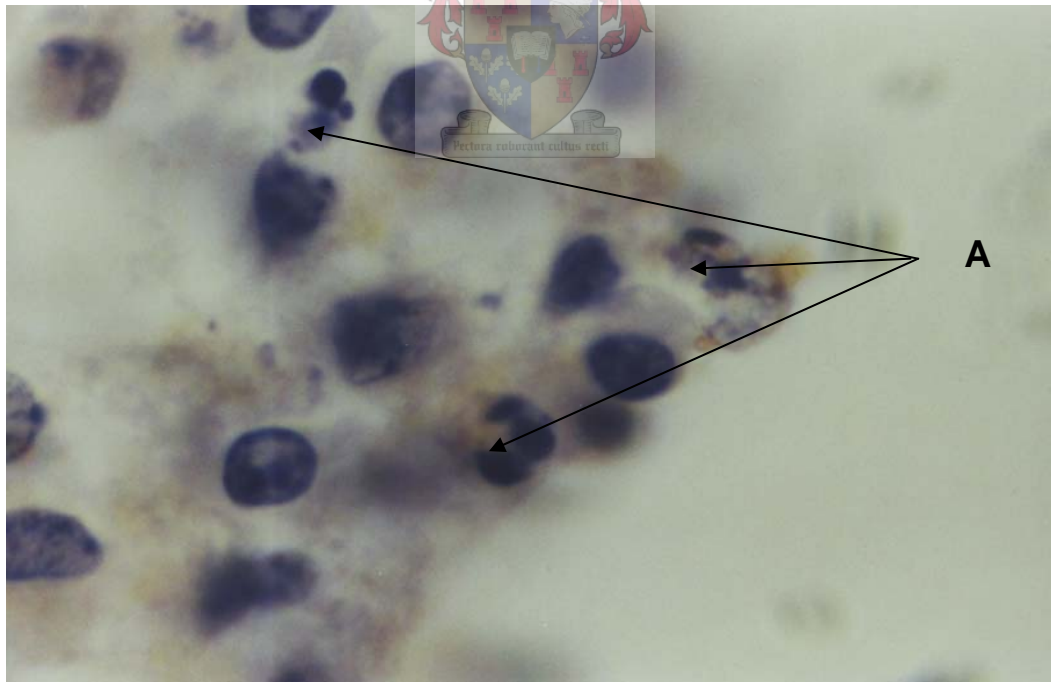


**Figure 75** Immunostaining for oER $\beta$  in a Mutton Merino with only normal functioning corpora lutea. There is no positive staining present. LM x 800 magnification





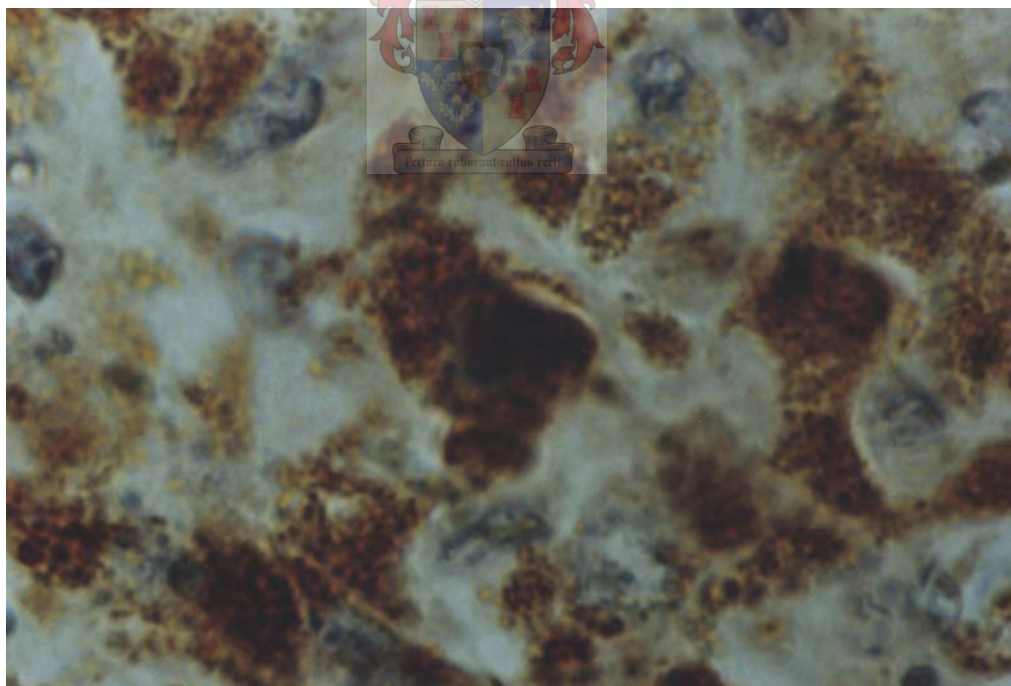
**Figure 76** Immunostaining for oER $\beta$  in a Mutton Merino with normal functioning and premature regressed corpora lutea. There is no positive staining present. LM x 800 magnification



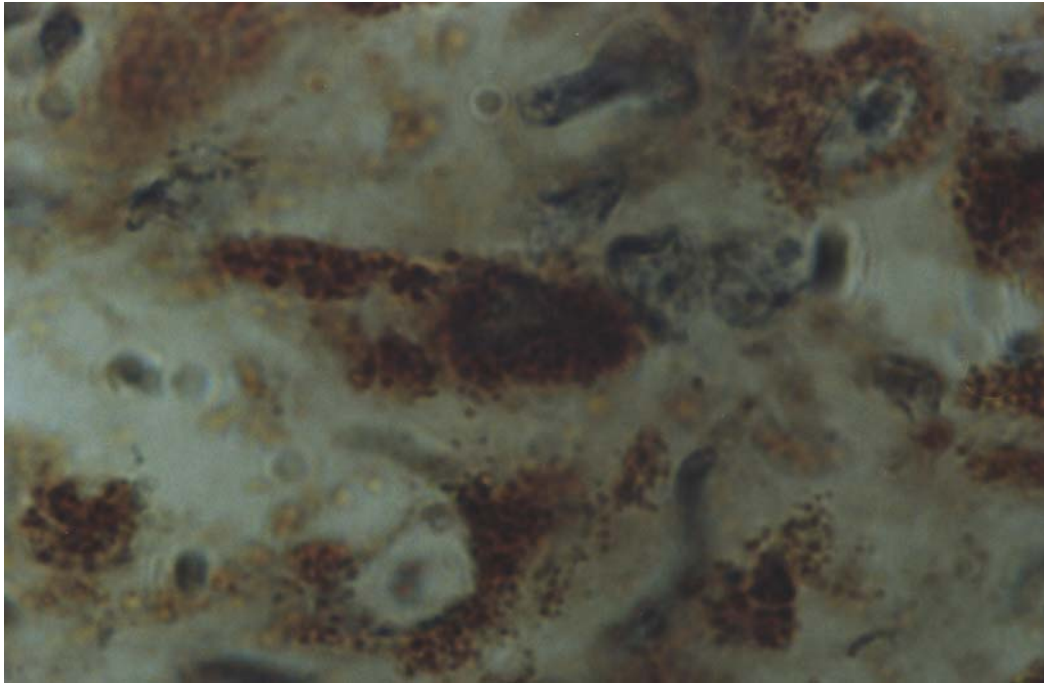
**Figure 77** Immunostaining for oER $\beta$  in a Mutton Merino where regression was induced by PGF $_{2\alpha}$ . There is no positive staining present. Note the clearly visible apoptotic bodies. LM x 800 magnification

In some cases normal functioning corpora lutea occurred with premature regressed corpora lutea on the same ovary. The normal functioning corpora lutea in these cases also showed no significant staining for the oER $\beta$  (figure 76). In Mutton Merino sheep where luteal regression was induced with PGF<sub>2 $\alpha$</sub> , there was also no significant staining although clearly visible apoptotic bodies were observed (figure 77).

In the corpora lutea from ewes with only premature regressed corpora lutea there were very pronounced positive nuclear and cytoplasmic staining for immuno-reactive oER $\beta$ . This was also observed in premature regressed corpora lutea that occurred with normal corpora lutea on the same ovary (see figures 78 and 79).



**Figure 78** Immunostaining for oER $\beta$  in a Mutton Merino sheep with only premature regressed corpora lutea. There is very pronounced nuclear and cytoplasmic staining for immuno-reactive oER $\beta$ . LM x 800 magnification



**Figure 79** Immunostaining for oER $\beta$  in a Mutton Merino sheep with premature regressed corpora lutea occurring with normal corpora lutea on the same ovary. There is very pronounced nuclear and cytoplasmic staining for immuno-reactive oER $\beta$ . LM x 800 magnification

#### 4.4 Discussion



It is known that in the sheep, staining intensity of the oER $\beta$  protein in granulosa cells on different days of the oestrous cycle is similar, suggesting that oER $\beta$  might be important for regulation of most stages of follicular growth [30]. Following the LH surge, oER $\beta$  is down regulated with oER $\beta$  mRNA levels declining from day 2 through 10 and increasing at the end of the estrous cycle [22]. It is thus expected that there will be very little to no oER $\beta$  present in the corpus luteum of the normal functioning corpus luteum on the 7<sup>th</sup> day of the oestrous cycle. It was significant to find oER $\beta$  expressed in premature regressed corpora lutea occurring in animals with only prematurely regressed corpora lutea

present on the ovaries and in animals where prematurely regressed corpora lutea occurred with normal functioning corpora lutea. Insightful as well, was the fact normal functioning corpora lutea that occurring with prematurely regressed corpora lutea had no oER $\beta$  expression. In this study, like in the previous study (Chapter 3) a second rise in E<sub>2</sub>-17 $\beta$  was observed on the 4<sup>th</sup> day of the cycle. This rise in E<sub>2</sub>-17 $\beta$  preceded the rapid decline in progesterone in animals with premature regressed corpora lutea.

As reported previously (Chapter 3) there are some distinct differences between premature luteal regression and PGF<sub>2 $\alpha$</sub>  induced regression. This study confirms the resistance of the early corpus luteum to the luteolytic actions of PGF<sub>2 $\alpha$</sub>  observed in the previous study (slower decline in progesterone) (Chapter 3) and reported in other studies [15-20]. It was also observed in the mentioned study (Chapter 3) that prematurely regressed corpora lutea sometimes occurred with normal functioning corpora lutea while this was never the case with PGF<sub>2 $\alpha$</sub>  induced regression. In the current study another difference was noted in that prematurely regressed corpora lutea express oER $\beta$  while no oER $\beta$  expression is found in corpora lutea where regression was induced by PGF<sub>2 $\alpha$</sub> .

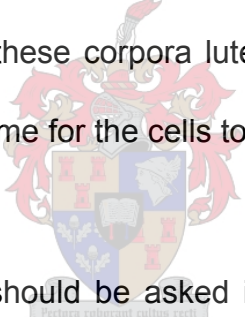
In the sheep as in many other species, the LH surge plays a vital role in initiating ovulation[31-38]. The LH surge represents a brief, massive outpouring of LH from the anterior pituitary gland. LH rise rapidly within 4 to 8 hours to a peak of 100 to 200 ng/ml (50 –100 times basal levels) and then decline just as

rapidly so that the surge lasts approximately 12 hours. The onset of the LH surge is usually closely coupled to the onset of estrus behaviour[37, 39, 40], except for breeds with high ovulation rates in which it begins 8 to 12 hours after the start of estrus[39, 41]. The interval between the LH surge and ovulation (22 to 26 hours) is remarkably constant[42].

In the current experiment ewes were synchronized with a progestagen-impregnated intravaginal sponge (Repromap™ 60 mg, Upjohn) for 12 days and the use of 5 mg ECP administered as an intra-muscular injection 24 hours after sponge induction, to induce luteolysis of any early corpora lutea. Ewes were superovulated by administering 1000 eCG 48 hours before sponge removal. In superovulated synchronized ewes, as in this experiment, the LH surge starts usually on average  $59 \pm 4.7$  hours after progestagen withdrawal[43]. In the Mutton Merino sheep used in this experiment estrus behaviour was observed on average 48 hours after sponge removal.

A unique property of eCG, the hormone used to superovulate the ewes, is its ability to express both FSH-like and LH-like biological properties in other mammalian species [44, 45]. In sheep eCG acts 60% as FSH and 40% as LH, thus 1000 IU of PMSG is equivalent to 400 IU of LH [46, 47](see section 2.11.1.1). eCG is a high molecular weight acidic glycoprotein (70 000 Da) with very high carbohydrate content, i.e 45% containing sialic acid. The high sialic acid content has been linked to the long biological half life of 7 days [46, 47]. The

exogenous long acting LH biological activity in the form of eCG, extends the LH surge (which in the normal oestrous cycle lasts only 12 hours) to a wave of LH lasting 7 days or longer. Follicular dynamic studies have shown that resulting from the extended exposure of biologically active LH in the form of eCG, is the fact that the time of ovulation is now also extended over a period of up to 120 hours, instead of the usual 22 to 26 hours after the endogenous LH surge in ewes not exposed to eCG [43, 48]. Confirming the extended time of ovulation, is the fact that embryos of various stages of development are usually collected spanning up to 36 hours apart in development. Taking the mentioned facts into account. A possible explanation for the fact that prematurely regressed corpora lutea expressed oER $\beta$ , is that these corpora lutea resulted from late ovulations and that there was insufficient time for the cells to down regulate their oER $\beta$ .



The next question that should be asked is, why did these corpora lutea regress and what was the signal initiating the event? It is very interesting that only corpora lutea expressing oER $\beta$ , underwent premature luteal regression, with the decline in progesterone starting the day after the second estrogen peak. This strongly suggest that estrogen could be the signal initiating premature luteal regression, not acting via PGF $_{2\alpha}$  released from the uterus, but by acting directly on the granulosa lutein cells by binding to the oER $\beta$ . This would then also answer the question why estrogen is luteolytic on the first two days of the oestrous cycle in the normal cycling sheep ewe. It probably takes two days to down regulate oER $\beta$ .

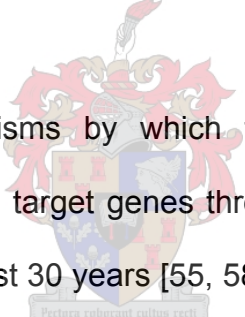
Estrogens are key molecules in development, differentiation, proliferation and growth. Their actions are mediated by specific cellular receptor proteins localized in the nucleus of the cell [49-53]. Estrogen signalling is mediated by one of two receptors, estrogen receptor alpha (ER $\alpha$ ) and estrogen receptor beta (ER $\beta$ ) [54]. It was demonstrated that ER $\beta$  was the predominant estrogen receptor expressed in the ovine ovary[22]. Estrogen receptors are members of the nuclear receptor family that represent the largest family of transcription factors. These are transcription factors that share similar structure homology [50, 53, 55-58]. The structure of these receptors is shown in figure 80.



**Figure 80** The schematic structure of the domains of the nuclear receptors [55]

As shown in figure 80, these receptors contain six regions of similarity that contribute to the functional domains of the receptors. Of the functional domains of the receptors, the three major domains are the ligand binding domain (LBD), the DNA binding domain (DBD), and the activation domain (AF). The LBD is located at the carboxyl terminus of the molecule, region E. The LBD gives the receptor specificity for the specific hormone or molecule that regulates the transcriptional activity of the receptor. The DBD gives the receptor specificity for the target genes the receptor will regulate. This specificity is determined by

which DNA sequences the DBD will recognize. These sequences or hormone response elements (HRE) (in the case of ER $\beta$  they are called “estrogen response elements” (ERE)), are located in enhancer/promoter regions of target genes. The classical ERE consists of a palindromic sequence of GGTC A in an inverted repeat separated by a three-base “spacer” (GGTCACAGTGACC), although most estrogen-responsive genes contain ERE sequences that diverge to varying degrees from this consensus sequence. The AF's are located throughout the molecule, regions A/B and E [58]. These domains are responsible for linking the receptors to the cellular transcriptional machinery and regulate transcription of target genes.



The molecular mechanisms by which the ovarian steroid hormones regulate the transcription of the target genes through their receptors have been actively investigated over the last 30 years [55, 58, 59]. In the absence of ligand, the receptors are associated with chaperone proteins that are transcriptionally inactive. The steroid hormone receptors can be activated by two mechanisms. The first, traditional ligand-dependent, mechanism by which receptors can be activated is by binding of the hormone to the LBD. The second mechanism by which receptors can be activated, is through a ligand-independent mechanism [60, 61]. The ligand-independent activation of the receptor is a result of the integration of other signalling pathways, usually membrane receptor signalling that results in the activation of kinases and ultimately the phosphorylation of the receptor. The activated receptor undergoes conformational change that results



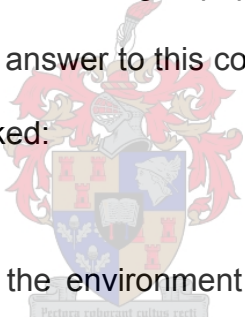
in dimerization of the receptors. In turn, these receptors attain the ability to bind specific DNA ERE's in the enhancer/promoter of the target gene.

ER $\beta$  does not always bind to DNA via the DBD. It was recently discovered that ER $\beta$  could mediate transcription via activator proteins. One such activator protein is called activator protein-1 (AP-1). AP-1 binds to the sequence (TGA GTC A) in the enhancer/promoter regions of some ERE's only after binding to a ligand-bound ER $\beta$ . In addition to binding of the ligand-bound ER $\beta$ , AP-1 also need the transcription factors *c-fos* and *c-jun*, which binds to DNA as a dimer at the AP-1 site mediating ER $\beta$  action [62-64].

The activator protein AP-1 should not be confused with coactivators also needed for successful transcription of estrogen responsive genes. The coactivators link the receptor to the transcription machinery, but also facilitate transcription by covalently modifying chromatin structure. These coactivators have histone acetyl transferase activity that function to acetylate histone proteins, allowing DNA to achieve a conformation that increases the accessibility of the promoter of the target gene to the activated receptor, and basal transcription machinery. This remodelling of the chromatin serves to facilitate transcription of specific genes. These coactivators include the Steroid Receptor Coactivator (SRC) family, CREB binding protein and related p300 (CBP/p300), High Mobility Group proteins (HMG's), and E3 ubiquitin protein ligases (E6-AP

and RPF-1) [65, 66]. It is also known that coactivators can bind dimers of nuclear receptors including heterodimers of ER $\alpha$ /ER $\beta$  [67, 68].

Estrogen, as mentioned earlier, is associated with growth and proliferation, but this study suggests involvement in the exact opposite cell death by apoptosis. In the growing follicle estrogen is one of the factors initiating unprecedented mitotic activity of granulosa cells. This is also seen in ER-positive mammary carcinomas [69-71]. Following ovulation under the influence of progesterone, among other, these cells differentiate into progesterone producing granulosa lutein cells. But why will these cells undergo apoptosis if they contain ER $\beta$  on the 4<sup>th</sup> day of the cycle? To find an answer to this compelling and intriguing question two other questions must be asked:

- 
- a) What is different in the environment between the estrogen-induced surge of mitosis and the initiation of apoptosis on the 4<sup>th</sup> day when the second estrogen rise is present?
  - b) Why is there a second estrogen rise?

During the mitotic surge in the final days of the growing follicle progesterone levels are very low. There should be virtually no ligand-bound PR present in cells. On the 4<sup>th</sup> day of the cycle when cells undergo apoptosis, cells are exposed to both progesterone and estrogen with both ligand-bound ER and PR

present in the cells. Secondly, very interestingly, on the 4<sup>th</sup> day apoptosis in atretic follicles that were not selected to ovulate, is pronounced. Could the second rise in estrogen function to remove unovulated antral follicles to prevent them of becoming cystic in the following cycle?

The following theory to explain the observations in this experiment. A coactivator protein exist capable of binding homodimers of either PR or ER, and heterodimers of ER/PR in the nucleus. If a homodimer of ER's binds, mainly mitogenic genes and the genes of growth factors are transcribed. If a homodimer of PR binds, genes necessary for the differentiation to granulosa lutein cells are transcribed. But if a heterodimer of ER/PR binds, it leads to transcription of apoptotic genes. This theory would explain why ER $\beta$  containing corpora lutea from late ovulating follicles will undergo premature luteal regression following the second rise of estrogen. This would also explain why prematurely regressed corpora lutea sometimes occur with normal functioning corpora lutea.

The *in vivo* experimental animal is a problematic model for investigating this hypothesis further, because of numerous influences on live animal. A tissue culture model will be necessary to explore this hypothesis.

## 4.5 Conclusions

There could therefore be a potential role for ER $\beta$  in premature luteal regression.

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## General Conclusion

It is clear from the research in this thesis and from the discovery by Sugimoto *et al* in 1997 that R-PGF<sub>2α</sub> knockout mice had normal estrous cycles, that PGF<sub>2α</sub> is not the only signal to induce luteal regression. The process is indeed complex and there are many factors affecting it. The precise efficiency of the reproductive system in animals is exceptional.

This thesis presents some interesting observations on premature regressed corpora lutea and provides a new hypothesis to explaining premature luteal regression in the superovulated sheep. (see discussion Chapter 4).

Severe shortcomings have been encountered in the *in vivo* study and the proposed hypothesis could not be proven conclusively. A more controlled tissue culture model is needed to study the complex interactions of multiple factors in this cellular process. Proving this hypothesis would present us with a better understanding of luteal regression in more evolved species like the human and primates. The model also presents us with some interesting options in the study of ER and PR positive breast cancers.