PROSTAGLANDINS AND STEROID HORMONES IN FOLLICULAR FLUID AS PREDICTORS OF OUTCOME IN IN-VITRO FERTILISATION

By

ELAINE MARJORY SMITH
M.B.Ch.B., M.R.C.O.G

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Department of Human Reproduction and Obstetrics,
University of Southampton.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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ABSTRACT

The evolution of in-vitro fertilisation over the last decade has produced major advances in ovarian hyperstimulation, cycle monitoring, oocyte retrieval and embryo transfer techniques. The methodology for culture of human oocytes and their fertilisation has been refined such that fertilisation rates of over 70% can now be obtained.

Nevertheless, pregnancy rates remain disappointingly lower, with only 33% being achieved by few of the most successful groups if as many as three embryos are replaced.

The present study attempted to determine any relationship between the concentrations of prostaglandins or steroid hormones in follicular fluid at oocyte retrieval with the outcome of the matched oocyte. If a strong relationship were established this could provide a further means of evaluating embryo potential prior to embryo transfer. The importance of steroid hormones, oestradiol and progesterone in the follicular and luteal phases of the menstrual cycle is well established but the significance of prostaglandins is really unknown.

Preliminary studies established the methodology required for accurate measurement of prostaglandins in follicular fluid. This provided improved techniques not previously applied to follicular fluid prostaglandin measurement and subsequently, interesting and reliable information was obtained in this field.

Studies were performed on select groups of patients in order to reduce the number of variables to a minimum (for instance all patients who had a purely mechanical barrier to conception, either tubal blockage, or mucus hostility with no male factor problems). Prostaglandins, PGE_2 and $PGF_{2\alpha}$ and oestradiol (E_2) and progesterone (P) were measured in follicular fluid obtained at the time of oocyte recovery.

Follicular fluids were assigned to one of four groups depending upon the outcome of the matched oocytes.

1. No fertilisation
2. Fertilisation but not transferred
3. Fertilisation, transfer but no pregnancy established
4. Fertilisation, transfer and pregnancy established

Within any group there was a highly significant correlation between PGE_2 and $PGF_{2\alpha}$ levels ($p<0.001$) and a significant correlation between E_2 and P levels ($p<0.001$) but the correlation between prostaglandins and steroids was poor. Progesterone levels were significantly lower in the unfertilised group compared to the other three groups ($p<0.001$) but no differences were shown for E_2 or the E_2:P ratio.

No significant differences were shown among the groups in the actual levels of either PGE_2 or $PGF_{2\alpha}$, but there were highly significant differences shown when the "PGE_2:$PGF_{2\alpha}$ ratios" were compared. The PGE_2:$PGF_{2\alpha}$ ratio fell within a much narrower range for the "pregnancy" group (0.46 to 2.00) compared with any of the other groups (group 1: 0.18 to 4.4; group 2: 0.15 to 7.66; group 3: 0.3 to 5.91). There was a highly significant difference between not only the "pregnancy" group and the unfertilised group ($p<0.001$) but also between the "pregnancy" group and the fertilised but "no pregnancy" group ($p<0.001$).

Therefore for oocytes that have the potential to establish a pregnancy there is a well defined range for the PGE_2:$PGF_{2\alpha}$ ratio. These studies show that measurement of PGE_2 and $PGF_{2\alpha}$ in follicular fluid could provide a valuable indicator for the selection of embryos with the optimal chance of establishing a pregnancy. Present findings have been made possible by achieving pregnancy rates as high as the best groups in the field and by applying convenient and precise methodology for the analyses of prostaglandins.

PRESENTATIONS AND PUBLICATIONS RELATING TO THESE STUDIES

Smith, E.M., Anthony, F., Gadd, S.C. & Masson, G.M. (1988) Prostaglandin E_2 and $F_{2\alpha}$ values in follicular fluid from patients undergoing in vitro fertilisation. Presented at Blair Bell Research Society Meeting, RCOG, London.

Smith, E.M., Anthony, F. & Masson, G.M. (1988) Oocyte development as assessed by steroid hormone and prostaglandin concentrations in follicular fluid. Presented at 4th Annual Conference, ESHRE, Barcelona, Spain.

Smith, E.M., Anthony, F., Gadd, S.C. & Masson, G.M. (1988) Prostaglandin E_2 and $F_{2\alpha}$ values in follicular fluid from patients undergoing in vitro fertilisation. Br J OG, 95,1211.

Smith, E.M., Anthony, F. & Masson, G.M. (1988) Oocyte development as assessed by steroid hormone and prostaglandin concentrations in follicular fluid. Human Reprod, 3, Suppl. 1,68.

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SECTION 1

INTRODUCTION

CHAPTER 1

STEROIDS AND PROSTAGLANDINS IN FOLLICULAR FLUID

1.1 PRELUDE

In vitro fertilisation (IVF) has become established over the last decade as a widely accepted clinical method for the treatment of many unrelated causes of infertility.

Since the birth of the first IVF baby in 1978 (Edwards & Steptoe, 1979) many advances and refinements have been made to both the clinical and the laboratory aspects of IVF, but success rates remain low. The methodology of fertilisation and culture of human embryos have been generally standardised. Fertilisation rates are upwards of 70% in the majority of established programmes (Laufer et al, 1986, Jones et al, 1983) but pregnancy rates remain considerably lower at around 33% for the best teams if as many as 3 embryos are replaced (Edwards, 1985).

Improved methods of assessment of the maturity and quality of oocytes recovered and the ability to recognise the potential of resultant embryos in terms of establishing a pregnancy are necessary.

Examination by microscopy forms the basis of present assessment of oocytes and embryos but this is not sufficient to produce a more acceptable pregnancy rate.

Although individual oocytes are often morphologically indistinguishable, they behave differently in terms of fertilisation, cleavage and implantation of the resultant embryos. Thus better markers for oocyte quality are required.

Analysis of the various components of follicular fluid, readily obtained during the process of oocyte recovery, could be considered a rational approach to acquiring further information on the quality of recovered oocytes and their subsequent pregnancy potential.

1.2 STEROID HORMONES IN FOLLICULAR FLUID

Follicular fluid is an important source of progestogens, oestrogens and androgens. Studies in various mammals, Bahr (1978) using rabbits and Goff & Henderson (1979) with rats, showed that changes in steroid concentrations, were indicative of the steroid secretory activity of the follicle. Ainsworth et al (1980) measured steroid hormones in porcine follicular fluid following administration of pregnant mare serum gonadotrophin and human chorionic gonadotrophin (hCG) and concluded that the ability of the follicle to ovulate a mature oocyte was associated with sequential changes in follicular fluid levels of progestins, androgens and oestrogens which were influenced by exposure of the developing follicle to gonadotrophic stimuli.

Studies on the natural cycle in humans (Fowler et al, 1977), showed that follicles, classified by appearance of the oocyte as pre-ovulatory or non-ovulatory had different hormonal concentrations. Pre-ovulatory follicles contained high levels of progesterone (P) and oestrogens (E_2) but low levels of androgens in contrast to non-ovulatory follicles. Oocytes from non-ovulatory follicles could not be fertilised.

Ovarian hyperstimulation is an integral part of IVF in order to induce multiple follicular maturation, allowing the retrieval of more than one oocyte as the pregnancy rate increases with the transfer of multiple embryos (Sharma et al, 1988). Fowler et al (1978) showed that hyperstimulation of the ovaries with human menopausal gonadotrophin (hMG) and hCG does not appear to alter the relationship between the various steroid hormones.

The earlier findings on the natural cycle were confirmed in the hyperstimulated cycle.

Coulam, Bustillo & Schulman (1986) first coined the term the "empty follicle syndrome". Tsuiki, Rose & Hung (1988) followed this up by comparing three IVF cycles in the same patient, of which two cycles yielded multiple oocytes and in one cycle no oocytes were obtained from 12 aspirated follicles. The steroid profiles of the follicular fluids from the empty follicle cycle showed a markedly increased E_2 level, a markedly decreased P level and an elevated androgen level.

Successful fertilisation depends on the collection of oocytes which are mature. Oocyte maturation depends on the constituents of the follicular fluid (McNatty et al, 1979). During follicular maturation the granulosa cell undergoes a change in steroidogenesis, which is augmented by luteinising hormone (LH) or hCG adminstration. The granulosa cell shifts from being primarily an E_2 producing cell to a P producer. Fishel, Edwards & Walters (1983) attempted to predict fertilisation on the basis of the steroid hormone levels in follicular fluid. The fertilised group showed higher P and E_2 levels and lower androgen levels than the non fertilised group. The concentration of P was the most accurate predictor of successful fertilisation of an oocyte, with a success rate which exceeded 90%. The mean value of P in the fertilised group was 35 μmol/L compared to 12.5 μmol/L in the unfertilised group. ($p<0.01$).

The importance of higher P concentrations for successful fertilisation was confirmed by Chikhaoui et al (1983) who reported that fertilisation only occurred when the follicular fluid P concentration exceeded 6.4 μmol/L. Reinthaller et al (1987), too, emphasised the importance of follicular fluid P levels and successful fertilisation, showing a significant increase in P concentration in follicular fluid from fertilised oocytes (mean P 27 μmol/L) compared to those from unfertilised oocytes (mean P21 μmol/L). Significantly lower androgen levels were also found in the fertilised group. Berger et al (1987), however showed no significant difference in either P or E_2 levels when comparing fertilisation with non-fertilisation.

Immature oocytes have a markedly decreased fertilisation rate. Botero-Ruiz et al (1984) showed that immature oocytes have a markedly reduced capacity to produce P in culture compared to mature oocytes suggesting that hCG reaches these follicles before they obtain a full complement of LH receptors and they are, therefore, unable to respond maximally to hCG stimulation.

The follicular fluid androgen/oestradiol (A/E_2) ratio may be more important in relation to fertilisation than the P concentration (Chabab et al, 1986). The A/E_2 ratio was significantly higher in their unfertilised group (mean 2.25) compared to their fertilised group (mean 0.9) whilst the P concentration did not show a significant difference. The increased A/E_2 ratio in the unfertilised group was caused by a significant increase in follicular fluid androgen levels, agreeing with the findings of Fishel et al.

Chikhaoui et al (1983), also considered a low A/E_2 ratio to be associated with high fertilisation rates. The highest fertilisation rate (85%) occurred when the A/E_2 ratio did not exceed 0.5, the fertilisation rate progressively decreasing as the ratio increased. (A/E_2 ratio more than 1, fertilisation rate = 25%).

The ratio of E_2 to P is also considered to be important. Follicular fluid E_2 and P levels increase as the follicle matures, P increasing more rapidly than E_2 (McNatty et al, 1975). Mature follicles thus have higher E_2 and P concentrations and relatively lower E_2/P ratios than immature follicles.

The major determination of the success of IVF is the stimulation of follicles to produce mature oocytes capable of producing normal pregnancies after fertilisation and culture in vitro. Botero-Ruiz et al (1984) evaluated the relationship between follicular fluid E_2, P and androgen levels and successful implantation of fertilised oocytes. There was an association between high follicular E_2 levels and subsequent pregnancy. Basuray et al (1988) however found no association between follicular fluid E_2 levels and pregnancy but did find significantly elevated P levels in the pregnancy group and consequently high $P:E_2$ ratios in this pregnancy group.

Kreiner et al (1987) analysed follicular fluid E_2 and P concentrations in a group of 19 single follicle, single oocyte, single embryo transfers that resulted in 19 pregnancies. A significant correlation ($p<0.01$) was found between follicular fluid, E_2 and the E_2/P ratio in this group. In those patients with multiple follicles, oocyte maturity was associated with higher follicular fluid E_2 and P concentrations and lower E_2/P ratios compared to immature oocytes. Follicular immaturity was characterised by a universally low P and variable E_2.

These studies would suggest that follicular fluid E_2 and P may be used as markers of oocyte viability by reflecting maturation and thus ability to fertilise. However no uniformity in previous findings has been established.

1.3 PROSTAGLANDINS IN FOLLICULAR FLUID

Our awareness of prostaglandins began in 1935 when a lipidic acid was shown to be responsible for the potent biological activity of human seminal fluid (von Euler, 1983).

Prostaglandins are derivatives of fatty acids. The group consists of prostaglandins, thromboxanes, some hydroperoxy and hydroxy fatty acid derivatives, and leukotrienes. Although their full physiological role is not completely known, they exert diverse biological actions.

The prostaglandins are a family of naturally occurring, biologically active, 20 carbon unsaturated fatty acids, incorporating a cyclopentane ring. By convention, prostaglandins are abbreviated PG, with the class designated by a capital letter (A,B,E,F,G,H and I), followed by a number and then in some cases a Greek letter. The letters refer to different ring structures. The number is used to designate the number of unsaturated bonds in the PG side -chains. The use of the Greek letter (α or β) applies only to the F series and refers to the hydroxyl group found at C-9. In the α-series, the hydroxyl group projects below the ring plane whilst in the β-series the hydroxyl at C-9 is above the plane of the ring.

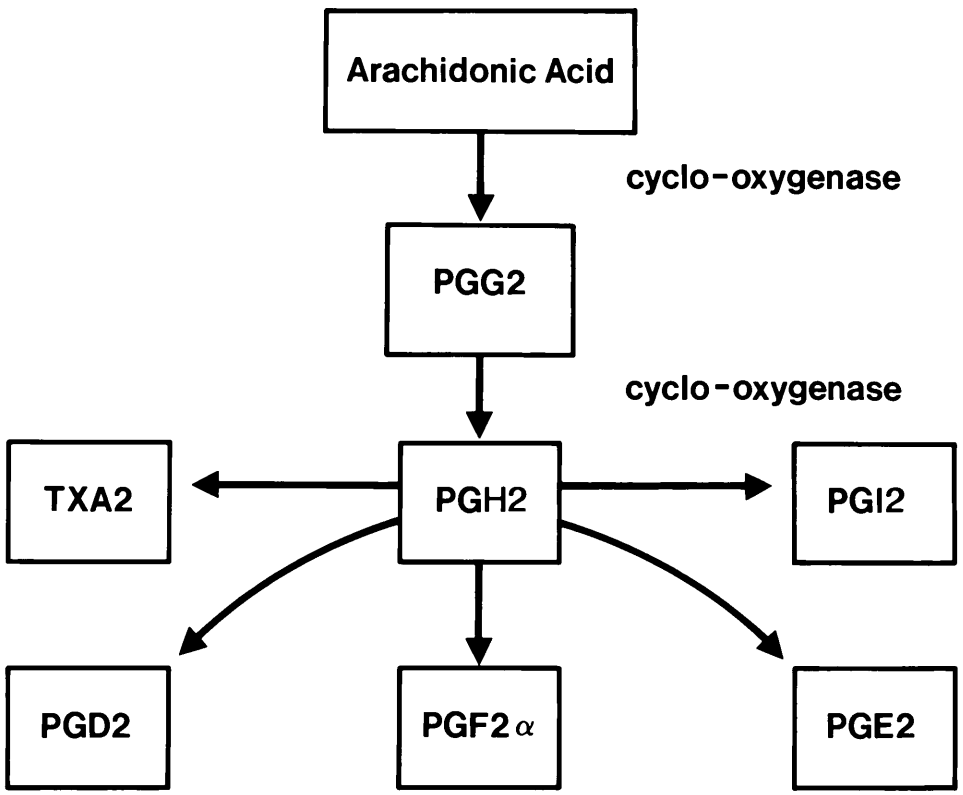
The predominant precursor of prostaglandins in man is arachidonic acid, incorporated into phospholipids in the cell membrane. Once formed, prostaglandins exert a very short lived effect and are rapidly catabolised. They are not stored preformed but are synthesised and secreted in response to an appropriate stimulus.

On demand, arachidonic acid is split from the cell membrane phospholipids by phospholipase A_2 and is metabolised by one of two pathways, the lipo-oxygenase or cyclo-oxygenase.

Cyclo-oxygenase converts arachidonic acid to unstable endoperoxides PGG_2 and PGH_2. These can isomerise to the stable prostaglandins - PGD_2, PGE_2 and $PGF_{2\alpha}$. The E and F type prostaglandins, after having reached the circulation, are rapidly broken down in the lung and liver, through the action of a dehydrogenase. Consequently their blood levels are low.

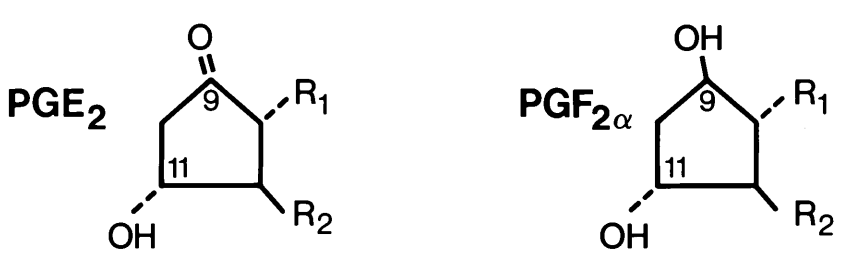
The cyclo-oxygenase pathway is shown in Diagram 1.A

DIAGRAM 1A CYCLO-OXYGENASE ENZYME PATHWAY



PG = Prostaglandin

TX = Thromboxane



R_1, R_2 = Prostaglandin side chains

Table 1.A lists some of the reported functions of the various prostaglandins.

TABLE 1.A PROSTAGLANDIN - MEDIATED EFFECTS

Site of Action	Physiological Response
Arterial smooth muscle	Alterations of blood pressure
Uterine muscle	Induces labour, therapeutic abortion
Lower gastrointestinal tract	Increases motility
Bronchial smooth muscle	Bronchospasm
Platelets	Increases coagulability
Stomach	Enhances gastric acid secretion
Capillaries	Increased permeability with flushing
Adipose tissue	Inhibits triglyceride lipolysis

The role that prostaglandins play in the process of ovulation has been investigated extensively. Comparisons have been made between ovulation and the inflammatory process, both of which involve prostaglandin production.

Espey (1980) proposed the hypothesis that an ovulatory surge of gonadotrophins induces an inflammatory reaction in mature follicles and this inflammatory condition leads to rupture of the ovarian surface. The hypothesis gives prostaglandins the central role as mediators of the inflammatory process and establishes a relationship between prostaglandins and ovarian proteolytic activity.

It was suggested that this group of inflammatory mediators stimulated the proliferation of thecal fibroblasts and production of proteolytic enzymes capable of disrupting the follicle wall during ovulation. Yoshimura & Wallach (1987) did not dismiss an inflammatory like reaction causing rupture of the follicle at ovulation even though other hypotheses were postulated as factors in the process of follicular rupture. These hypotheses included an increase in the follicular pressure and enhanced steroidogenesis.

The absence of an increase in intrafollicular pressure preceding follicle rupture and the non-explosive nature of ovulation supported the conclusion that rupture is not a forcible phenomenon but rather is due to a degenerative process within the follicle wall. Yoshimura et al (1987) also demonstrated in the rabbit ovary that inhibition of steroidogenesis, oestradiol and progesterone, did not affect ovulation.

Parr (1975) demonstrated in rat follicles, the similarity between inflammation and ovulation. Changes in the follicle wall, following administration of hCG, were consistent with the notion that ovulation involved its degradation. In further support that follicle rupture may involve the early phase of an inflammatory process, indomethacin inhibition of ovulation was found to be quantitatively similar to the effect of indomethacin on model systems of inflammation in rats.

Normal ovulation not only comprises changes in the follicle leading to its rupture but to at least two other distinct physiological processes: the completion of the maturation division of the oocyte and changes in the mode of contact between granulosa cells, followed by their transformation into luteal cells.

Ovulation in mammals occurs as the culmination of a sequence of maturational changes within the ovarian follicle in response to a co-ordinated series of hormonal actions and interactions. Complex alterations including steroid and prostaglandin metabolism, enzymatic pathways and inflammatory mediators appear to be closely correlated with both the ovulatory and oocyte maturation processes.

The relative amounts of locally produced hormones, which act on the different components of the follicle simultaneously or in a particular sequence appear to determine the maturational stage of the oocyte and cumulus differentiation during the process of ovulation (Yoshimura et al, 1987).

The ovulatory process is controlled by the release of gonadotrophins from the pituitary. A direct involvement of prostaglandins in this process has been established by animal studies.

Brown & Poyser (1984) noted increased concentrations of PGE_2 and $PGF_{2\alpha}$ in rat follicles at the time of ovulation with PGE_2 showing the greater increase, some three fold more than the rise in $PGF_{2\alpha}$.

The major role of prostaglandins in rat ovulation was also attributed to PGE_2 by Tsafriri et al (1972). Further evidence for this was provided when administration of PGE_2 produced follicular rupture where ovulation had been blocked by indomethacin.

The role of prostaglandins in oocyte maturation was unclear, as although indomethacin blocked follicular rupture, the entrapped oocytes completed their maturation division. Tsafriri et al concluded from this that prostaglandins must act on the ovary itself rather than by stimulating LH release and oocyte maturation must be attributed to the availability of endogenous LH. Nembutal blockade of LH caused non-maturation of oocytes implying that prostaglandins are not essential for induction of oocyte meiosis by LH. But it was also noted that the addition of PGE_2 to cultured follicle enclosed oocytes induced maturation.

Administration of LH causes prostaglandin levels in follicular fluid to increase. Koos et al (1983) demonstrated increased prostaglandin levels in follicular fluid from rabbit ovaries perfused in vitro in response to LH. Prostaglandin levels were variable and it was therefore postulated that large follicles that failed to respond to LH with increased prostaglandin synthesis and that do not ovulate may be overly mature or atretic.

Munalulu, Hillier & Peddie (1987) showed the onset of ovulation to be closely related to the peak levels of prostaglandin synthesis. Yang, Marsh & Le Maire (1974) found the average levels of $PGF_{2\alpha}$ and PGE_2 to be 5 fold higher in ovulated follicles compared to non-ovulated follicles.

Clark, Marsh & Le Maire (1976) in rat follicles and Marsh, Yang & Le Maire (1974) in rabbit follicles showed a continuing increase in prostaglandin levels at five hours incubation following administration of LH.

The magnitude of the ovarian response to LH is greatest on the day of proestrus when follicular development is maximal (Bauminger, Lieberman & Lindner, 1975). Follicles explanted from proestrus rats before the pre-ovulatory gonadotrophin surge responded to the addition of LH to the culture medium with a 10-30 fold increase in PGE_2 and a 5 fold increase in $PGF_{2\alpha}$ over a 4 hour period. Stimulation of follicular prostaglandin accumulation was unimpaired during suppression of progesterone and oestrogen synthesis by aminoglutethimide. Steroids therefore appear to play no part in the mediation of the LH effect on follicular prostaglandin formation.

$PGF_{2\alpha}$ levels in rabbit follicles rise in the immediate pre-ovulatory period and through the time of follicular rupture but then progressively decline to reach near pre-ovulatory levels by 48 hours. PGE_2 levels, though, remain elevated for some time after ovulation before declining (Yang et al, 1974).

Similar increases in prostaglandin levels were recorded by Tsang et al (1979) in follicular fluid collected from the follicles of prepubertal gilts. These increases were maximal around the expected time of ovulation.

There is a pronounced increase in follicular fluid $PGF_{2\alpha}$ levels as ovulation approaches but in follicles not destined to ovulate this increase does not occur (Ainsworth, Baker & Armstrong, 1975). Although these latter follicles are stimulated to grow to pre-ovulatory size, they eventually undergo atresia. At about the time of ovulation, the concentration of $PGF_{2\alpha}$ in follicular fluid from follicles designated as pre-ovulatory reaches levels about 30 times greater than those from follicles which were not destined to ovulate.

Armstrong et al (1974) established a role for $PGF_{2\alpha}$ in rabbit ovulation. Microinjection into the follicle of antiserum against $PGF_{2\alpha}$ blocked LH induced ovulation. Intrafollicular injection of indomethacin under conditions in which the drug blocked ovulation prevented or markedly reduced the expected increase in $PGF_{2\alpha}$ levels in the follicles.

$PGF_{2\alpha}$ was shown to be involved in ovulation in the Rhesus monkey (Wallach et al, 1975). The monkeys were treated with hMG-hCG to induce ovulation together with indomethacin. Ovulation occurred only once in 9 cycles and then it was delayed. With the addition of $PGF_{2\alpha}$ to the hMG-hCG and indomethacin, ovulation was detected in 13 of 19 cycles. This suggested that indomethacin inhibition of ovulation was mediated through the action of this drug on prostaglandin synthesis, probably at the ovarian level. Progesterone continued to be produced in the absence of follicle rupture implying that indomethacin does not locally inhibit luteinisation.

Thus $PGF_{2\alpha}$ restored ovulation which was suppressed when indomethacin was administered together with hMG-hCG, suggesting that locally available $PGF_{2\alpha}$ is a pre-requisite for the ovulatory process.

Rhodes et al (1985) found that indomethacin blockade of LH induced ovulation in "does" was associated with inhibition of both PGE_2 and $PGF_{2\alpha}$.

The speed with which indomethacin affects follicular prostaglandin levels has been demonstrated by the work of Espey, Norris & Saphire (1986) on rabbit follicles. 2 hours after hCG administration, there was a significant increase in PGE_2 reaching a peak, 10 hours after hCG, which is the expected time of ovulation. There was a 5.9 fold increase in PGE_2 during the ovulatory process. $PGF_{2\alpha}$ levels followed the same pattern of increase, 6.8 fold during the ovulatory process.

Indomethacin significantly reduced prostaglandin levels within 5 minutes of its administration. Ovulation was suppressed if indomethacin was given 2 hours pre-ovulation but if indomethacin was given 1 hour later, ovulation occurred. This was despite significant reduction in prostaglandin production.

Thus prostaglandins do not need to be produced in any significant amount during the hour preceding follicle rupture. This suggests that there may be substances other than PGE_2 and $PGF_{2\alpha}$ that can mediate ovulation and may be less sensitive to indomethacin than the prostaglandins.

The conclusions drawn from these animal studies are that prostaglandins act at the level of the follicle. Evidence for this lies in measurements of prostaglandins in follicular fluid and the effect on prostaglandin levels of direct intra-follicular injection of indomethacin with consequent blockade of LH induced ovulation. Antisera against $PGF_{2\alpha}$: and PGE_2 administered intra follicularly also blocks ovulation.

As reviewed by Armstrong (1986) prostaglandins appear to play a major role in the pre-ovulatory processes in mammals. PGE_2 and $PGF_{2\alpha}$ are synthesized locally in the ovarian follicle, accumulating at high concentrations in follicular fluid pre-ovulation. Blockade either by prostaglandin synthetase inhibitors or antisera to prostaglandins prevents ovulation without affecting most of the endocrinological changes and oocyte maturation.

However, although the presence of prostaglandins in pre-ovulatory follicles has been well documented in animals, the findings have not been consistent between species.

Investigations into the role played by PGE_2 and $PGF_{2\alpha}$ in human ovulation have been aided by the advent of laparoscopy, providing access to ovarian follicular sampling at various stages in the menstrual cycle.

Darling, Jogee & Elder (1982) found increasing concentrations of $PGF_{2\alpha}$ in human follicular fluid leading up to ovulation with a rapid decline post ovulation. $PGF_{2\alpha}$ increases in a time-related sequence from the onset of the LH surge implying that LH plays a role in the follicular prostaglandin accumulation (Seibel et al,1984).

Lumsden et al (1986) induced follicular development with clomiphene followed by hCG and also found that $PGF_{2\alpha}$ rose significantly between 0 and 36 hours after hCG. Both $PGF_{2\alpha}$ and PGE_2 showed peaks at 12 and 36 hours but unlike $PGF_{2\alpha}$ concentrations, increase in PGE_2 between 0 and 36 hours was not significant. Follicles which yielded oocytes contained more $PGF_{2\alpha}$ and PGE_2 than those which did not yield oocytes. The increasing levels of $PGF_{2\alpha}$, 30 or more hours after the onset of the LH surge or the administration of hCG and associated advancing stages of oocyte maturation suggest prostaglandins may play a role in ovulation, oocyte maturation and/or the maintenance of healthy oocytes.

Seibel et al (1984) demonstrated a similar trend of increasing $PGF_{2\alpha}$ concentration with resumption of meiosis and advancing stages of oocyte maturation. There was no similar association for PGE_2 implying that the significant changes in $PGF_{2\alpha}$ do not represent cell death and non-specific release but rather a specific intra-follicular event utilising a locally active prostaglandin synthetase system.

The localisation to particular follicle components of these rises in prostaglandins has been investigated by Triebwasser et al (1978) in rabbits. Graafian follicles, 9 hours after hCG administration, were dissected into a follicular fluid component, a granulosa cell - oocyte component and a thecal cell component.

The follicular fluid contained 4-10 times more PGE_2 and $PGF_{2\alpha}$ than the other two components but in-vitro biosynthesis of PGE_2 and $PGF_{2\alpha}$ showed that the follicular fluid synthesised no prostaglandins. The cell type responsible for prostaglandin synthesis in the follicles appeared to be the granulosa cells or possibly the oocyte. As the follicular fluid represents a major site of accumulation of prostaglandins it may be that prostaglandins must pass from the granulosa cells into the antrum of the follicle before prostaglandins are capable of expressing their role in the process of ovulation.

Again interspecies differences were demonstrated, firstly by Evans et al (1983) and later by Ainsworth et al (1984) in studies on porcine pre-ovulatory follicles. Both found thecal tissue to be the predominant producer of PGE_2 and $PGF_{2\alpha}$. The concentrations of both prostaglandins increased with the maturity of the oocyte. These findings are consistent with the concept that an increase in intra-follicular production of prostaglandins is a pre-requisite for rupture of the follicle and release of a fertilisable oocyte.

Patwardhan & Lanthier (1981) examined human pre-ovulatory follicles and found the concentrations of PGE_2 and $PGF_{2\alpha}$ in follicular fluid to be higher than in theca and granulosa cells, thus similar to the findings of Triebwasser et al (1978) in rabbits. Although theca showed more capacity to form PGE_2 and $PGF_{2\alpha}$ in vitro than the corresponding granulosa cells, exposure to gonadotrophins stimulated PGE_2 and $PGF_{2\alpha}$ formation in the granulosa cells and not in the theca.

Therefore, although thecal tissue showed a greater intrinsic capacity to form PGE_2 and $PGF_{2\alpha}$ the contribution of granulosa cells may be more important under acute gonadotrophin stimulation.

The presence or absence of an oocyte within the follicle may determine the prostaglandin production but conflicting results have been produced in humans. Heinonen et al (1986) found no difference in prostaglandin concentrations in follicles yielding oocytes compared to follicles without oocytes, in cycles hyperstimulated with clomiphene and hMG. Conversely Lumsden et al (1986) showed the concentration of both PGE_2 and $PGF_{2\alpha}$ to be significantly greater in follicles yielding oocytes, thus implying an association with oocyte maturation.

Lumsden's findings were supported by Jeremy et al (1986). Following clomiphene and hMG hyperstimulation, follicular fluid collected at the time of oocyte recovery contained consistently low concentrations of PGE_2 and $PGF_{2\alpha}$ when arising from non-ovulatory follicles.

Jeremy et al also examined the relationship between prostaglandin concentrations and oocyte maturity in these women and found a direct relationship. Mature oocyte-corona-cumulus complexes were derived from follicles that contained higher concentrations of PGE_2 and $PGF_{2\alpha}$ than did follicles from which immature and intermediate oocyte-corona-cumulus complexes were obtained. Thus there appears to be not only a relationship between prostaglandin concentrations and the process of ovulation but also with oocyte maturation.

The fertilisation rate of oocytes is a reflection on oocyte maturity. However Chabab et al (1986) found higher concentrations of both PGE_2 and $PGF_{2\alpha}$ in the follicular fluid from oocytes that did not fertilise compared to those that did fertilise. The ratio of PGE_2 to $PGF_{2\alpha}$ was similar in both the unfertilised (n = 120) and the fertilised (n = 131) groups. There was a relationship between higher concentrations of PGE_2 and $PGF_{2\alpha}$ when hMG was given for ovarian hyperstimulation.

Reinthaller et al (1987) reported PGE_2 and $PGF_{2\alpha}$ levels to be similar in follicular fluid from both fertilised oocytes and unfertilised oocytes.

Follicular fluid levels of prostaglandins were measured by Chikhaoui et al (1983) and related to the evolution of the matured oocyte in terms of fertilisation in spontaneous cycles and also in cycles induced with clomiphene and hMG. The results from spontaneous cycles differed to those of induced cycles. Spontaneous cycles showed $PGF_{2\alpha}$ concentrations in follicular fluid to be higher than those of PGE_2. The reverse was the case in induced cycles. In spontaneous cycles, the follicular fluid $PGE_2/PGF_{2\alpha}$ ratio correlated with fertilisation. Fertilisation was associated with a ratio 0.8 or higher whilst fertilisation did not occur if the ratio was less or equal to 0.4.

Cristol et al (1985) measured prostaglandin levels in follicular fluid from induced cycles. The range of both PGE_2 and $PGF_{2\alpha}$ concentrations was too wide to assess any correlation with fertilisation and the PGE_2:$PGF_{2\alpha}$ ratio was similar in both favourable and unfavourable outcomes. But the observation made was that for fertilisation to occur the PGE_2:$PGF_{2\alpha}$ ratio should be greater than 0.8.

Studies in humans, as with animal studies, have shown increasing concentrations of prostaglandins in follicular fluid approaching the time of ovulation. $PGF_{2\alpha}$ appears to play the more important role.

Contradictory evidence has been produced for prostaglandin levels in ovulatory and non-ovulatory follicles. The relationship between oocyte maturity and follicular fluid prostaglandin levels is also not established.

Fertilisation of oocytes is related to oocyte maturity but embryo quality is the vital factor for a successful outcome. The establishment of a pregnancy is the ultimate test of oocyte/embryo quality but thus far no consistent association has been made between follicular fluid prostaglandin levels and pregnancy outcome for the matched oocyte.

1.4 METHODOLOGY FOR PROSTAGLANDIN ANALYSIS

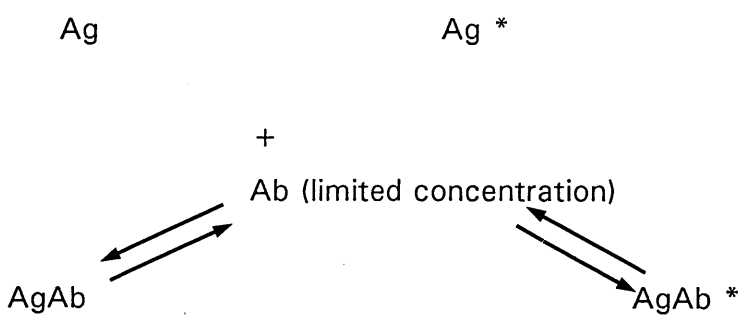
Prostaglandins are measured by radioimmunoassay, this being the most sensitive analytical method. The method combines the sensitivity of radioactive measurements and the specificity of the antigen-antibody reaction (Felber, 1982).

The radio-immunoassay method is a competitive binding technique and was devised originally for the measurement of insulin (Yalow & Berson, 1960). A radioactive tracer competes for antibody sites with the compound to be measured in radioimmunoassay. The amount of tracer bound to the antibodies is inversely proportional to the amount of compound being measured.

In fact, the system is based on the interrelationship between three substances: the antibody, the unlabelled and the labelled antigen, which are in equilibrium in the incubation medium. (Diagram 1.B)

DIAGRAM 1.B

BASIS OF RADIOIMMUNOASSAY



A brief synopsis of the methodology used by other authors to measure prostaglandins is given in Table 1.B.

TABLE 1.B

METHODOLOGY OF VARIOUS AUTHORS IN MEASUREMENT OF PROSTAGLANDINS

AUTHOR & YEAR	MATERIAL ANALYSED	EXTRACTION METHOD	CHROMATO-GRAPHY	RIA
Ainsworth 1975	Pig Follicular Fluid	Ethanol	No	Yes
Armstrong 1974	Rabbit Crushed Follicles	Ethanol	No	Yes
Bauminger 1975	Rat-Homogenised Follicles	Ether	No	Yes
Brown 1975	Rat Homogenised Follicles	Ethylacetate Acid	Silicic Chrom Mass Spec	Gas
Chabab 1986	Human Follicular Fluid	No	No	Yes
Clark 1976	Rat Homogenised Follicles	Ethylacetate Isopropanol	Silicic Acid	Yes
Darling 1982	Human Follicular Fluid	No	No	Yes
Espey 1984	Rabbit Homogenised Follicles	No	No	Yes
Evans 1983	Pig Granulosa Cells	No	No	Yes
Heinonen 1986	Human Follicular fluid	Isopropanol Ethyl-acetate	XAD-2 Resin	Yes
Jeremy 1987	Human Follicular fluid	Ethanol Resin	XAD-2	Yes
Koos 1983	Rabbit Follicular fluid	Ethylacetate	No	Yes
Lumsden 1986	Human Follicular fluid	No	No	Methyloxime Derivatives Yes

AUTHOR & YEAR	MATERIAL ANALYSED	EXTRACTION METHOD	CHROMATO-GRAPHY	RIA
Marsh 1974	Rabbit Homogenised Follicles	Ethylacetate Acid	Silicic	Yes
Patwardhan 1981	Human Homogenised Follicles	Ethylacetate Isopropanol	Micro-Silicic Acid	Yes
Reinthaller 1987	Human Follicular Fluid	Ethylacetate	Slurry of BIO-SILA	Yes
Rhodes 1985	Rabbit Follicular Fluid	No	No	Yes
Seibel 1984	Human Follicular Fluid	No	No	Yes
Triebwasser 1978	Rabbit Follicular Fluid	No	No	Yes
Tsang 1979	Pig Follicular Fluid	Ethanol	No	Yes
Yang 1974	Rabbit Homogenised Follicles	No	No	Yes

Table 1.B clearly demonstrates that radioimmunoassay is the accepted method for measurement of prostaglandins but that there is no overall agreement on sample preparation methods prior to assay.

1.5 AIM OF THE THESIS

Animal and human studies have indicated a role for prostaglandins in the process of ovulation. The relationship between follicular fluid prostaglandin levels and the outcome of the matched oocyte remains unclear in both animal and human studies. The main aim of these studies was "to establish the relationship between prostaglandins and oocyte potential" at the time of human IVF oocyte recovery.

Reliable methods for preparation of samples and the establishment of sound radioimmunoassays for PGE_2 and $PGF_{2\alpha}$ were to be developed.

The studies would initially consider all follicular fluids for analysis and then be refined to include only those from patients where there was a mechanical indication for IVF. Patients with evidence of endometriosis, a male factor problem or unexplained infertility would be excluded from the in depth analysis. Any differences in the concentrations of PGE_2 and $PGF_{2\alpha}$ in follicular fluid would also be studied for oocyte containing and non-oocyte containing follicles. Oocyte outcomes evaluated would be the subsequent fertilisation, or non fertilisation, cleavage rates and the grade of resultant embryos.

PGE_2 and $PGF_{2\alpha}$ and the steroid hormones, E_2 and P, would be measured in follicular fluid. Steroid results would also be analysed in relation to the outcome of the matched oocytes. Any correlation between steroid and prostaglandin levels would be investigated.
The pregnancy rate achieved during the programme was such that 'the pregnancy outcome of embryos could be assessed in relation to the above biochemical parameters'.

CHAPTER 2

IN VITRO FERTILISATION: GENERAL CONSIDERATIONS

2.1 OVARIAN HYPERSTIMULATION

Adequate ovarian stimulation is a pre-requisite for IVF. Multiple follicles must be stimulated to guarantee a sufficient number of recovered oocytes for the transfer of around three good quality embryos to achieve the highest pregnancy rates. The stimulation regime administered must provide the response required to meet these objectives.

'Clomiphene citrate (CC) and human menopausal gonadotrophin (hMG)' was considered to be the conventional treatment to stimulate the ovaries for in-vitro fertilisation. Many patients on this regime display an endogenous LH surge and this must be monitored continually to maintain the highest chances of success (Edwards, 1985).

Endogenous LH surges, if strong enough, may cause spontaneous ovulation and thus loss of oocytes or if weaker, and therefore more difficult to detect, may result in follicles that are luteinised at the time of oocyte recovery. This luteinisation causes abnormal fertilisation and embryonic growth with dispermic oocytes and fragmenting embryos.

Lejeune et al (1986) reported a significant difference in the number of oocytes retrieved after a spontaneous LH surge compared with the number retrieved if hCG was given, 57% of follicles yielding oocytes after an LH surge compared with 71% after hCG. The pregnancy rate was also significantly lower after an LH surge. Cycles, with a spontaneous LH surge were characterised by impaired follicular growth and E_2 secretion, dysmaturity of the oocyte - cumulus complex and reduced P levels in the early luteal phase.

Wang et al (1987) observed the same adverse effects. There was a significant reduction in the number of oocytes recovered and in turn the number of embryos transferred when an LH surge occurred compared with hCG administration with no LH surge occurring. These adverse effects combined with the difficulties both to patients and staff of continual monitoring for endogenous LH surges, encouraged us to seek an alternative stimulation regime.

Luteinising hormone releasing hormone analogues (LHRH-A) given in excess abolish endogenous LH production by inducing endogenous hypogonadotrophism, which can be exploited by induction of follicular growth with exogenous gonadotrophins (Fleming & Coutts, 1986). Therefore cycles do not have to be cancelled because of premature luteinisation of follicles and can be more easily monitored and controlled.

Serafini et al (1988), reported a significant improvement using the LHRH-A, leuprolide acetate and hMG, in patients who previously had responded poorly to CC and hMG. Oocyte recovery was successful in only 17.5% of these patients when stimulated with CC and hMG compared to 92.6% when leuprolide acetate and hMG were used.

The number of oocytes recovered is also significantly improved using an LHRH-A and gonadotrophins. Rutherford et al (1988) compared results in women receiving CC and hMG and women receiving the LHRH-A buserelin and hMG. The mean number of oocytes recovered was significantly higher with buserelin and hMG, 9.5 compared to 5.5 with CC and hMG. In addition the mean number of embryos obtained was significantly higher with buserelin and hMG, 4.3 compared to 2.9 with CC and hMG.

Neveu et al (1987) found similar improvements when comparing the use of buserelin and follicle stimulating hormone (FSH) with FSH alone. The number of oocytes recovered was significantly higher with buserelin and FSH.

Both Rutherford et al and Neveu et al reported higher pregnancy rates in patients receiving buserelin and gonadotrophins.

Thus buserelin in combination with gonadotrophins produces good quality fertilisable oocytes allowing a higher degree of selection of those embryos suitable for transfer. This was the regime adopted for ovarian stimulation in this programme.

2.2 CYCLE MONITORING

Ultrasound (U/S) measurement of follicular diameter and serial serum oestradiol levels are the standard monitoring modalities in IVF. Scanning can be performed transabdominally or transvaginally.

The success of IVF depends on the collection of mature, fertilisable oocytes. Therefore accurate monitoring methods for follicular development are essential to ensure the optimal timing of oocyte recovery.

All cycles are monitored biochemically, serum oestradiol concentrations being the most reliable steroid hormone indicator of an appropriate ovarian response.

Hackeloer et al (1979) showed a good correlation between peripheral serum E_2 levels and U/S measurement of follicular diameter in the unstimulated cycle, but Ritchie (1985) showed a deteriorating correlation with increasing numbers of stimulated follicles. Nevertheless, it is generally accepted that knowledge of the number of follicles is necessary for adequate interpretation of E_2 levels and it is not the absolute value of E_2 that is a good predictor of success (Jones et al, 1983).

Transvaginal U/S provides an improved quality of image over transabdominal U/S. This is attributed to the relatively short distance of 2-4cm between the vaginal vault and the ovaries compared to that between the abdominal wall and the ovaries with the intervening full bladder necessary for abdominal scanning.

Yee et al (1987) compared transabdominal and transvaginal U/S measurements with the findings at laparoscopy. The transvaginal route produced increased visualisation and resolution of follicles compared with the transabdominal route.

These are the reasons for this programme changing from transabdominal ultrasound monitoring to transvaginal monitoring during the first year of operation.

2.3 OOCYTE RECOVERY

When IVF became established as a recognised treatment for various forms of infertility, laparoscopy was the only method used for oocyte recovery. This was the technique developed by Steptoe & Edwards (1979).

Ultrasound - guided methods were later developed for patients with limited ovarian access and found to provide a viable alternative to laparoscopy (Lenz, Leeton & Renou, 1987, Wikland et al, 1987). Excellent results for the transabdominal, transvesical approach have been reported by Lewin et al (1986) and Riddle et al (1987).

Feichtinger & Kemeter (1986) and Wikland et al (1987) reported their results using the transvaginal route. Oocyte recovery rates were at least as good as other methods of recovery but with substantial benefits to patients.

General anaesthesia and a prolonged pneumoperitoneum as required for laparoscopic recovery were avoided. Thus the recovery time for the patient and the length of hospitalisation were shortened. In addition, Boyers et al (1987) has suggested a decreased fertilisation rate and Hayes et al (1987) a detrimental effect on cleavage associated with either general anaesthesia or CO_2 pneumoperitoneum.

The transvaginal route confers advantages over other ultrasound routes in the avoidance of a full bladder, the high acceptance rate, light sedation with ovarian access rarely a problem, the short puncturing distance and a decreased length of time for oocyte recovery (Janssen-Caspers et al, 1988). (Diagram 2.A)

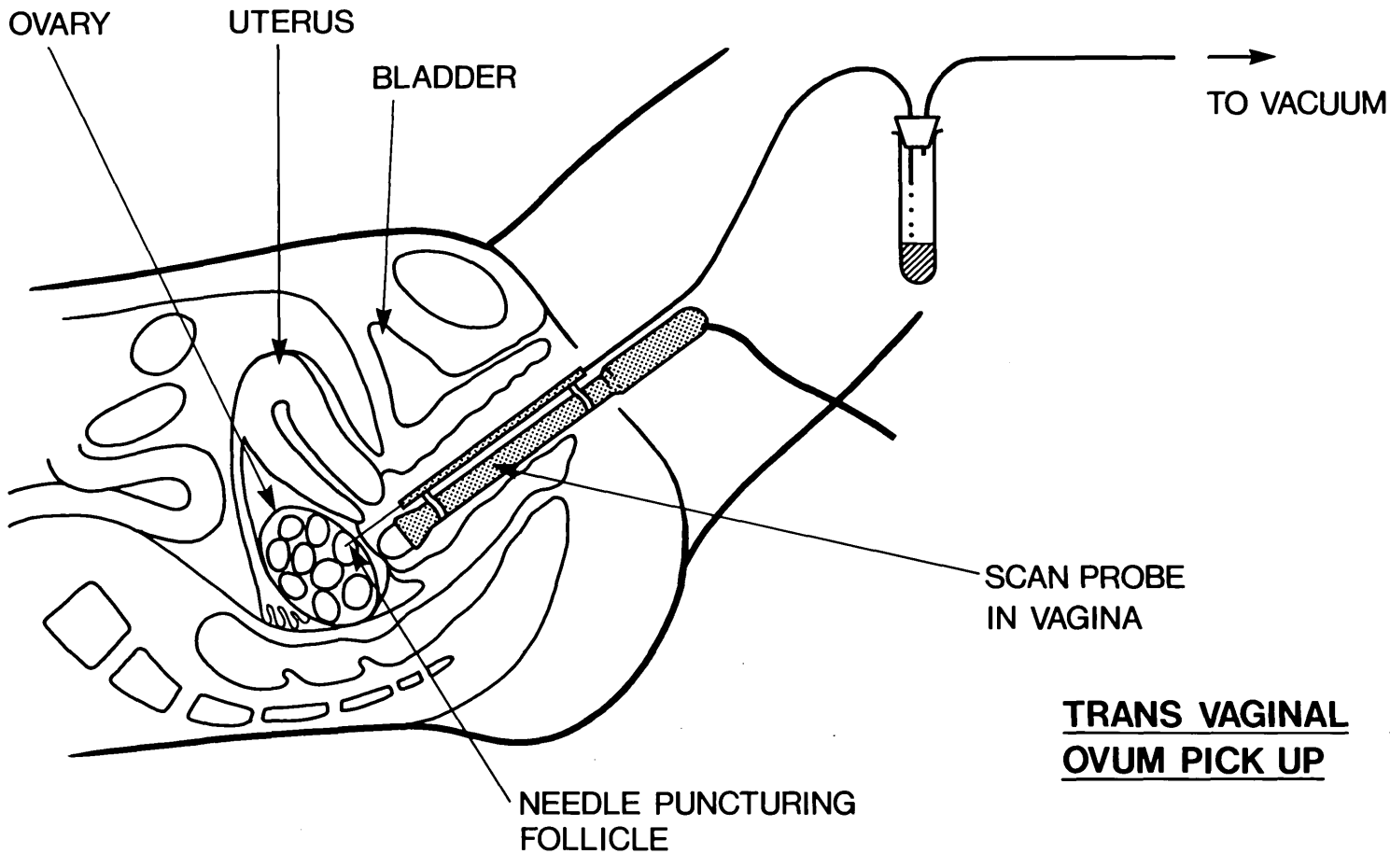


DIAGRAM 2A METHOD OF FOLLICULAR FLUID COLLECTION

2.4 LUTEAL PHASE

Luteal phase support following ovarian hyperstimulation and follicular aspiration for IVF has been the subject of great debate. Adequate production of progesterone by the corpus luteum is necessary for implantation of a pregnancy and its early maintenance (Csapo et al, 1972). It has been suggested that follicular aspiration, by removing granulosa cells during oocyte recovery, may cause deficiency in luteal phase function or, alternatively, that the ovulatory dose of hCG may provide inadequate stimulation of the corpora lutea. Yet it has been shown that follicular aspiration does not lead to lower P levels (Varygas, Kletzky & Marrs, 1986) and, because of the presence of multiple corpora lutea, progesterone levels after in-vitro fertilisation/embryo transfer (IVF/ET) are higher than in the natural cycle (Huang et al, 1986).

Pregnancy cycles are associated with higher P levels in the luteal phase (Jones et al, 1984) and many centres support the luteal phase on an empirical basis in an attempt to mimic the hormonal profiles of conception cycles. Hyperstimulation regimes consisting of hMG alone or in combination with CC have been followed by luteal phase supplementation with either P (Dlugi et al, 1985), hCG (Mahadevan, Leader & Taylor, 1985), or both P and hCG (Forman et al, 1988).

Pregnancy rates have shown no significant improvement although hCG did prolong the luteal phase (Bayly et al, 1985, Buvat et al, 1988), which was thought to exert a beneficial effect as poor pregnancy rates have been associated with regimes that lead to short luteal phases (Quigley, 1985).

Acceptable pregnancy rates are achieved with no luteal supplementation (Russell, Polan & de Cherney, 1986, Van Steirteghem et al, 1988). Thus, there is no clear evidence in the literature as to the beneficial effect of systematic supplementation of the luteal phase in IVF cycles where ovarian stimulation was achieved by CC plus hMG or hMG alone.

The combined use of gonadotrophin releasing hormone agonists (GnRH-A) and gonadotrophins is an effective and more controlled hyperstimulation regime (Rutherford et al, 1988). The endogenous hypogonadotrophism during induction of follicular growth with exogenous gonadotrophins, eliminates premature luteinisation (Fleming et al, 1986). This reversible pituitary suppression may influence the adequacy of the luteal phase in establishing a uterine environment suitable for nidation.

Some programmes using this form of therapy have supported the luteal phase, either with hCG (Neveu et al, 1987, Zorn et al, 1988) or with P (Serafini et al, 1988), but without using a control population. Similar pregnancy rates have been achieved with the use of either hCG or P (Van Steirteghem et al, 1988), again without a control population.

In order to evaluate the effect of support with hCG in the luteal phase following hyperstimulation with buserelin and hMG, patients in this programme were randomly allocated to receive support or no support according to whether the maturational dose of hCG was given on an odd or an even date. The pregnancy rate was significantly higher in the women who received support with hCG, 41% compared to 15% for those who were not supported (Smith et al, 1989).

All patients included in this study received luteal phase support in the form of hCG, 2500 IU administered on day +3 and day +6 (Day 0 = maturational dose of hCG).

CHAPTER 3

IN VITRO FERTILISATION: TECHNIQUES AND PROCEDURES, UNIVERSITY OF SOUTHAMPTON AND CHALYBEATE HOSPITAL IVF PROGRAMME

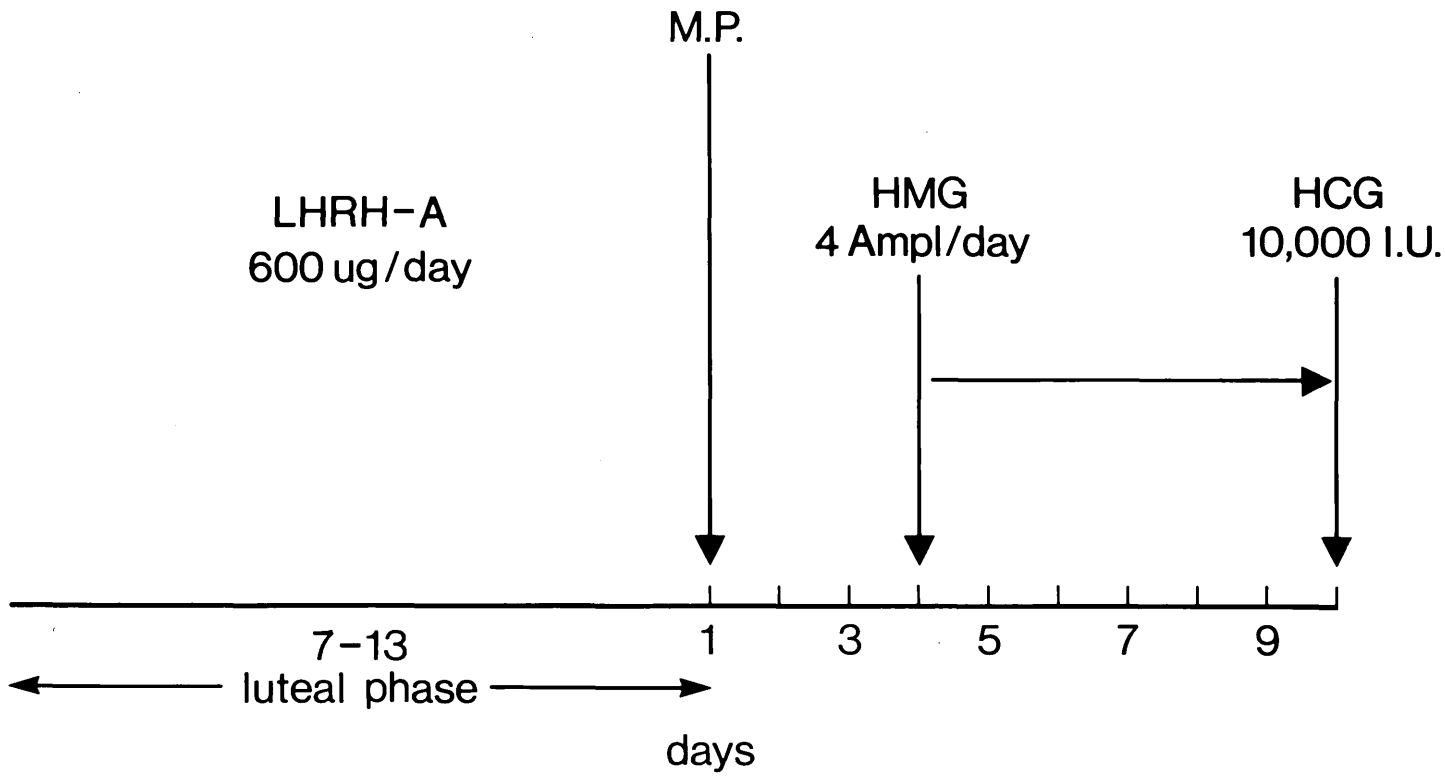
3.1 OVARIAN HYPERSTIMULATION

All patients whose follicular fluids were analysed for the purpose of these studies received the same stimulation regime.

The LHRH-A, buserelin was administered by nasal spray (200 μg three times daily) commencing midway through the luteal phase of the pre-treatment cycle. Following menstruation, hMG was given, 300 I.U. daily, from day 4 and continued until an adequate ovarian response was elicited. On average, this required 7 days treatment with hMG. If an adequate response had not been achieved after 9 days treatment, the cycle was abandoned. (Diagram 3.A).

Final oocyte maturation was induced with hCG (10,000 I.U.) and oocyte recovery performed 34 hours later.

DIAGRAM 3A OVARIAN HYPERSTIMULATION REGIME



3.2 CYCLE MONITORING

All treatment cycles were monitored biochemically and ultrasonically.

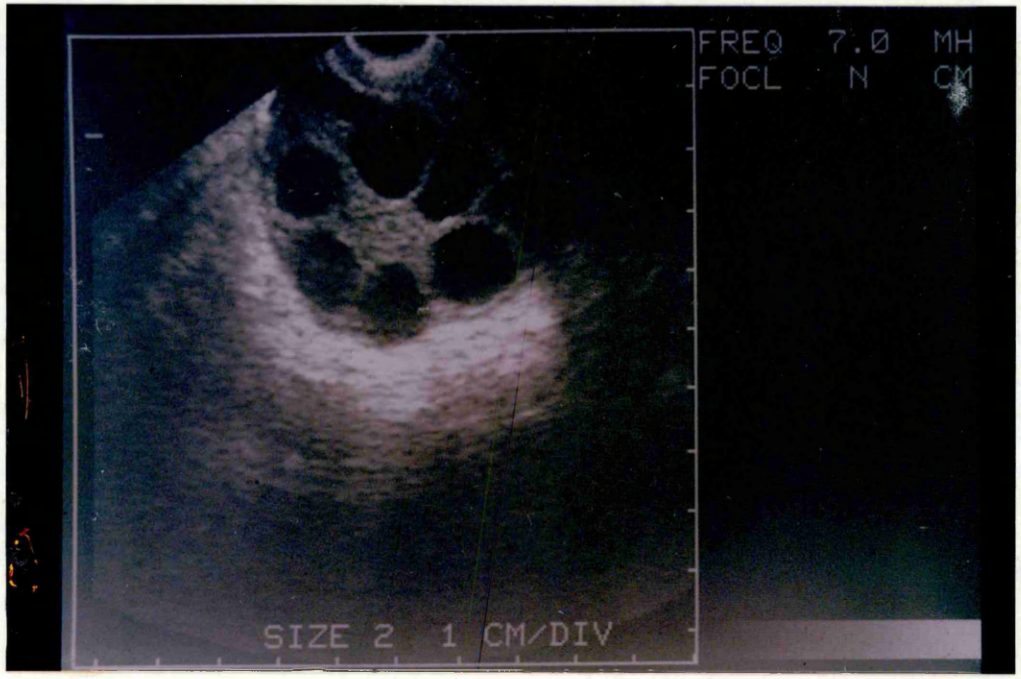
Biochemical monitoring consisted of measurement of serial serum E_2 levels. Blood samples were taken on days 4, 7 and daily from day 9 onwards. E_2 was measured by a commercially available radioimmunoassay (Serono Diagnostics, E_2 MAIA product number 12264).

Ultrasound monitoring for the earlier patients in the preliminary study was performed by the transabdominal transvesical route, using a Phillips SDU 7000,3.5 MHZ transducer.

Later patients in the preliminary study and all patients in the major study were monitored with transvaginal U/S, using a Bruel and Kjaer, 7 MHZ transducer.

An example of mature follicles as visualised with transvaginal U/S is shown in Figure 3.A.

FIGURE 3.A MATURE FOLLICLES USING TRANSVAGINAL U/S



3.3 OOCYTE RECOVERY

When the programme first started all oocyte recoveries were performed laparoscopically. The abdomen was cleaned with 0.015% chlorhexidine. Under general anaesthesia, a 2L CO_2 pneumoperitoneum was induced. Percutaneous aspiration of follicles was performed using a 14 gauge double lumen needle (Casmed UK, Surrey, UK). Follicles were aspirated using mechanical suction controlled by a foot pedal (Craft pump, Rocket, UK). If no oocyte was recovered in the aspirate, follicles were flushed with heparinised, serum-free culture medium (Earle's Gibco, UK) until an oocyte was detected or the flushes became cell-free. Oocytes (immediately following detection) were transferred to sealed tubes containing 1.5ml culture medium and placed in the incubator (Heraeus B5061 EK/CO_2).

Vaginal ultrasound was introduced during the first year of operation. Thereafter all patients underwent oocyte recovery by ultrasound guided transvaginal needle aspiration. Light premedication with oral lorazepam (1mg) was given 1 hour before the procedure. Bolus doses consisting of a combination of diazepam (2.5mg) and pethidine (25mg) were given intravenously during the procedure, if there was any patient discomfort.

The vagina was cleaned with 0.015% chlorhexidine and then washed out with culture medium. Contact gel was applied to the vaginal transducer and the transducer encased in a sterile sheath. A sterilised needle guide was attached on one side of the transducer and the puncturing needle, a 15 gauge, double lumen needle (Casmed UK, Surrey, UK) inserted through the guide.

The needle was advanced through the vaginal wall and into the ovary. Follicles were aspirated in turn without withdrawal of the needle from the ovary, using the same mechanical suction and flushing medium as for laparoscopic recoveries.

3.4 LABORATORY PROCEDURES

All flushing medium used was Earle's Balanced Salt Solution supplied by Gibco UK, 10 x concentrate (Cat. No. 042-04050 H) with additions as follows:

Bicarbonate: 4 mmol/L (Sigma, UK)
Pyruvate: 0.1 mmol/L (Sigma, UK)
HEPES: 21 mmol/L (Sigma, UK)
Penicillin: 60 mg/ml
Streptomicin: 50 mg/ml
Heparin: 10,000 U/L

Osmolality 284 $\pm$ 2 mOsm/kg.

Flushing medium was made up in batches, filtered using Sterivex Filter 0.22Xm filter unit (Millipore, UK) and stored up to a maximum of 14 days.

The aspirated fluid from follicles, either initial aspirate or subsequent flushes, was poured into sterile Falcon tissue culture dishes and examined microscopically.

The recovered oocyte was placed in a 4ml Falcon tube containing 1.5ml culture medium which had been equilibrated overnight under 5% CO_2 and placed in the incubator.

The culture medium was supplied by Gibco, UK. Earle's Balanced Salt Solution, 10 x concentrate with the following additions:

Bicarbonate: 25 mmol/L (Sigma, UK)
Albumin: 10% (Albuminar 5, Armour Pharmaceutical Company Ltd, UK)
Pyruvate: 0.1 mmol/L
Penicillin: 60 mg/ml
Streptomicin: 50 mg/ml

Osmolality 284 $\pm$ 2 mOsm/kg.

At the end of the oocyte recovery, the oocytes were transferred to organ culture dishes containing 1ml culture medium and covered with 0.8ml light liquid paraffin (Kirby Warrick, UK). These dishes had been equilibrated overnight with 5% CO_2.

Oocytes were pre-incubated for 6 hours prior to insemination.

After waiting for semen liquefaction to occur, (approximately 10 minutes in the majority of cases) 3 volumes flushing medium containing 10% albumin were added to the fresh semen sample. This was centrifuged at 200xg for 10 minutes. The supernatant was discarded and the pellet resuspended in 0.5ml culture medium.

100μl of the suspension was layered under 1.5ml culture medium in Falcon tubes which had been pre-equilibrated. The spermatozoa were allowed to swim-up (approximately 30 minutes) and the top two thirds removed and used for insemination.

Approximately 1 x 10^5 normal motile sperm were added to each oocyte containing dish. The following morning (approximately 18 hours later) oocytes were examined microscopically for the presence of pronuclei.

The cumulus around the oocyte was stripped off using fine pipettes, hand-drawn, and the number of pronuclei noted. The fertilised oocytes were each then transferred to a fresh culture dish, which had been pre-equilibrated.

On the day of embryo-transfer (approximately 42 hours post-insemination) the fertilised oocytes were examined microscopically to establish cleavage and the embryos graded in terms of quality.

GRADE 1

All blastomeres have equal size without anucleate fragments.

GRADE 2

Not all blastomeres have an equal size and anucleate fragments present in at most 20% of the embryo.

GRADE 3

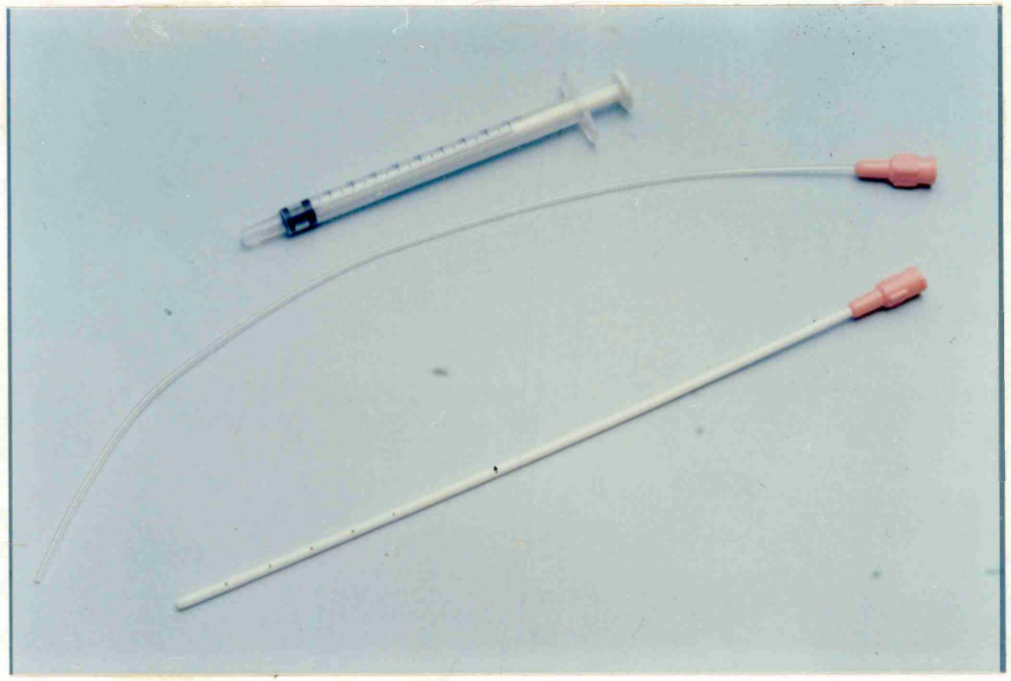
Not all blastomeres have an equal size and anucleate fragments are present in 20-50% of the embryo.

GRADE 4

Totally fragmented embryo present.

Immediately prior to the embryo-transfer, embryos selected for the transfer were placed in a Falcon tube, containing 0.6ml albumin and 0.4ml culture medium. The transfer catheter (Wallace, UK) was washed through with culture medium. The embryos were drawn up into the catheter in 20 μl medium with 10 μl air bubbles at either end. A 1ml syringe was used on the end of the catheter. Figure 3.B shows a Wallace Transfer Catheter.

FIGURE 3.B WALLACE EMBRYO TRANSFER CATHETER

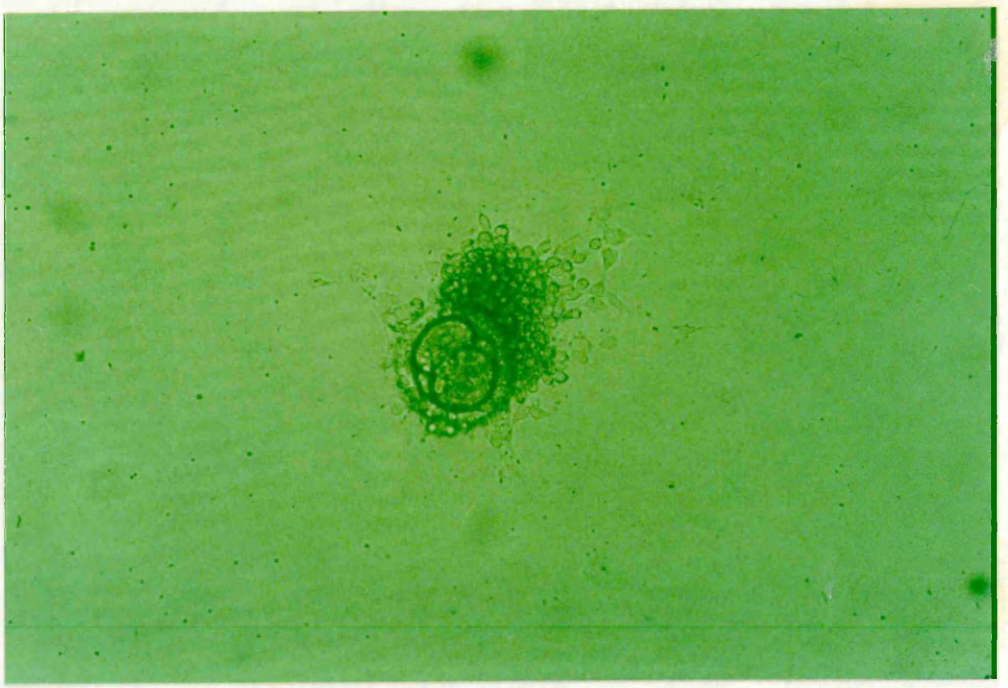


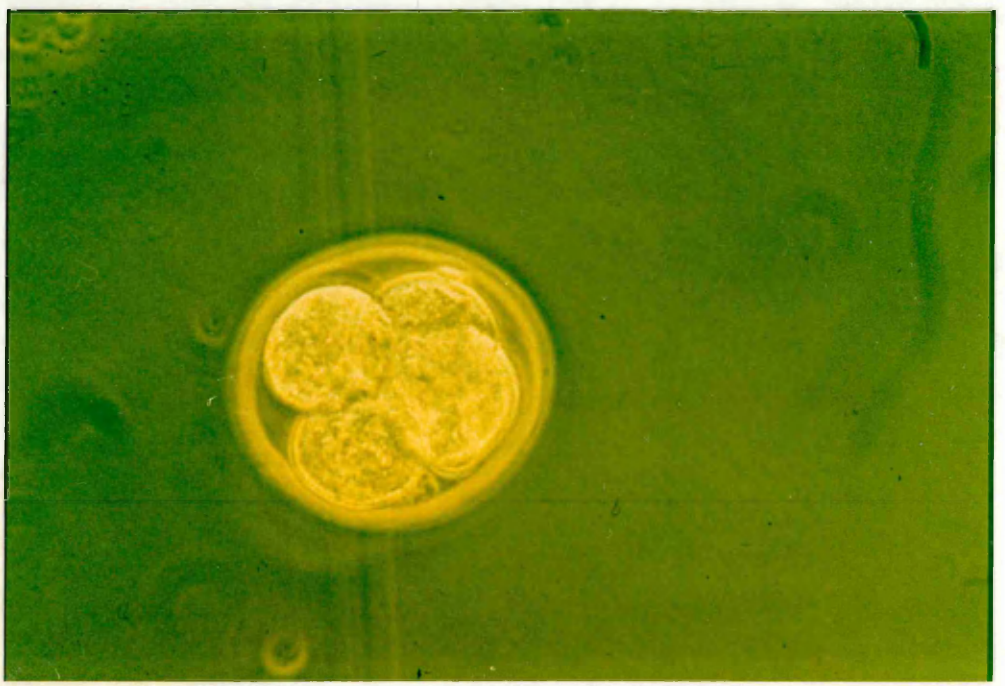
3.5 EMBRYO TRANSFER

Embryo transfers were performed 42-46 hours post insemination. The patient was placed in the lithotomy position. A bivalve speculum, soaked in culture medium, was inserted into the vagina and adjusted to obtain the optimal view of the cervix. The cervix was cleaned with culture medium. The end of the outer sheath of the catheter was placed at the external os and the inner catheter passed through the internal os into the uterus. The preset syringe was depressed and the embryo(s) transferred.

The catheter was rotated and then withdrawn. The catheter was examined under the microscope to ensure all embryos had been transferred. Figure 3.C shows typical 2 cell and 4 cell grade 1 embryos.

FIGURE 3.C 2 CELL AND 4 CELL GRADE 1 EMBRYOS





3.6 LUTEAL PHASE

All patients undergoing oocyte recovery received hCG luteal phase support.

hCG support was given in a dose of 2500 I.U. on day +3 and on day +6 from the initial hCG (day = 0).

Blood samples were taken on days +1, +6 and +12 for estimation of serum E_2 and P. P was measured by a commercially available radioimmunoassay (Amersham International, Product No. 1M.3221).

SECTION 2

DEVELOPMENT

CHAPTER 4

ASSAY DEVELOPMENT AND EVALUATION

4.1 OBJECTIVES

a) To set up a reliable extraction process for PGE_2 and $PGF_{2\alpha}$ in follicular fluid.

b) To develop radio-immuno assays (RIA) for $PGF_{2\alpha}$ and PGE_2.

c) To examine the effect of storage of samples at -20°C prior to assay.

4.2 EXTRACTION MATERIALS AND METHODS

SAMPLE STORAGE

Prior to analysis all samples were stored at -20°C (except for a proportion of samples to establish effects of freezing).

METHOD

In the past the most common method for extracting prostaglandins has been to extract acidified aqueous solutions with organic solvents, such as diethyl ether or ethyl acetate. Prostaglandins can be extracted from body fluids, such as plasma or urine, by chromatography on columns of XAD-2 resin. (See Chapter 1.4). Both methods are time-consuming and require evaporation of relatively large amounts of solvent.

The procedure developed by Powell (1982) using octadecylsilyl (ODS) silica has been modified for extraction of prostaglandins from follicular fluid. ODS silica is a hydrophobic material formed by allowing silicic acid to react with octadecyltrichlorosilane.

Prostaglandins are adsorbed by reversed phase chromatography followed by elution by normal phase chromatography using a series of increasingly polar organic solvents that are not miscible with water. Extraction of prostaglandins from aqueous media using ODS silica has two main advantages. A relatively clean extract is obtained and secondly prostaglandin fractions can be obtained in relatively small volumes of volatile organic solvents that can be readily evaporated under a stream of nitrogen.

MATERIALS

Radiolabelled eicosanoids were obtained from Amersham International [5,6,8,11,12,14,15(n) -^{3}H] PGE_2 (Code Number TRK.431, Batch 77) [5,6,8,9,11,12,14,15(n) -^{3}H] $PGF_{2\alpha}$ (Code Number TRK. 464, Batch 63). Cartridges of ODS silica were obtained from Waters Associates, UK (Sep-pak C18 cartridges).

Prior to extraction, follicular fluid samples were acidified to pH 3-4 using 0.5M citric acid. Water was added to each 1-2ml sample to increase the volume to 5ml. The radiolabelled eicosanoids, 10 μC_i/ml, were diluted 1 in 400. 100 μl of either eicosanoid, approximately 5000 ^{3}H dpm, was added to each sample for the determination of procedural losses (mass 5pg).

It is necessary to wet the ODS silica with an organic solvent prior to use. Ethanol activates the column and the excess ethanol is then removed by passing water through the column.

To - ODS Silica
add
10ml Ethanol
then
10ml Water

Following the column washing process, the samples were applied to ODS silica with these initial and subsequent steps:

Fresh or thawed follicular fluid
samples to be extracted

are

Centrifuged in a Damon IEC centrifuge
200 x g for 10 mins

then samples are

Acidified with citric acid

before application to

ODS Silica, Sep-pak column

to the column is then applied

10ml 15% Ethanol:85% Water

followed by

10ml Petroleum Ether (40-60% fraction)

and the prostaglandins are eluted with

10ml Diethyl Ether

See Figure 4.A.

FIGURE 4.A APPARATUS FOR EXTRACTION OF PROSTAGLANDINS

Follicular fluid was applied directly to the column after acidification. 15% aqueous ethanol elutes materials more polar than prostaglandins. Water remaining in the column is removed by passing through petroleum ether. Finally the prostaglandins were eluted from the ODS with diethyl ether.

The diethyl ether fraction was evaporated to dryness under nitrogen at 30°C and the residue reconstituted in 2ml RIA buffer.

The reagents used to make up the RIA buffer were:

K_2HPO_4 (0.1M)	17.4 g litre -1
Gelatine (0.1%)	1.0 g litre -1
EDTA (20 mM)	7.5 g litre -1
Sodium Azide (0.1%)	1.0 g litre -1
NaCl (0.9%)	9.0 g litre -1

The final pH was adjusted with NaOH to pH 7.5.

4.3 DETERMINATION OF PROCEDURAL LOSSES

For every sample:-

Recovery values for the extraction were determined for each of the two eicosanoids assayed. 0.1ml of a 1 in 400 dilution of tracer, 10 μC_i/ml was added to the sample before extraction, (approximately 5000 dpm). Two count controls consisting of 100 μl diluted isotope solution in 5ml scintillant (Beckman's Ready Safe Solution) were prepared with each extraction. A blank sample (1ml RIA buffer made up to 5ml with water) was included with each set of 24 follicular fluid samples. For each extracted sample (Section 4.2) reconstituted in 2 ml RIA buffer, 20% of the volume (i.e. 0.4ml) was counted for ^3H, with 5ml scintillant in an LKB Minibeta Liquid Scintillation Spectrometer (Model 1211). Generally the volume of follicular fluid extracted was 1-2ml. The minimum volume required was 0.5ml.

In addition:-

A known quantity of PGE_2 (5000pg) was added to 10 follicular fluids previously extracted and assayed. This was repeated for $PGF_{2\alpha}$. 82% (+/- 3%) and 84% (+/- 4%) respectively of the known quantity of prostaglandin added was recovered.

4.4 RADIO-IMMUNO ASSAY MATERIALS AND METHODS FOR PROSTAGLANDINS

The principal of RIA depends on the competitive binding of radio-actively labelled antigen with unlabelled antigen to immunological sites of a specific antibody.

The concentration of the antigen in an unknown sample is determined by calculating the degree of inhibition of label bound to antibody that the antigen causes and comparing it to that caused by a known concentration of the same substance used as a standard.

MATERIALS

STANDARDS:

PGE_2 and $PGF_{2\alpha}$ were supplied by Sigma, UK.

PGE_2 (Product Code No. P5640)

$PGF_{2\alpha}$ (Product Code No. P3023)

Both standards were diluted in RIA buffer to provide a working range of 6.25 pg/ml to 1600 pg/ml. In addition it was possible to measure higher concentrations of both prostaglandins by diluting samples appropriately in assay buffer.

ANTIBODIES:

Both antibodies were supplied by Dr Keith Hillier, Department of Pharmacology, University of Southampton.

PGE_2 (Code Number KHE9)

$PGF_{2\alpha}$ (Code Number KHF10)

Antibodies were raised in rabbits using the method of Salmon and Karim (1976) after conjugation of eicosanoids to BSA using 1-ethyl-3 (3-dimethyl aminopropyl) carbodiimide (Caldwell, Burstein & Brock, 1971).

An antiserum titration curve was constructed to choose the adequate antiserum dilution, the antiserum being added in increasing dilution to a series of tubes all containing the same concentration of the tracer. (See Figures 4.B and 4.C)

The antibodies were stored in aliquots at -20°C and diluted on the day of the assay in RIA buffer to give 35-50% maximum binding of labelled eicosanoid per 0.1ml antibody, after overnight incubation. This percentage binding is generally that giving the highest sensitivity and reproducibility.

The cross reactivity for PGE_2 antiserum was 29.6% for PGE_1, 2.5% for PGB_2 and 2.1% for 15-keto - PGE_1. Cross reactivity for other prostaglandins and their metabolites or fatty acids was less than 1%.

FIGURE 4B ANTISERUM-TITRATION CURVE - PGE_2

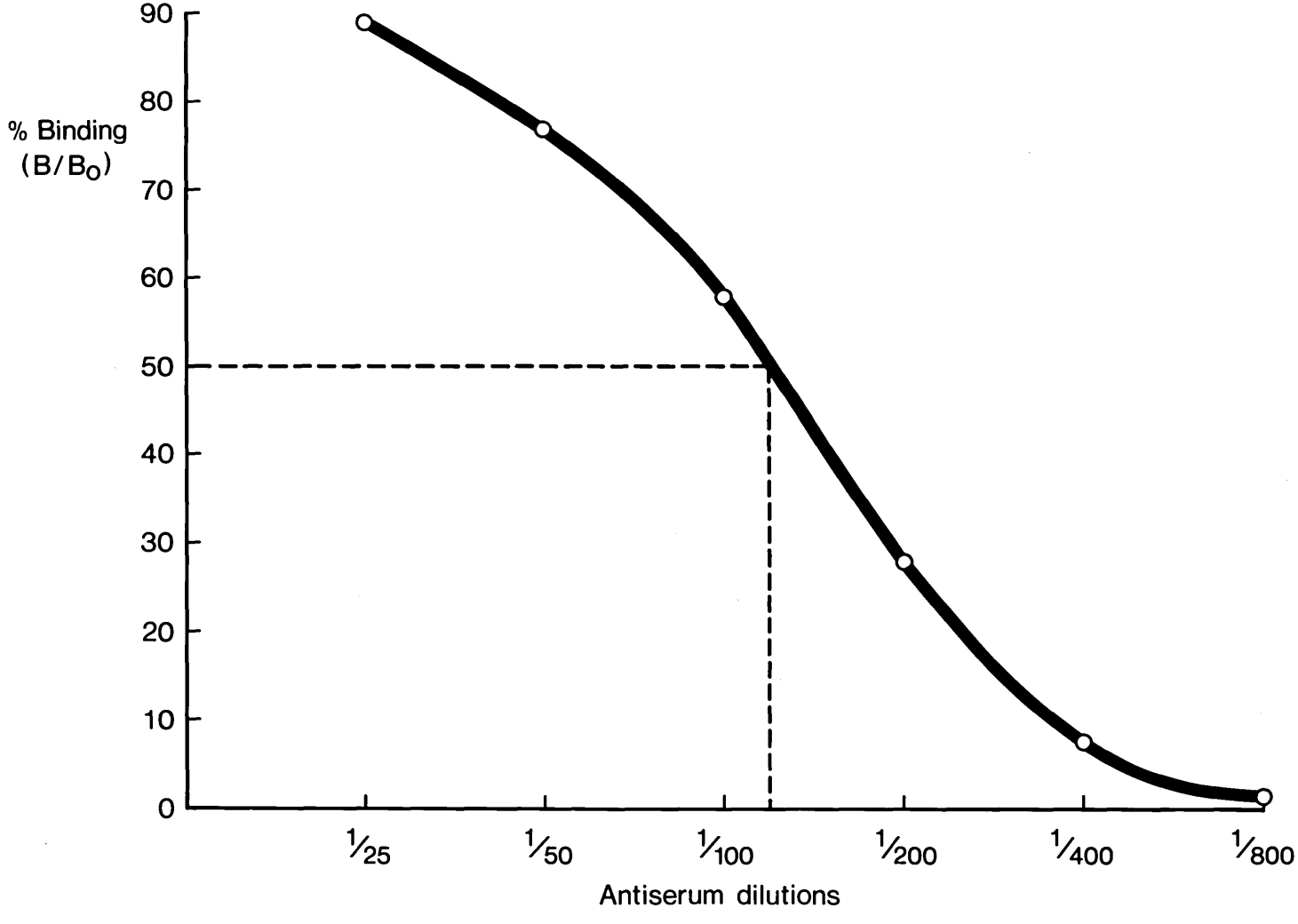
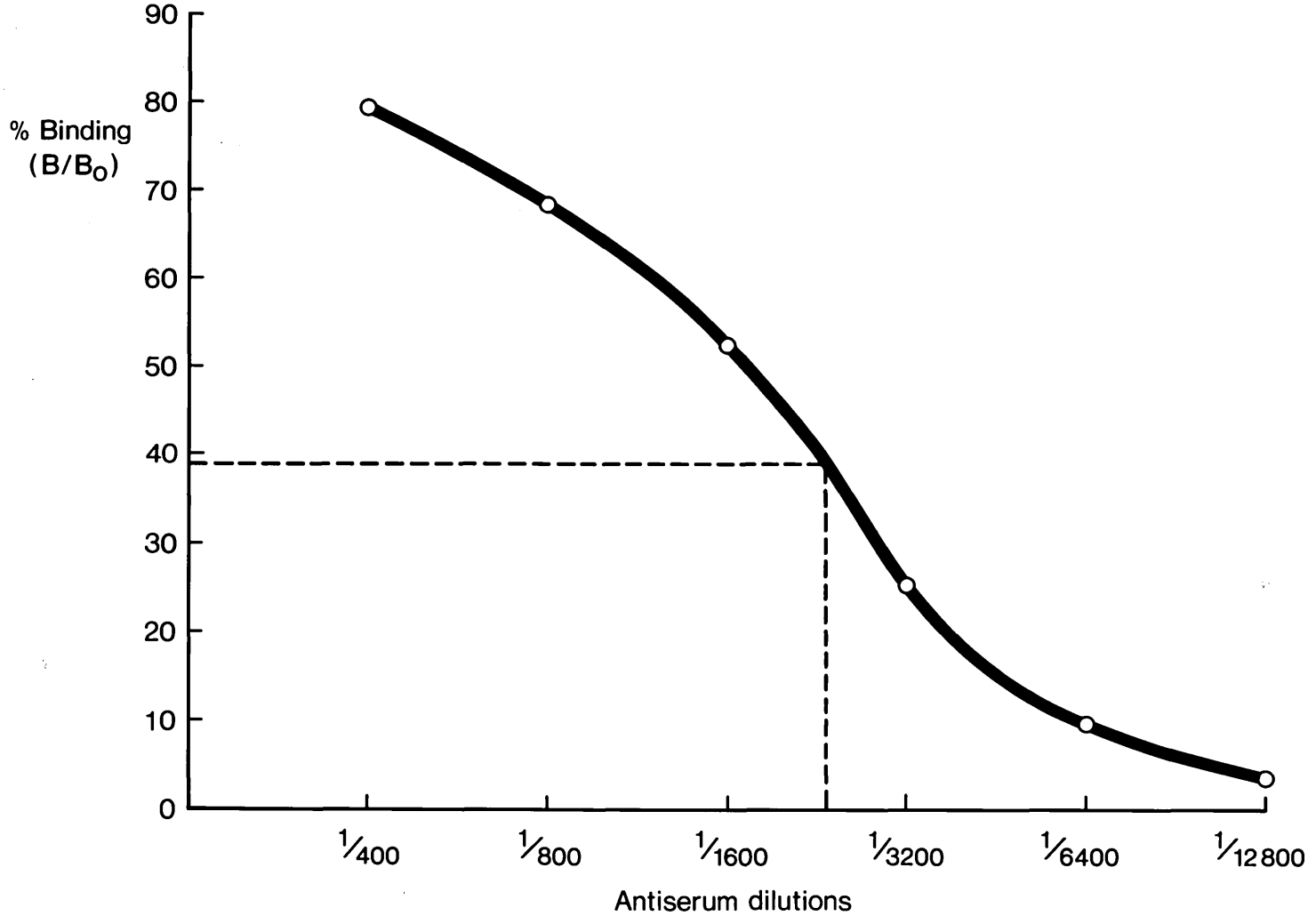


FIGURE 4C ANTISERUM-TITRATION CURVE - $PGF_{2\alpha}$



The cross reactivity for $PGF_{2\alpha}$ antiserum was 6.7% for PGD_2, 3.8% for $PGF_{1\alpha}$ and less than 1% for other prostaglandins and their metabolites or fatty acids.

RADIOLABELLED EICOSANOIDS

[5,6,8,11,12,14,15(n)-^{3}H] PGE_2 (Code Number TRK.431, Batch 77) and [5,6,8,9,11,12,14,15(n)-^{3}H] $PGF_{2\alpha}$ (Code Number TRK.464, Batch 63) were both obtained from Amersham International.

Both isotopes were diluted in ethanol:water, 7:3 to 10 μC_i/ml and stored under nitrogen at -20 °C. The specific activity was 185 C_i/mmol to achieve maximum radioactivity with the least mass. Both isotopes were diluted in RIA buffer on the day of the assay to give a 1 in 100 dilution, which was approximately 20,000 dpm per 0.1ml. (This was equivalent to 7,000 cpm in the beta counter, i.e. efficiency ~ 35%.) (Mass 20pg)

METHODS

The assay consists of three steps: incubation, separation and radioactivity measurement.

Incubation was performed in a series of test tubes (See Table 4.A).

In summary, a series of tubes, in duplicate, were set up for the different controls and the standard curve:- 1. total radioactivity, with no antibody; 2. measurement of non-specific binding, with the antibody replaced by buffer; 3. maximum binding for set antibody amount (zero standard), with buffer in place of unlabelled antigen; 4. antibody and the unlabelled antigen in serial dilution for the standard curve; 5. sample of known concentration for quality control and finally 6. the unknowns were measured after this series of controls and standards.

TABLE 4.A

RIA INCUBATION: CONTROLS, STANDARD CURVE AND UNKNOWNS

TUBES FOR:	STANDARD OR UNKNOWN	ANTIBODY OR BUFFER	TRACER
Total Count	Buffer	Buffer	Tracer
Non-Specific Binding	Buffer	Buffer	Tracer
Zero Standard Binding	Buffer	Antibody	Tracer
Standard Curve	Standards in Serial Dilution	Antibody	Tracer
Control	Known Sample	Antibody	Tracer
Unknowns	Unknown Sample	Antibody	Tracer

Following overnight incubation at 4°C, separation was performed using 0.25ml of dextran-coated charcoal (1.25% charcoal, Sigma, Product Code Number C5260 with 0.25% dextran, Sigma, Product Code Number D5501, in RIA buffer) added to all tubes except the total counts. The tubes were vortexed and left for 10 minutes at 4°C to allow adsorption by the charcoal of the eicosanoid not bound to the antibody.

The samples were then centrifuged at 500xg for 10 minutes at 4°C in a Damon IEC centrifuge and the supernatant decanted into 5ml scintillation fluid. These were then counted for ^{3}H as for recoveries.

The final assay protocol for the RIA of both prostaglandins was as follows:

To: 0.1ml assay buffer alone (for blank and zero standard) or standards S1-S9 in assay buffer (6.25 to 1600pg/ml) or sample to be measured (control and unknowns)

Add

0.1ml tracer

and

0.1ml 1:125 dilution of anti-PGE_2

or

0.1ml 1:2500 dilution of anit-$PGF_{2\alpha}$

Incubate overnight at 4°C

Add

0.25ml dextran-coated charcoal
Centrifuge at 500xg for 10 minutes at 4°C
Decant supernatant into 5ml scintillation fluid
Count solution in a beta counter

(Every determination was run in duplicate.)

4.5 CALCULATION OF RESULTS

PROCEDURAL LOSSES:

Recovery values were determined for each of the prostaglandins assayed.

20% of the reconstituted sample was counted for ^{3}H with 5ml scintillant.

Recoveries were calculated as:

$$\% \text{ recovery for each sample} = \text{cpm} \times \frac{\text{total volume}}{\text{volume assayed}} \times \frac{1}{\text{TC}} \times \frac{100}{1}$$

TC = total counts added

Recoveries all fell within the range 80-95%.

Median = 83.35

RADIOIMMUNOASSAY:

The zero standard binding is the maximum binding between antibody and tracer in the absence of unlabelled antigen.

This was calculated as:

$$\frac{Bo-Bns}{TC} \times \frac{100}{1}$$

This was kept within the range 35-50%.

Non specific binding (NSB) was calculated as:

$$NSB(\%) = \frac{NSB}{TC} \times \frac{100}{1}$$

Percentage bound in standards and samples (B/Bo) was calculated as:

$$B/Bo(\%) = \frac{B - Bns}{Bo - Bns} \times \frac{100}{1}$$

NSB	:	Non-specific binding
B/Bo	:	Percentage maximum binding
Bo	:	Maximum binding counts
TC	:	Total Counts
Bns	:	Non-specific binding counts
B	:	Sample or standard counts

A standard curve (B/Bo plotted against known prostaglandin concentrations) was constructed. The sample prostaglandin concentration was calculated from the B/Bo. Results were expressed using the equation shown in Diagram 4.A.

DIAGRAM 4.A EQUATION FOR EXPRESSION OF PROSTAGLANDINS RESULTS

$$\text{TOTAL ng/ml} = \frac{\text{pg}}{1000} \times \frac{\text{total volume}}{\text{assayed volume}} \times \frac{100}{\%\ \text{recovery}} \times \frac{1}{\text{original volume}}$$

Conversion to ng

dilution factor

procedural losses factor

volume factor

ASSAY CHARACTERISTICS

Composite standard curves calculated from 45 assays are shown in Figures 4.D and 4.E. The mean and standard deviations are given in Tables 4.B and 4.C. The assay detection limit (blank sample value minus 2 standard deviations) was less than 100 pg/ml. 50% displacement of radiolabelled tracer occurred at 1600 pg/ml for PGE_2 and 700 pg/ml for $PGF_{2\alpha}$. The intra-assay coefficient of variations were 4% and 5% for PGE_2 and $PGF_{2\alpha}$ respectively and the inter-assay coefficient of variations were 7% and 8% for PGE_2 and $PGF_{2\alpha}$ respectively.

TABLE 4.B

BINDING OF STANDARD PGE_2

STANDARD pg/ml	B/Bo (%)	SD
16000	9.2	0.8
8000	16.4	0.7
4000	28.2	0.5
2000	46.3	0.8
1000	60.2	0.8
500	74.5	0.6
250	88.1	0.6
125	92.1	0.4
62.5	94.0	0.7

FIGURE 4D COMPOSITE STANDARD CURVE FOR PGE_2

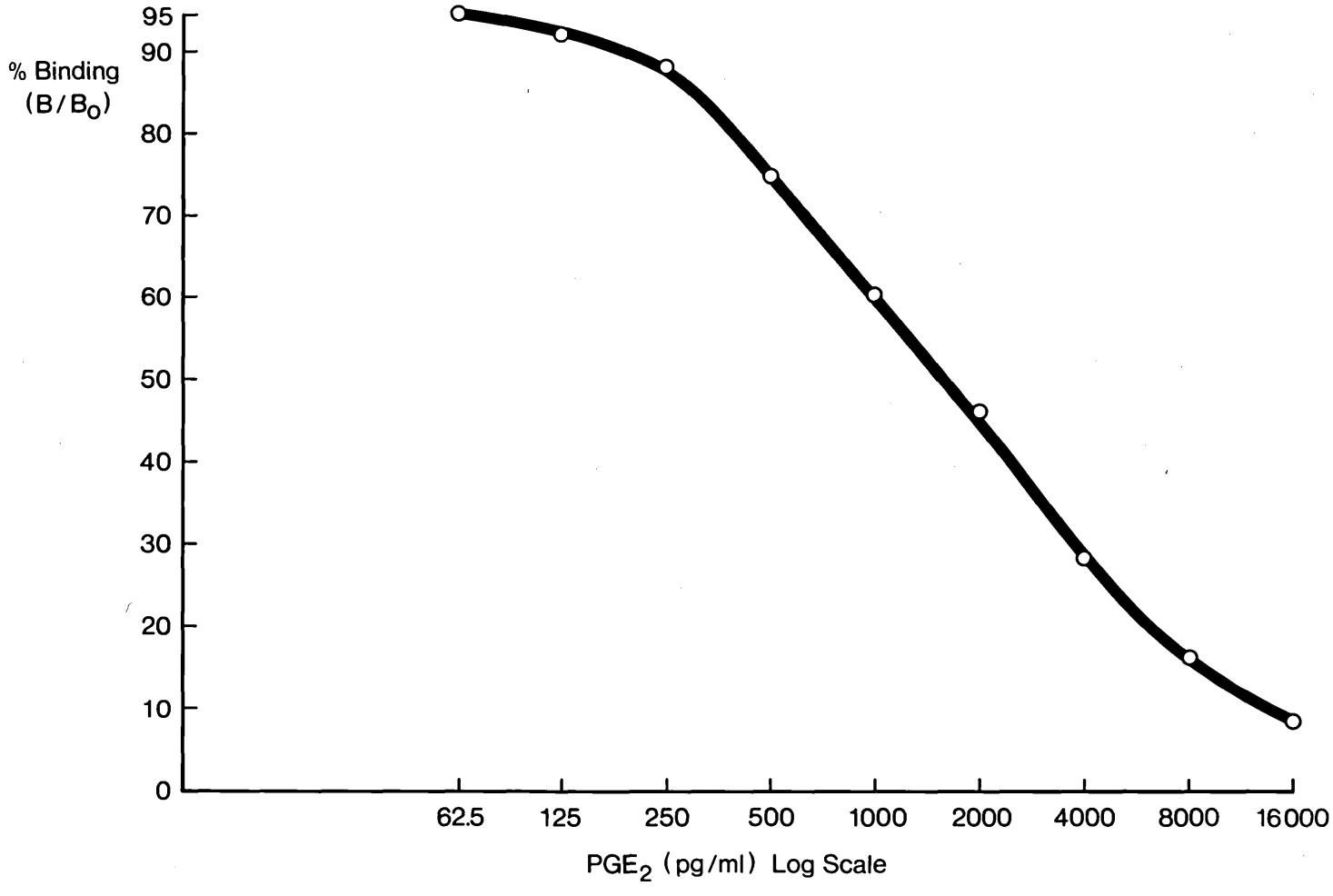


FIGURE 4E COMPOSITE STANDARD CURVE FOR $PGF_{2\alpha}$

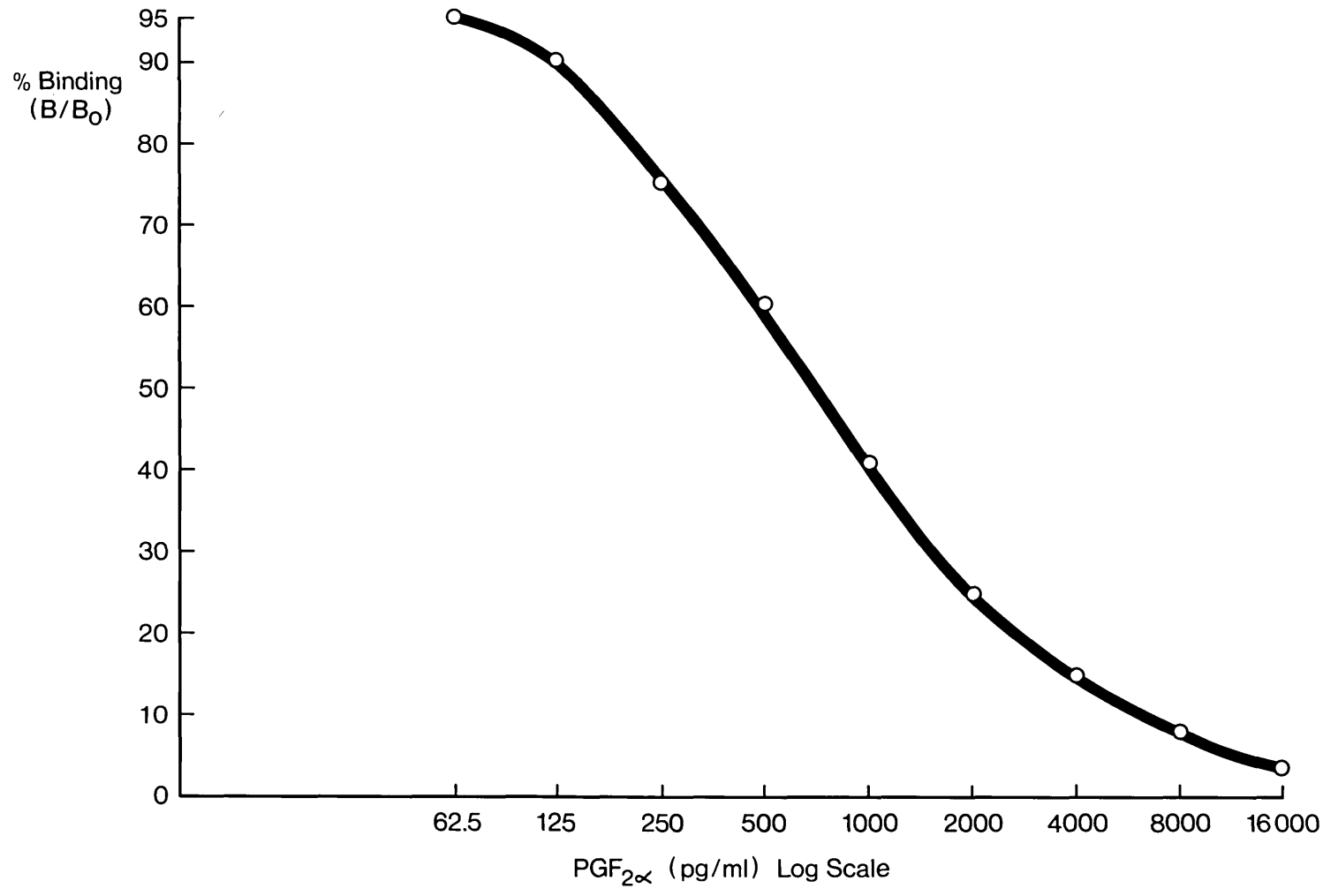


TABLE 4.C

BINDING OF STANDARD $PGF_{2\alpha}$

STANDARD pg/ml	B/Bo (%)	SD
16000	4.3	0.8
8000	8.2	0.7
4000	15.1	0.7
2000	25.6	0.5
1000	41.2	0.7
500	60.3	0.8
250	75.1	0.8
125	90.2	0.6
62.5	94.8	0.7

Extracted prostaglandins were serially diluted in assay buffer between 1:2 and 1:32. The binding of extract serial dilutions (Figures 4.F and 4.G) ran parallel to the standard curves for PGE_2 and $PGF_{2\alpha}$.

FIGURE 4F SERIAL DILUTION OF PGE_2

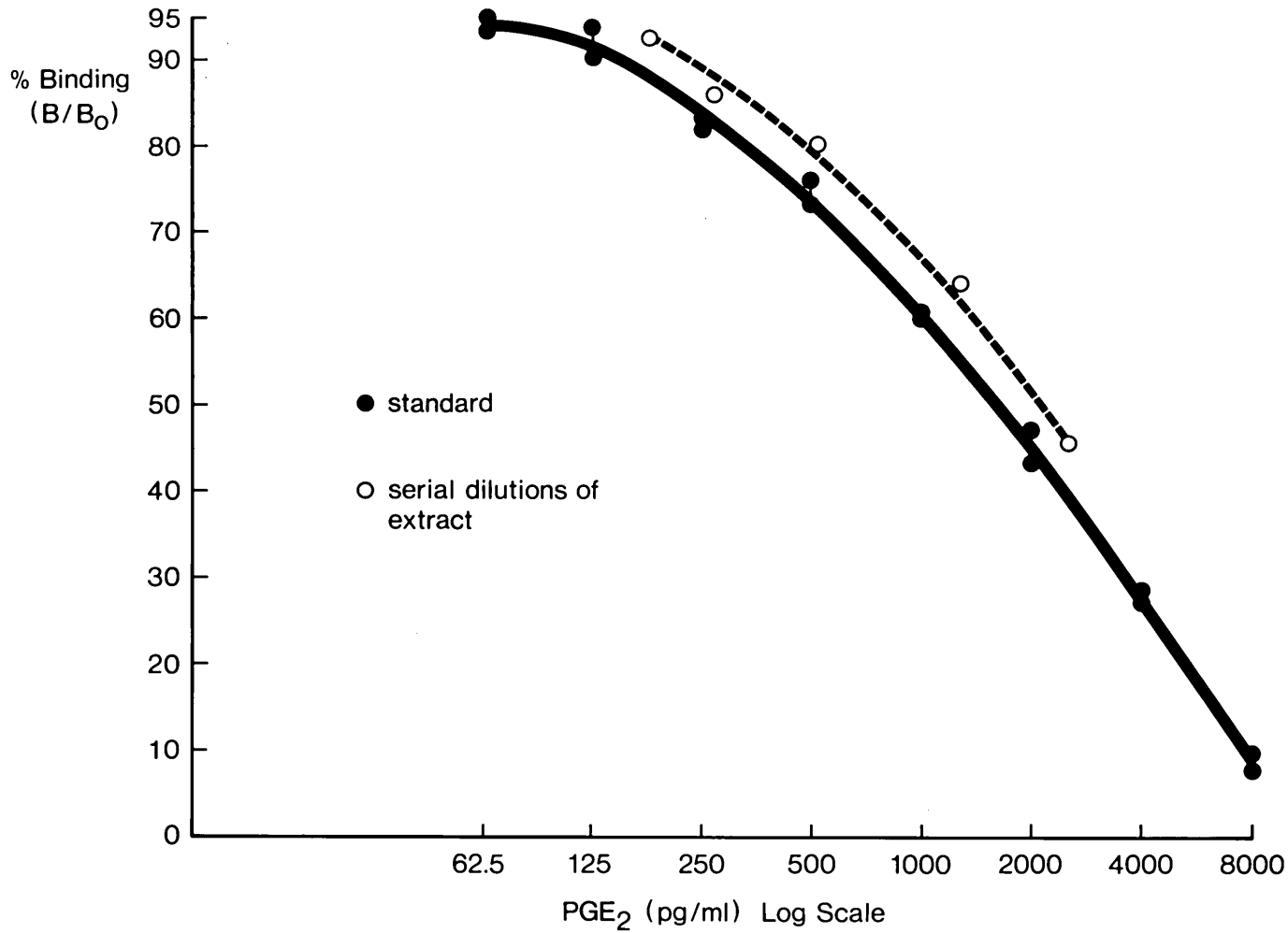
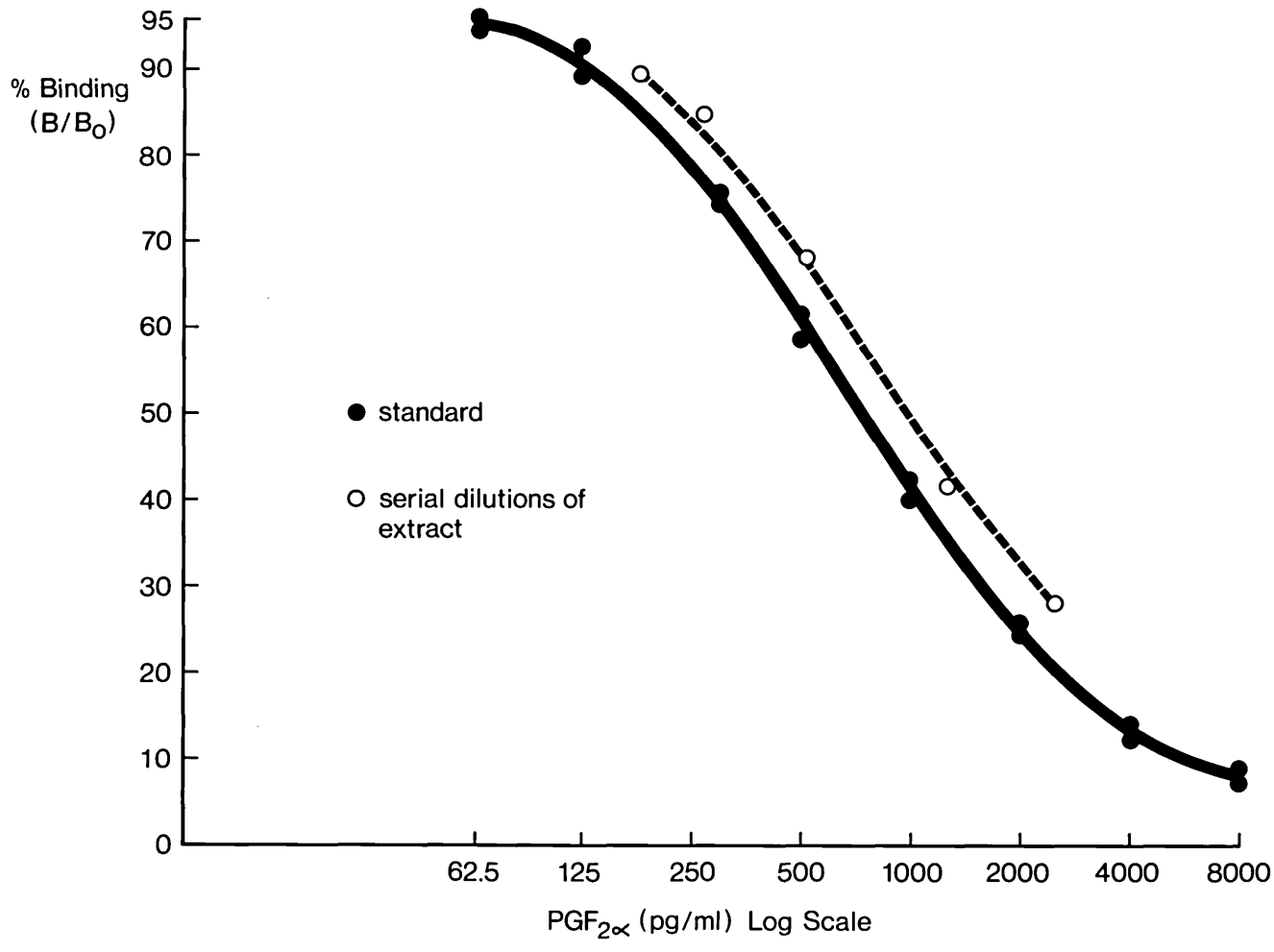


FIGURE 4G SERIAL DILUTION OF $PGF_{2\alpha}$



4.6 COMPARISON OF EXTRACTION AND NON EXTRACTION OF FOLLICULAR FLUID

To establish whether an extraction process is necessary prior to radioimmunoassay of prostaglandins 10 samples were equally divided. One half of each sample was extracted and assayed and compared with the other half assayed without prior extraction on mini columns (Sep-pak). (Table 4.D)

TABLE 4.D

UNEXTRACTED: EXTRACTED RATIO FOR $PGF_{2\alpha}$

SAMPLE	NGML-1 EXTRACTED (E)	NGML-1 UNEXTRACTED (U)	%U/E X 100
1	0.65	<0.1	<15%
2	0.68	<0.1	<15%
3	1.32	0.39	29.5%
4	0.90	<0.1	<11%
5	1.32	<0.1	<8%
6	1.32	0.81	61.3%
7	3.03	1.17	32%
8	1.09	<0.1	<9%
9	1.29	<0.1	<8%
10	1.51	<0.1	<7%

Unextracted samples assayed for $PGF_{2\alpha}$, showed markedly lower concentrations when compared with the same sample assayed after extraction. Results ranged between <7% and 61.3% of the concentrations measured in the extracted samples. (Figure 4.H)

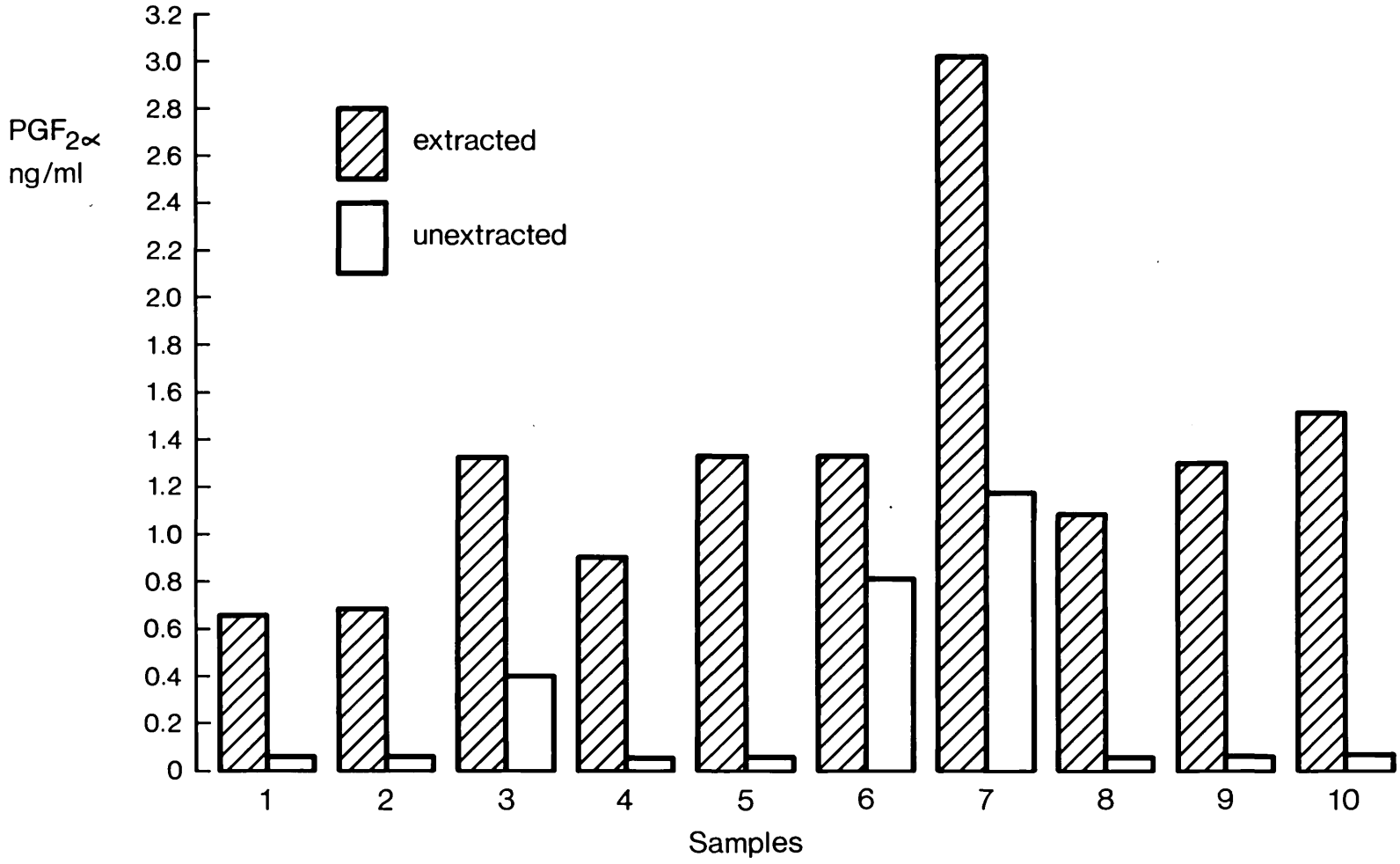
TABLE 4.E

UNEXTRACTED: EXTRACTED RATIO FOR PGE_2

SAMPLE	NGML-1 EXTRACTED (E)	NGML-1 UNEXTRACTED (U)	%U/E X 100
1	0.71	<0.1	<14%
2	0.67	0.39	58%
3	2.12	0.86	40.6%
4	0.77	<0.1	<14%
5	0.55	<0.1	<8%
6	1.16	<0.1	<9%
7	2.45	<0.1	<4%
8	2.25	<0.1	<5%
9	1.32	0.56	42.4%
10	0.50	<0.1	<20%

A similar pattern emerged when extracted samples were compared with unextracted samples for the PGE_2 assay. (Table 4.E)

FIGURE 4H COMPARISON OF EXTRACTION & NON-EXTRACTION FOR $PGF_{2\alpha}$



The value in measured prostaglandin concentration for unextracted samples ranged between <4% and 58% of the concentrations measured in extracted samples. (Figure 4.I)

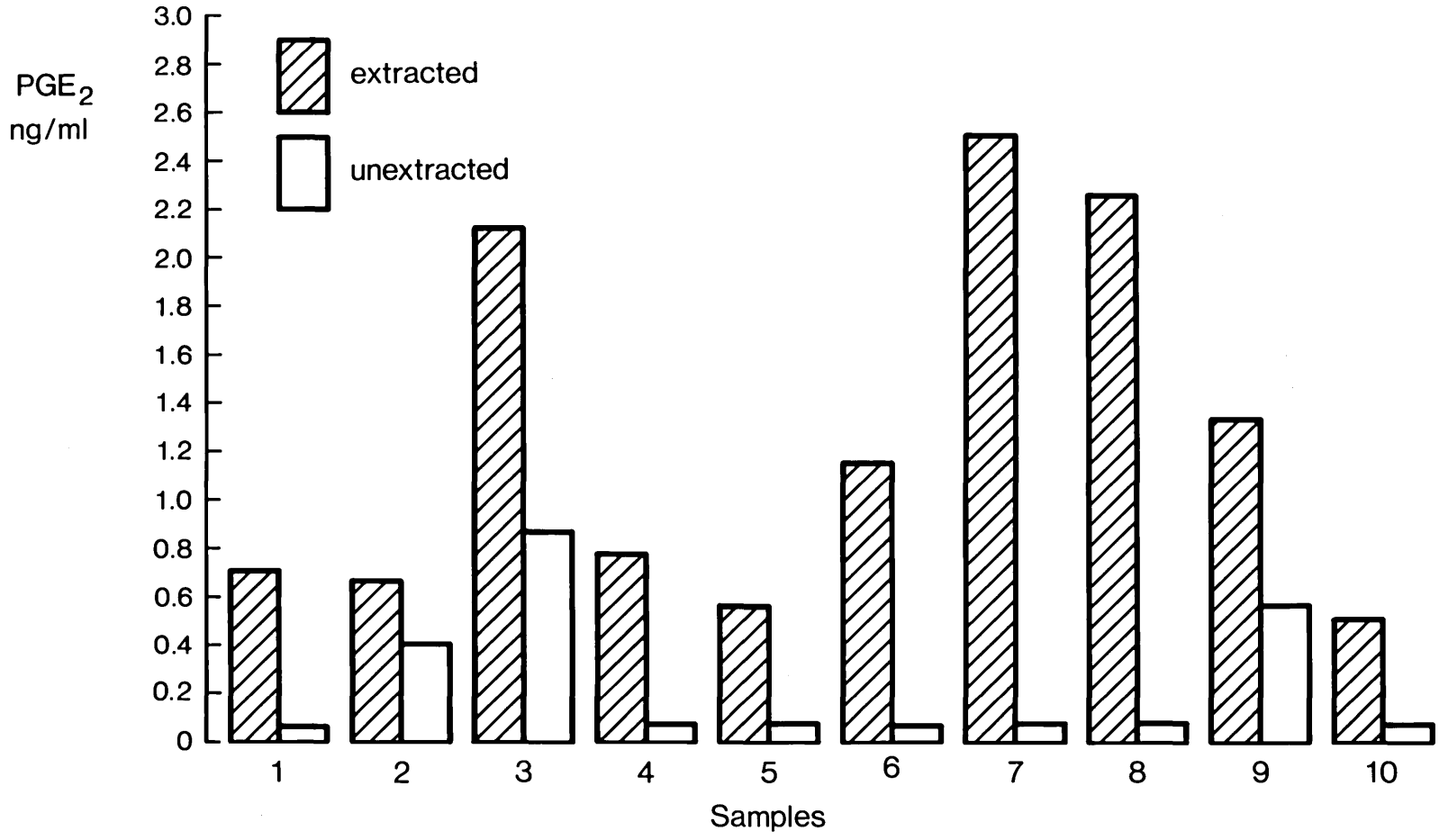
CONCLUSIONS

To obtain an accurate estimate of the prostaglandin concentration in follicular fluid, it is necessary for all samples to be taken through the extraction process described.

Recoveries varied from 80-95% with the used extraction method whereas in some cases as little as <4% of prostaglandin could be detected, when no extraction was carried out. A sensitive and reproducible method was developed for preparation of follicular fluid prior to RIA for prostaglandins.

All results presented hereafter are on extracted follicular fluids.

FIGURE 4 I COMPARISON OF EXTRACTION & NON-EXTRACTION FOR PGE_2



4.7 EFFECT OF FREEZING SAMPLES

Follicular fluids, immediately following the oocyte recovery were centrifuged at 200xg for 10 minutes. The supernatant was removed and stored at -20°C until extracted and assayed.

To assess the effects of storage at -20°C on the prostaglandin concentration, 10 follicular fluids were assayed fresh and also after storage (3 months). (Tables 4.F and 4.G)

TABLE 4.F

% CHANGE IN $PGF_{2\alpha}$ CONCENTRATION
IN FOLLICULAR FLUID ON STORAGE AT -20°C

SAMPLE	NGM-1 FRESH	NGML-1 FROZEN	% CHANGE FRESH-FROZEN
1	1.8	1.85	+3%
2	0.15	0.15	0%
3	4.2	4.45	+6%
4	3.2	3.3	+3%
5	3.0	3.1	+3%
6	2.4	2.5	+4%
7	2.85	3.0	+5%
8	3.4	3.15	-8%
9	1.4	1.3	-8%
10	1.8	1.8	0%

TABLE 4.G

% CHANGE IN PGE_2 CONCENTRATION
IN FOLLICULAR FLUID ON STORAGE AT -20°C

SAMPLE	NGM-1 FRESH	NGML-1 FROZEN	% CHANGE FRESH-FROZEN
1	33.5	34.3	+2%
2	20.6	20.9	+1%
3	11.3	12.1	+7%
4	43.1	40.6	-6%
5	65.8	69.8	+6%
6	4.5	4.3	-4%
7	2.5	2.5	0%
8	11.5	11.0	-4%
9	1.2	1.15	-4%
10	3.3	3.4	+3%

The variation in results between assays on fresh samples and frozen samples did not exceed 7% for PGE_2 and 8% for $PGF_{2\alpha}$. (Figures 4.J and 4.K)

CONCLUSIONS

Study results showed an acceptable rate of variation (within the inter-assay variation) to allow frozen storage of samples for up to 3 months before assay.

FIGURE 4J COMPARISON OF FRESH SAMPLES & STORAGE SAMPLES AT -20°C – $PGF_{2\alpha}$

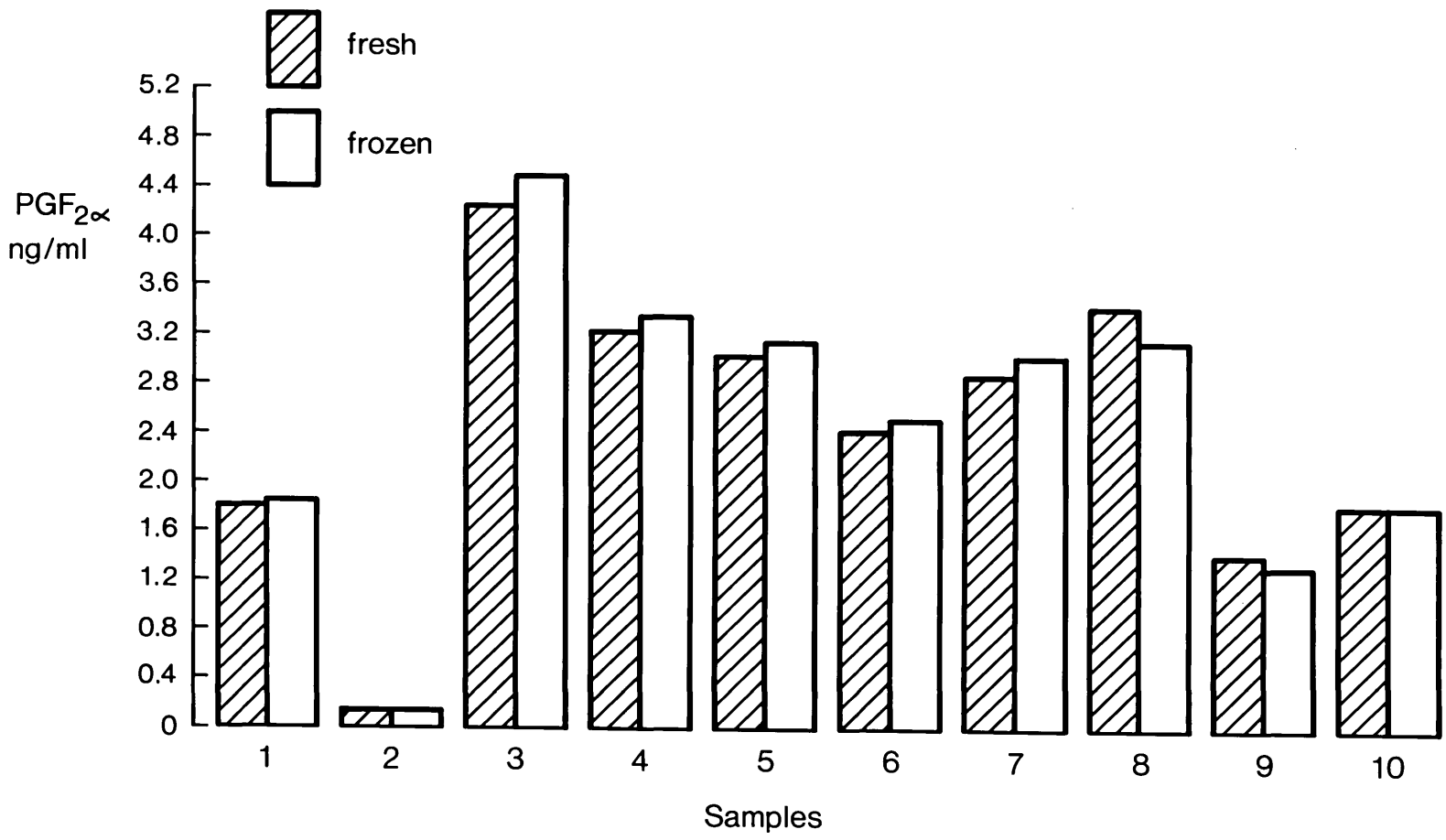
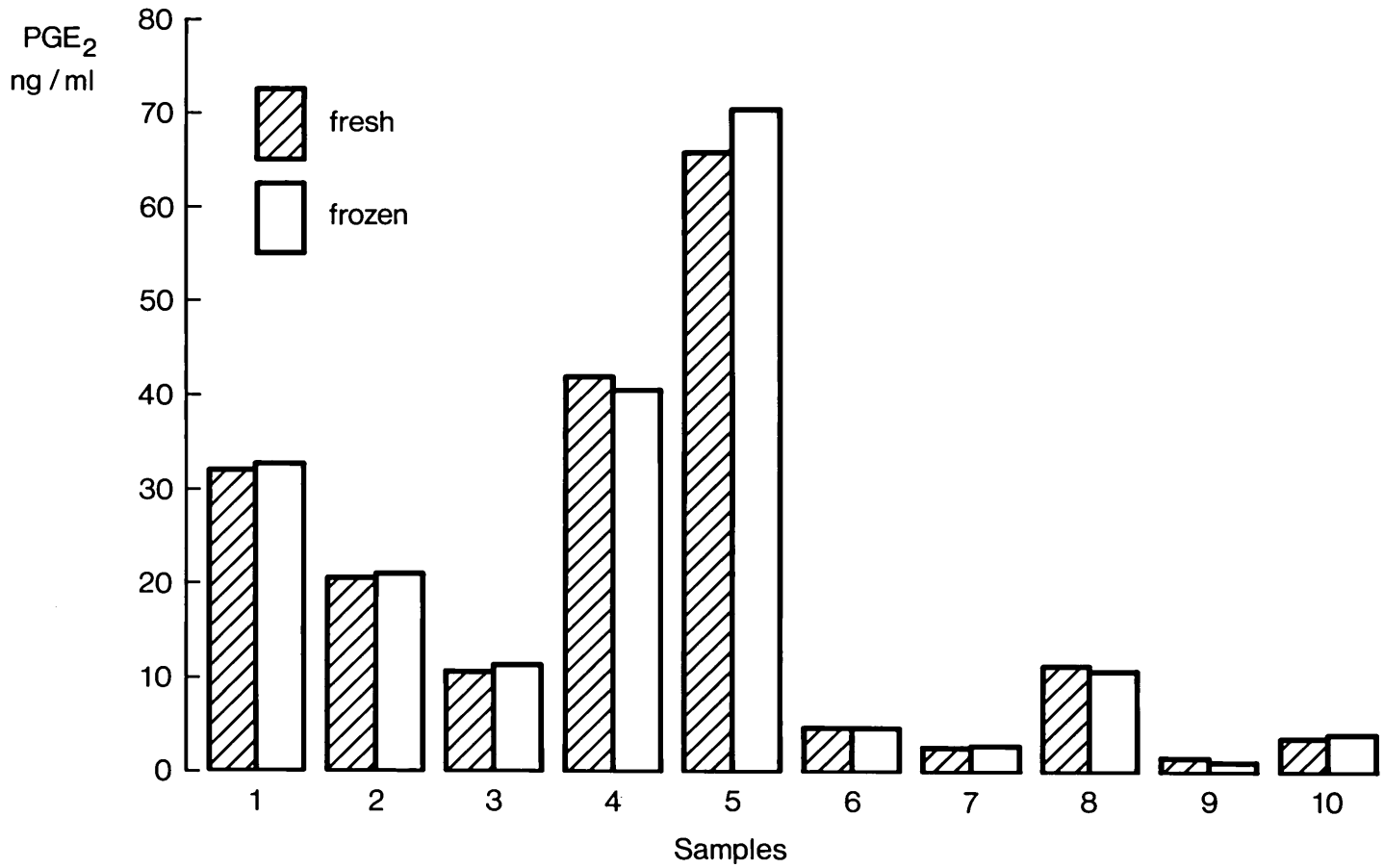


FIGURE 4K COMPARISON OF FRESH SAMPLES & STORAGE SAMPLES AT −20°C − PGE_2



4.8 RIA FOR OESTRADIOL AND PROGESTERONE

MATERIALS AND METHODS

Oestradiol MAIA Kit, Code Number 12264 supplied by Serono Diagnostics. The follicular fluid was assayed without any preliminary treatment of the sample. The kit has been designed for determination of oestradiol as a direct assay.

The main steps of the assay are:

Sample dilution 1 in 1,000 in assay buffer

then

Incubation of reaction mixture — Sample/Standard

3 hours at room temperature — + tracer

+ antiserum

then

Addition of second antibody

coupled to magnetic particles

20 minutes incubation

then

Placed on magnetic separator

then

Decantation of supernatant

then

Radioactivity counting in gamma counter

The cross reactivity was 1% for oestrone and less than 1% for other steroid hormones and their metabolites.

The intra-assay variation was 5%. The inter-assay variation was 8.5%. The minimal detectable concentration of oestradiol was 45 pmol/L.

Amerlex -M Progesterone RIA Kit, Code Number IM 3220/IM 3221/IM3224 supplied by Amersham. Follicular fluid was assayed without any preliminary treatment of the samples. The kit has been designed for direct assay.

The main steps of the assay are:

Sample dilution 1 in 1,000 in assay buffer

then

Incubation of reaction mixture — Sample/Standard
2 hours at room temperature — + tracer
+ antibody coupled to magnetic particles

then

Magnetic Separator
15 minutes

then

Discard supernatant/retain pellet

then

Radioactivity counting in gamma counter

The cross reactivity was 3.3% for 5α pregnan -3, 20-dione and less than 3% for other steroid hormones and their metabolites. The intra-assay co-efficient of variation was 5%. The inter-assay co-efficient of variation was 9%. The mimimum detectable concentration of progesterone was 0.25nmol/L.

STATISTICAL ANALYSES

The analysis of data in this thesis has often had to take the form of non parametric analysis (often the Mann-Whitney Test for comparison) because of skewness of data or non-normally distributed data. This has partly resulted from the type of populations studied but may also be inherent to some extent in the use of radioimmunoassays. The medians and ranges therefore defined the data much more appropriately. Where possible, means and S.D. and parametric tests have been used for comparison with other studies and if these have been the accepted methods of analysis.

Statistical analysis has been carried out using the University Statgraphics package on an IBM PCAT computer. This package has been used for the regression analyses and the best lines of fit (regression lines) plotted on graphs associated with this, are plotted with the 95% confidence limits for this line. Logarithmic transformations or other models did not fit all data and were therefore not adopted.

4.9 SUMMARY

It was possible to develop a fast, convenient and moderately inexpensive method of extraction for the removal of PGE_2 and $PGF_{2\alpha}$ from follicular fluids before assay. These follicular fluids could be stored at -20°C for at least 3 months before assay. The PGE_2 and $PGF_{2\alpha}$ radioimmunoassays on extracts were reliable and reproducible and made the following studies possible. P and E_2 were measured using commercially available assays.

SECTION 3

STUDY FINDINGS

CHAPTER 5

STUDY 1: PRELIMINARY ANALYSIS OF PROSTAGLANDINS AND STEROID HORMONE CONCENTRATIONS IN FOLLICULAR FLUID IN RELATION TO OOCYTE RETRIEVAL AND INITIAL OOCYTE OUTCOME

5.1 OBJECTIVES

An analysis of the PGE_2 and $PGF_{2\alpha}$ concentrations in follicular fluid to study the following:

Prostaglandin levels associated with

a) oocyte containing and non-oocyte containing follicles.
b) fertilisation and non-fertilisation of matched oocytes.
c) rates of cleavage of fertilised matched oocytes.
d) grades of embryo.

(The steroid hormones, E_2 and P, in follicular fluid were also assayed and analysed.)

5.2 OOCYTE CONTAINING AND NON-OOCYTE CONTAINING FOLLICLES

PATIENTS:

Follicular fluids from 18 patients who had both oocyte containing and non-oocyte containing follicles at oocyte recovery were analysed.

The age range was 23-40 years with a mean of 30.8 years. Table 5.A shows the indications for IVF.

TABLE 5.A

INDICATIONS FOR IVF

	n = 18
Tubal	10
Tubal/Sperm	2
Tubal/Endometriosis	1
Unexplained	2
Mucus Hostility	1
Endometriosis	1

5 patients underwent oocyte recovery using laparoscopy and 13 patients using transvaginal ultrasound guided oocyte recovery.

The numbers of oocyte containing follicles analysed were more than double the numbers not yielding oocytes due to the preponderance of oocyte containing follicles in all patients (83 compared to 42).

RESULTS

Table 5.B shows the range and median for the follicular fluid prostaglandin levels.

TABLE 5.B

FOLLICULAR FLUID PGE_2 AND $PGF_{2\alpha}$ IN OOCYTE CONTAINING AND NON-OOCYTE CONTAINING FOLLICLES

	MEDIAN ng/ml	RANGE ng/ml	N
Oocyte Containing Follicles PGE_2	3.40	0.4-86.3	83
Non-oocyte Containing Follicles PGE_2	4.21 (ns)	0.18-80.3	42
Oocyte Containing Follicles $PGF_{2\alpha}$	1.35	0.1-22.5	83
Non-oocyte Containing Follicles $PGF_{2\alpha}$	1.45 (ns)	0.2-16.5	42

ns = not significant when compared by Non-parametric analysis (Mann-Whitney).

However, PGE_2 levels, showed a significant correlation with $PGF_{2\alpha}$ levels in both oocyte containing ($p<0.001$, $r=0.87$) and non oocyte containing ($p<0.001$, $r=0.53$) follicles.

No significant difference in the $PGE_2/PGF_{2\alpha}$ ratio was shown. (See Table 5.C.)

TABLE 5.C

FOLLICULAR FLUID PGE_2:$PGF_{2\alpha}$ RATIO IN OOCYTE CONTAINING AND NON-OOCYTE CONTAINING FOLLICLES

	MEDIAN	RANGE
Oocyte Containing Follicles	3.00	0.24-16.4
Non-oocyte Containing Follicles	2.89 (ns)	0.18-15.48

ns = not significant when compared by Non-parametric analysis (Mann-Whitney).

When these results were analysed on an individual basis wide variations were seen both within the non-oocyte containing and the oocyte containing follicles.

Table 5.D lists the results for one individual who had transvaginal ultrasound guided egg recovery.

TABLE 5.D

FOLLICULAR FLUID PGE_2, $PGF_{2\alpha}$ AND THE PGE_2:$PGF_{2\alpha}$ RATIO IN ONE SUBJECT

	ORDER OF ASPIRATION	PGE_2 ng/ml	$PGF_{2\alpha}$ ng/ml	PGE_2:$PGF_{2\alpha}$
Non-oocyte	1	9.1	1.5	6.07
Containing	4	1.9	1.4	1.36
Follicles	8	0.7	0.2	3.5
	10	0.9	1.05	0.86
	12	1.9	1.0	1.9
Oocyte	2	13.8	0.9	15.3
Containing	3	0.4	1.35	0.3
Follicles	5	6.2	1.35	4.59
	6	4.5	10.35	0.43
	7	0.7	2.4	0.29
	9	2.7	0.8	3.38
	13	8.2	0.5	16.4
	14	8.4	1.05	8.0

The concentrations of PGE_2 and $PGF_{2\alpha}$ were not related to order of aspiration and therefore not influenced by the aspiration procedure. There was no statistical difference for the PGE_2 and $PGF_{2\alpha}$ concentrations or the PGE_2:$PGF_{2\alpha}$ ratio in one individual when non oocyte containing follicles were compared with oocyte containing follicles.

Table 5.E shows the follicular fluid E_2 and P levels for oocyte containing and non-oocyte containing follicles. Once again no statistical differences were shown.

TABLE 5.E

FOLLICULAR FLUID E_2 AND P LEVELS AND E_2:P RATIO IN OOCYTE CONTAINING AND NON-OOCYTE CONTAINING FOLLICLES

	MEDIAN		RANGE	N
Oocyte Containing Follicles				85
E_2 (nmol/L)	680		11-3400	
P (μmol/L)	15		1.5-55	
E_2:P (x 10^{-3})	51		13-767	
Non-oocyte Containing Follicles				31
E_2 (nmol/L)	680	(ns)	68-2300	
P (μmol/L)	13	(ns)	1.5-42	
E_2:P (x 10^{-3})	49.5	(ns)	10-110	

ns = not significant when compared by Non-parametric analysis (Mann-Whitney).

Follicular fluid E_2 concentration correlated significantly with the P concentration for both non-oocyte containing ($p<0.001$, $r=0.71$) and oocyte containing ($p<0.001$, $r=0.46$) follicles. (Figures 5.A and 5.B.)

No significant correlation was shown between follicular fluid PGE_2 levels and either E_2 or P levels. Also no significant correlation was shown between follicular fluid $PGF_{2\alpha}$ and E_2 or P.

FIGURE 5A CORRELATION BETWEEN E_2 & P FOR NON-OOCYTE CONTAINING FOLLICLES

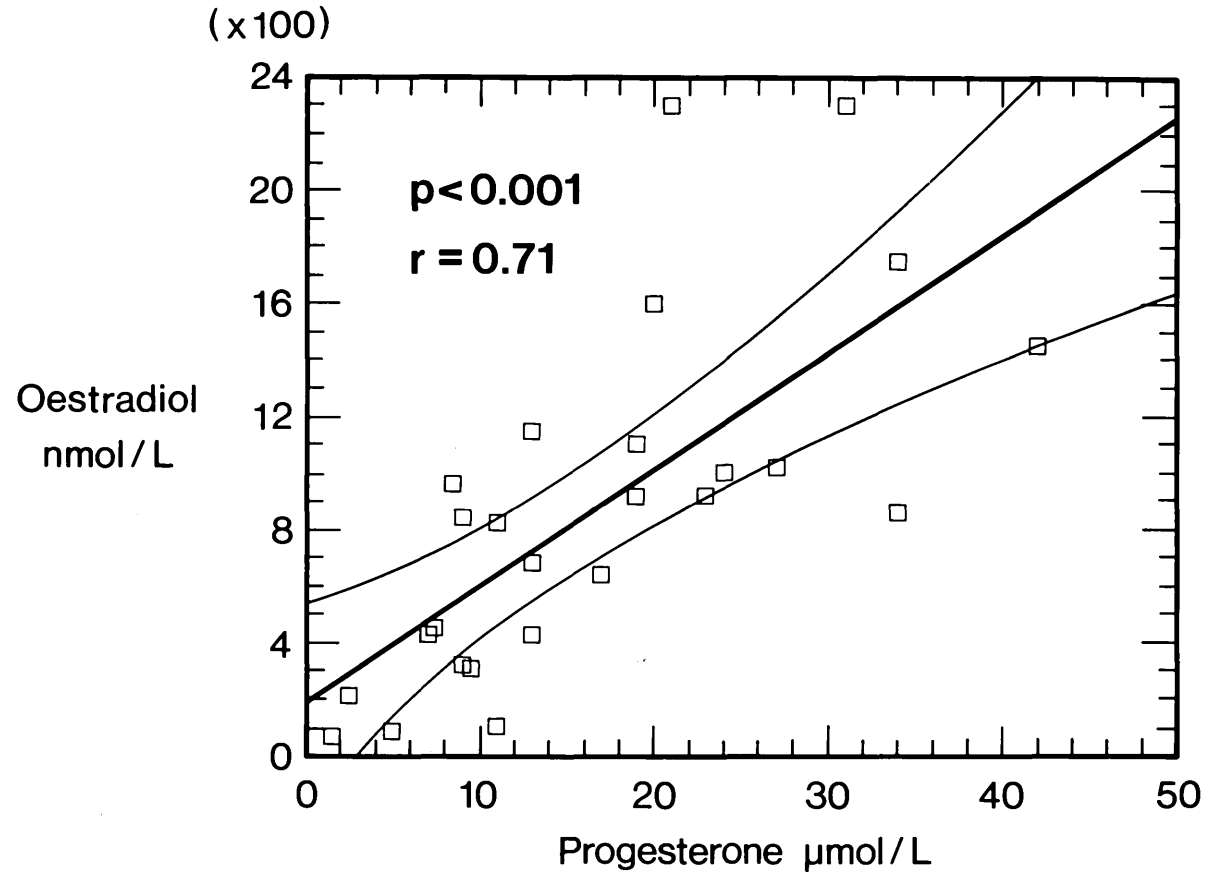
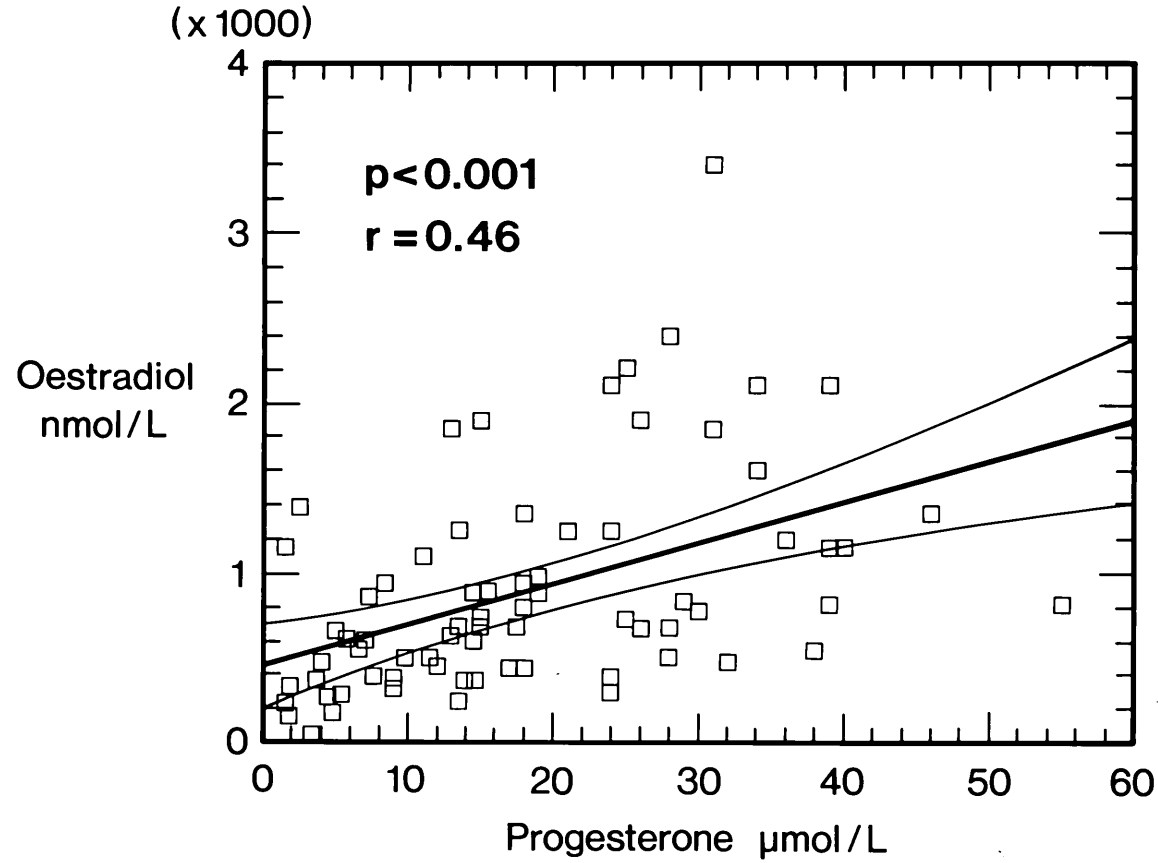


FIGURE 5B CORRELATION BETWEEN E_2 & P FOR OOCYTE CONTAINING FOLLICLES



5.3 FERTILISATION AND NON-FERTILISATION OF THE MATCHED OOCYTE

PATIENTS

The same patient group as in 5.2 was included in this study. Forty three follicular fluids from which the matched oocyte did not fertilise were compared with 82 follicular fluids from which the matched oocyte did fertilise.

RESULTS

Concentrations of PGE_2 and $PGF_{2\alpha}$ are shown in Table 5.F.

TABLE 5.F

FOLLICULAR FLUID PGE_2 AND $PGF_{2\alpha}$ FROM FERTILISED AND UNFERTILISED OOCYTES

	MEDIAN ng/ml	RANGE ng/ml	N
Fertilised Oocyte PGE_2	3.25	0.5-327	82
Unfertilised Oocyte PGE_2	4.34 (ns)	0.4-337	43
Fertilised Oocyte $PGF_{2\alpha}$	1.33	0.1-31.5	82
Unfertilised Oocyte $PGF_{2\alpha}$	1.40 (ns)	0.2-52.5	43

ns = not significant when compared by Non-parametric analysis (Mann-Whitney).

There was no significant difference whether or not fertilisation occurred in the matched oocyte. The PGE_2:$PGF_{2\alpha}$ ratio was also not significantly different. (Table 5.G.)

TABLE 5.G

FOLLICULAR FLUID PGE_2:$PGF_{2\alpha}$ RATIO FROM FERTILISED AND UNFERTILISED OOCYTES

	MEDIAN	RANGE
Fertilised Oocytes	2.53	0.67-16.4
Unfertilised Oocytes	3.33 (ns)	0.21-10.0

ns = not significant when compared by Non-parametric analysis (Mann-Whitney).

PGE_2 levels correlated significantly with $PGF_{2\alpha}$ levels for both fertilised ($p<0.001$, $r=0.95$) and unfertilised ($p<0.001$, $r=0.96$) oocytes. (Figures 5.C and 5.D)

The follicular fluid E_2 and P levels were not significantly different. (Table 5.H.) The ratio of E_2 to P also did not differ significantly.

FIGURE 5C CORRELATION BETWEEN FOLLICULAR FLUID PGE_2 & $PGF_{2\alpha}$ FOR THE FERTILISED GROUP

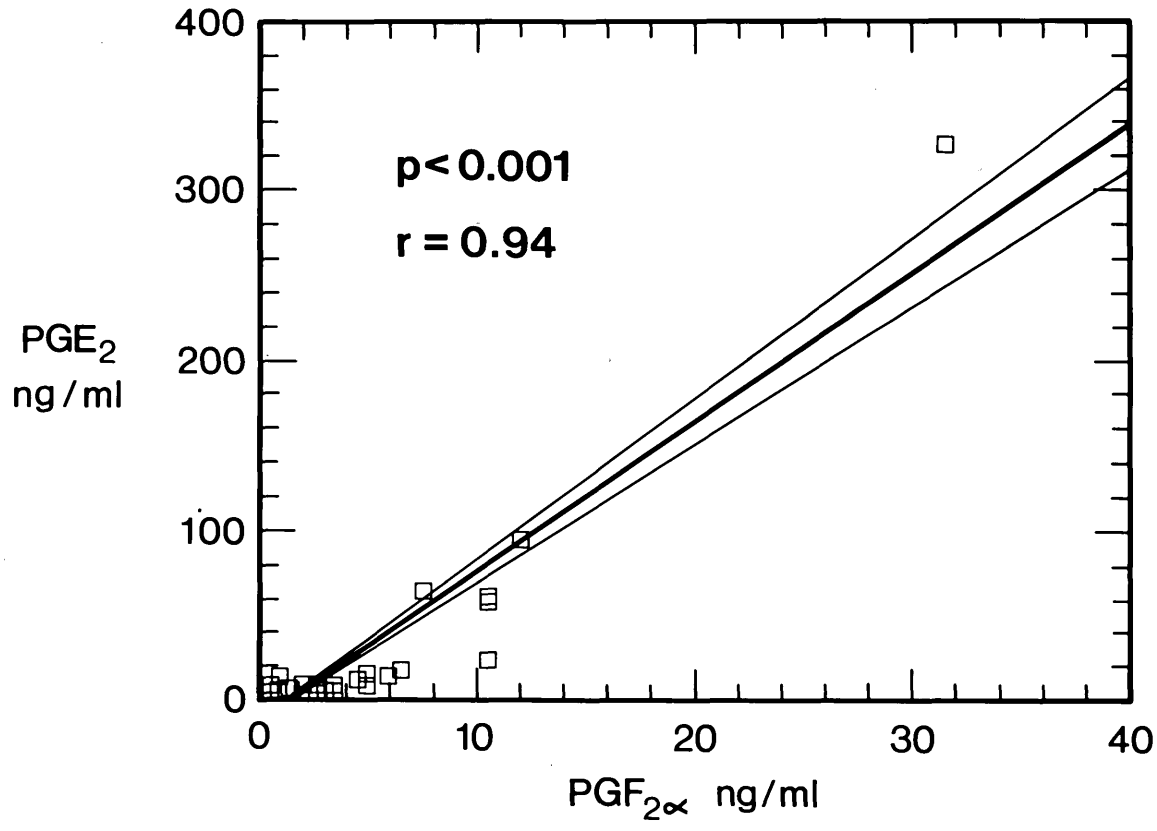


FIGURE 5D CORRELATION BETWEEN FOLLICULAR FLUID PGE_2 & $PGF_{2\alpha}$ FOR THE NON-FERTILISED GROUP

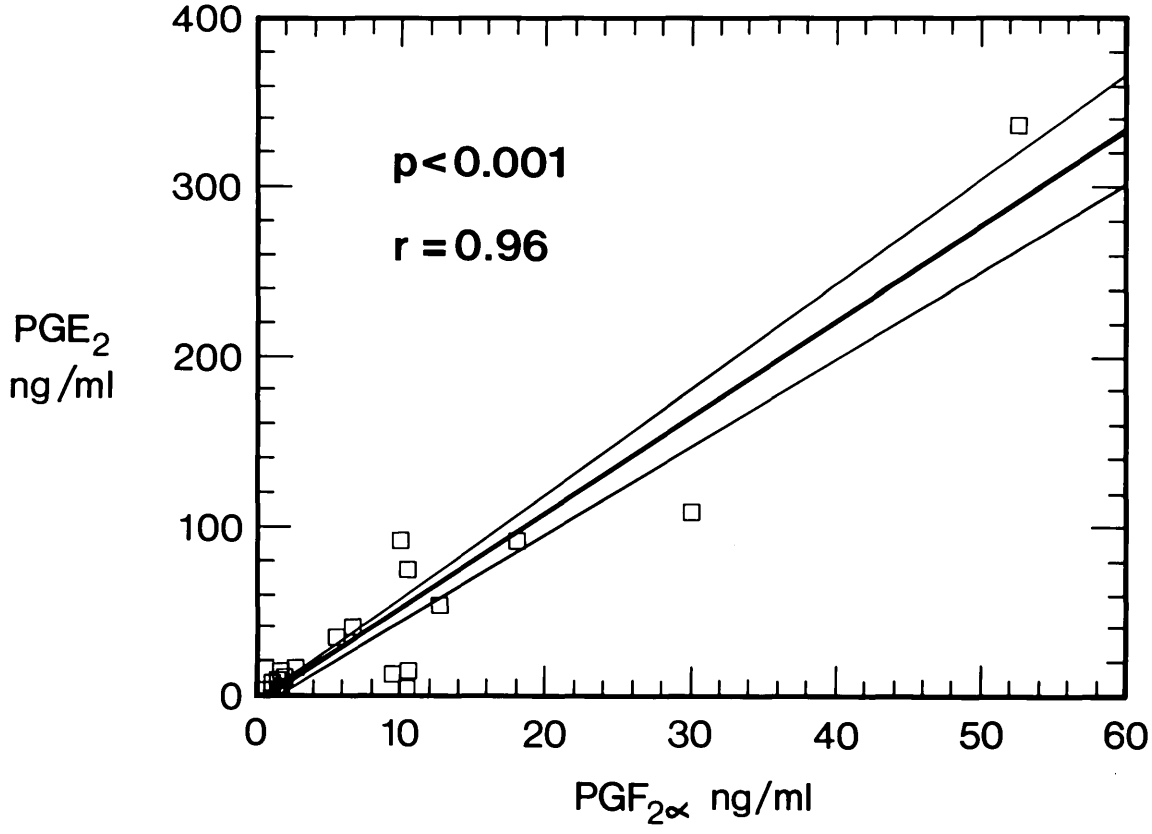


TABLE 5.H

FOLLICULAR FLUID E_2 AND P LEVELS AND E_2:P RATIO FROM FERTILISED AND UNFERTILISED OOCYTES

	MEDIAN	RANGE	N
Fertilised Oocytes			82
E_2 (nmol/L)	730	82-3400	
P (μmol/L)	19	1-55	
E_2:P (x 10^{-3})	49	10-767	
Unfertilised Oocytes			43
E_2 (nmol/L)	800 (ns)	52-3400	
P (μmol/L)	20 (ns)	2-43	
E_2:P (x10^{-3})	35 (ns)	15-174	

ns = not significant when compared by Non-parametric analysis (Mann-Whitney).

There was a significant correlation between follicular fluid E_2 and P levels in both the fertilised group ($p<0.001$, $r=0.53$) and the unfertilised group ($p<0.05$, $r=0.39$).

As seen in the previous analysis no significant correlations were shown between either PGE_2 or $PGF_{2\alpha}$ and either E_2 or P in follicular fluid.

5.4 RATES OF CLEAVAGE OF THE FERTILISED MATCHED OOCYTE

PATIENTS

The same patient group as in 5.2 was used to compare follicular fluids from oocytes resulting in 2 cell embryos and oocytes resulting in 4 cell embryos in the same time period.

RESULTS

No significant difference was seen for either PGE_2 or $PGF_{2\alpha}$ levels in follicular fluid for either 2 cell or 4 cell embryos. (Table 5.I.)

TABLE 5.I

FOLLICULAR FLUID PGE_2 AND $PGF_{2\alpha}$ ASSOCIATED WITH 2 CELL EMBRYOS AND 4 CELL EMBRYOS

	MEDIAN ng/ml	RANGE ng/ml	N
2 cell embyro PGE_2	3.2	0.5-327	27
4 cell embryo PGE_2	3.4 (ns)	0.5-60.9	40
2 cell embryo $PGF_{2\alpha}$	1.5	0.15-31.5	
4 cell embryo $PGF_{2\alpha}$	1.15 (ns)	0.1-10.5	

ns = not significant when compared by Non-parametric analysis (Mann-Whitney).

The only difference observed was some inidividually high results for both prostaglandins with 2 cell embryos. The PGE_2:$PGF_{2\alpha}$ ratio was not significantly different. (Table 5.J).

TABLE 5.J

FOLLICULAR FLUID PGE_2:$PGF_{2\alpha}$ RATIO FROM 2 CELL AND 4 CELL EMBRYOS

	MEDIAN	RANGE	N
2 cell embyros	2.51	0.67-16.4	27
4 cell embryos	2.46 (ns)	0.67-14.4	40

ns = not significant by Non-parametric analysis (Mann-Whitney).

A significant correlation was shown between PGE_2 and $PGF_{2\alpha}$ levels in follicular fluid from both 2 cell embryos ($p<0.001$, $r=0.96$) and 4 cell embryos ($p<0.001$, $r=0.85$).

E_2 and P concentrations did not differ significantly whether the embryo was at the 2 or the 4 cell stage at the time of transfer. (Table 5.K.)

TABLE 5.K

FOLLICULAR FLUID E_2 AND P LEVELS AND THE E_2:P RATIO ASSOCIATED WITH 2 CELL EMBRYOS AND 4 CELL EMBRYOS

	MEDIAN	RANGE	N
2 Cell Embyros			27
E_2 (nmol/L)	705	130-3400	
P (μmol/L)	23	2-47	
E_2:P (x 10^{-3})	39.5	10-228	
4 Cell Embyros			40
E_2 (nmol/L)	680(ns)	105-3400	
P (μmol/L)	18 (ns)	1-55	
E_2:P (x 10^{-3})	50 (ns)	13-220	

ns = not significant when compared by Non-parametric analysis (Mann-Whitney).

The follicular fluid E_2 levels correlated highly significantly with the P level in both the 2 cell embryos ($p<0.001$, $r=0.41$) and the 4 cell embryos ($p<0.001$, $r=0.53$).

There were no significant correlations shown between either PGE_2 or $PGF_{2\alpha}$ and either E_2 or P levels.

5.5 GRADE OF EMBRYO FROM THE FERTILISED MATCHED OOCYTE

PATIENTS

Follicular fluids from the same patient group as in 5.2 were analysed. 42 follicular fluids from oocytes that fertilised and resultant embryos graded as 1 and 2 were compared with 22 fluids from oocytes that resulted in 3 and 4 embryos.

RESULTS

Table 5.L shows the median and range of PGE_2 and $PGF_{2\alpha}$. There was no significant difference between different grades of embryo quality.

TABLE 5.L

FOLLICULAR FLUID PGE_2 AND $PGF_{2\alpha}$ FROM GRADE 1 AND 2 AND GRADE 3 AND 4 EMBRYOS

	MEDIAN ng/ml	RANGE ng/ml	N
Grade 1 & 2 PGE_2	4.7	0.7-94.4	41
Grade 3 & 4 PGE_2	2.8 (ns)	0.5-86.3	22
Grade 1 & 2 $PGF_{2\alpha}$	1.35	0.1-12.0	41
Grade 3 & 4 $PGF_{2\alpha}$	1.4 (ns)	0.1-27.0	22

ns = not significant when compared by Non-parametric analysis (Mann-Whitney).

TABLE 5.M

FOLLICULAR FLUID PGE_2:$PGF_{2\alpha}$ RATIO FROM GRADE 1 AND 2 AND GRADE 3 AND 4 EMBRYOS

	MEDIAN	RANGE	N
Grade 1 & 2	3.09	0.93-16.4	41
Grade 3 & 4	3.43 (ns)	0.67-8.57	22

ns = not significant when compared by Non-parametric analysis (Mann-Whitney).

As with the other outcomes analysed the PGE_2:$PGF_{2\alpha}$ ratio was also not significantly different.

Once again PGE_2 correlated significantly with $PGF_{2\alpha}$ for both embryo categories. (Grade 1 & 2, $p<0.001$, $r=0.85$) (Grade 3 & 4, $p<0.005$, $r=0.62$)

Table 5.N shows the median and range for E_2 and P in these embryos. No significant differences were shown.

TABLE 5.N

FOLLICULAR FLUID E_2 AND P LEVELS AND THE E_2:P RATIO ASSOCIATED WITH GRADE 1 AND 2 AND GRADE 3 AND 4 EMBRYOS

	MEDIAN	RANGE	N
Grade 1 and 2			41
E_2 (nmol/L)	510	105-3400	
P (μmol/L)	13	1-55	
E_2:P (x 10^{-3})	31	10-132	
Grade 3 and 4			22
E_2 (nmol/L)	740 (ns)	170-2200	
P (μmol/L)	15 (ns)	1-39	
E_2:P (x 10^{-3})	50 (ns)	11-112	

ns = not significant when compared by Non-parametric analysis (Mann-Whitney).

The follicular fluid E_2 levels correlated significantly with P levels for both Grade 1 and 2 embryos ($p<0.001$, $r=0.42$) and Grade 3 and 4 embryos ($p<0.01$, $r=0.63$).

No correlation was seen between either follicular fluid PGE_2 or $PGF_{2\alpha}$ and either E_2 or P.

5.6 SUMMARY

PGE_2, $PGF_{2\alpha}$, E_2 and P were measured in follicular fluids obtained at oocyte recovery of patients undergoing IVF for various indications. Considerable variation for prostaglandins and steroids was found in the majority of individuals. Comparisons were made in regard to the presence or absence of an oocyte, fertilisation, cleavage and grade of embryos. No significant differences were shown for any comparison between groups for this non select group of patients and differing IVF practices.

There were no correlations between the prostaglandins and the steroid hormones. Nevertheless there were significant correlations in all groups for PGE_2 vs $PGF_{2\alpha}$ and E_2 vs P.

These studies showed that no obvious differences in prostaglandin levels could be shown with such diverse groups. Therefore if any important relationships existed further studies were necessary with more discrete groups and with IVF success in terms of pregnancies.

Study 2 was therefore set up to investigate more select groups.

CHAPTER 6

STUDY 2: A MORE EXTENSIVE ANALYSIS OF PROSTAGLANDINS IN FOLLICULAR FLUID IN RELATION TO FERTILISATION OF THE MATCHED OOCYTE AND PREGNANCY OUTCOME

PATIENT RECRUITMENT, OOCYTE FERTILISATION AND PREGNANCY

6.1 INTRODUCTION

Study 1 failed to establish a significant relationship between the levels of the measured follicular fluid components and the initial outcome of the matched oocyte. But there were a number of variables in that study that may have affected the results.

Refinements were made in this continuing study to attempt to exclude any factor influencing the quality of oocytes or spermatozoa. The patient group selected for this study had a purely mechanical indication for IVF. Those with any evidence of endometriosis, present or past, were excluded. Patients with so far unexplained infertility were also excluded as it has been our experience that previously unsuspected male or female problems have come to light in a significant percentage of those patients whilst undergoing IVF.

The method of ultrasound monitoring and the technique used for oocyte recovery were not standardised in the previous study. Five out of the 18 patients underwent laparoscopic oocyte recovery using general anaesthesia. The 100% CO_2 pneumoperitoneum required for laparoscopy may have adverse effects on oocyte quality because rapid uptake of CO_2 may decrease follicular fluid pH. General anaesthesia may also exert adverse effects. However, comparisons of the two methods of IVF in the preliminary study were not able to yield significant differences between TVU methods and laparoscopic methods. The PGE_2:$PGF_{2\alpha}$ ratio for laparoscopy follicular fluids ranged from 0.24 to 15.48 and TVU from 0.18 to 16.4.

Boyers et al (1987), in a paired analysis of in vitro fertilisation and cleavage rates of first versus last recovered oocytes, showed a significantly decreased fertilisation rate in last recovered oocytes when the time between first and last oocytes exceeded 5 minutes. Separation of CO_2 and general anaesthetic effects was not possible as both were used concurrently. Cleavage rates were similar in both first and last recovered oocytes.

In contrast, Hayes et al (1987) showed no differences in fertilisation rates between early and late recovered oocytes but cleavage rates were decreased.

All patients included in this study 2, underwent oocyte recovery by ultrasound guided transvaginal aspiration. Any possible influence of a CO_2 pneumoperitoneum and the effects of general anaesthesia were removed from this study.

The IVF programme had by the second year of operation achieved acceptable pregnancy rates and therefore this was now a further factor to be studied in relation to follicular fluid prostaglandin and steroid hormone levels.

Selection criteria for Study 2 were therefore much more refined and this would be reflected in results found.

6.2 OBJECTIVES OF STUDY 2

To study the relationship between PGE_2 and $PGF_{2\alpha}$, E_2 and P concentrations in follicular fluid and the outcome of the matched oocyte in the following terms:

a) Fertilisation or non-fertilisation

b) Establishment or non-establishment of pregnancy

6.3 PATIENT CRITERIA

Patients included in this study had a purely mechanical problem causing their infertility. Only patients with tubal blockage or mucus hostility were included. Patients with endometriosis, unexplained infertility and male factor problems were excluded. (Table 6.A).

TABLE 6.A

NUMBER OF PATIENTS, CYCLES AND INDICATIONS FOR IVF

	No Of Patients	No Of Cycles
Tubal Blockage	40	47
Mucus Hostility	5	5

33 patients were undergoing their 1st IVF attempt, 10 their 2nd attempt, 1 patient her 3rd attempt and 1 patient her 4th attempt.

The age of patients fell within the range, 24-40 years, mean 33 years.

6.4 OVARIAN HYPERSTIMULATION

All patients in the study received the same drugs, buserelin and hMG. Buserelin was administered as previously stated in Chapter 3. HMG was administered, commencing on day 4 of the treatment cycle, in a daily dosage of 4 ampoules until an adequate response was achieved. Only 2 patients received a higher daily dose of hMG, i.e. 6 ampoules because of previous failure to respond adequately on the lower dose. Both conceived on the higher dosage.

The dosage of hMG required are shown in Table 6.B.

TABLE 6.B

NUMBER OF CYCLES FOR A PARTICULAR TOTAL hMG DOSAGE

AMPS hMG	NO. OF CYCLES
24	6
28	31
32	9
36	4
42	1*
48	1*

* 6 amps daily

1 amp hMG = 75 IU FSH, 75 IU LH

The mean and mode for the number of ampoules of hMG required was 28.

6.5 CYCLE MONITORING

Patients were monitored by biophysical and biochemical methods. All patients had, on vaginal ultrasound, at least 2 follicles measuring more than 17mm for the oocyte maturation dose of hCG to be given.

The serum oestradiol levels on the same day were in the range 1800 - 25,000 pmol/L, median 4250 pmol/L. Adequate E_2 levels were considered to be around 400 pmol/L per mature follicle.

6.6 OOCYTE RECOVERY

All recoveries were performed using transvaginal ultrasound guided needle aspiration as detailed in Chapter 3.

Table 6.C shows the average numbers of follicles aspirated per patient, the average number of oocytes recovered per patient and the oocyte yield for the number of follicles aspirated.

TABLE 6.C

NUMBERS OF FOLLICLES ASPIRATED, NUMBERS OF OOCYTES RECOVERED AND OOCYTE YIELD

	MEAN	RANGE
Follicles Aspirated	12.6	4-26
Oocytes	11.04	3-26
Oocyte Yield	89.6%	

6.7 EMBRYO TRANSFER

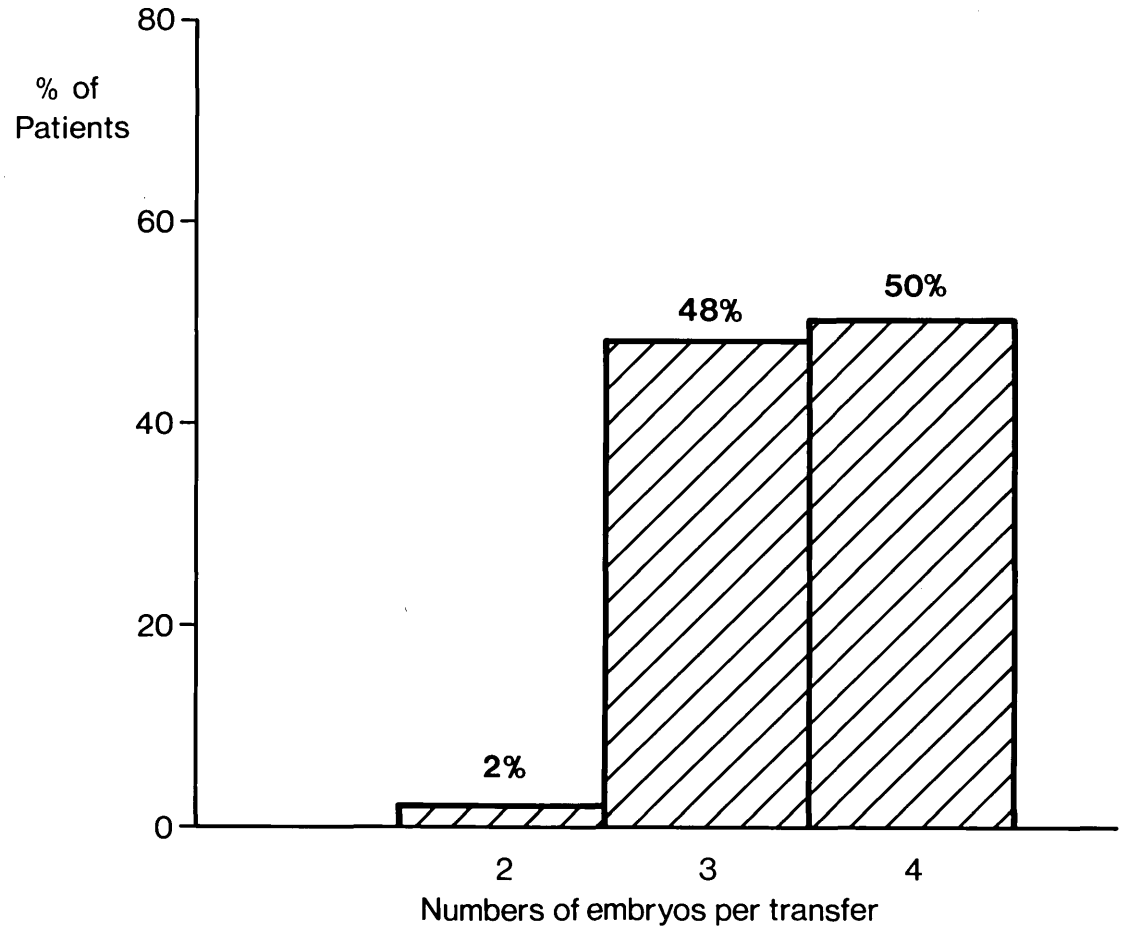
All embryo transfers were performed using a 1ml syringe and a Wallace catheter.

The mean fertilisation rate was 77.7%.

Figure 6.A shows the percentage of patients receiving 2, 3 or 4 embryos per transfer.

All patients received luteal phase support as hCG, 2500 I.U. on days +3 and +6 (oocyte maturational dose of hCG given on day 0).

FIGURE 6A NUMBERS OF EMBRYOS TRANSFERRED PER PATIENT



6.8 PREGNANCIES

22 of the 52 cycles analysed resulted in pregnancy. Pregnancy was diagnosed by a positive fetal heart beat on ultrasound. Biochemical pregnancies were excluded.

Table 6.D shows the indications for IVF in these patients.

TABLE 6.D

INDICATION FOR IVF IN THOSE PATIENTS ESTABLISHING A PREGNANCY

	N = 22
Tubal	17
Mucus	5

The ages ranged between 26 and 40 years with a mean of 32.5 years.

All patients had either 3 or 4 embryos transferred. Table 6.E shows the implantation rate per embryo transferred.

TABLE 6.E

IMPLANTATION RATE PER EMBRYO TRANSFERRED

	NO OF EMBRYOS	NO IMPLANTING	IMPLANTATION RATE
Overall	78	31	39.7%
3 Embryos	30	11	36.7%
4 Embryos	48	20	41.7%

One multiple pregnancy, twins, occurred in the 10 patients who had 3 embryos transferred and 7 multiple pregnancies occurred in the 12 patients who had 4 embryos transferred, 6 sets of twins and 1 set of triplets.

All pregnancies resulted from the transfer of at least one grade 1 embryo. There were no significant differences in the numbers of transferred grade 1 embryos between the 'pregnancy' and the 'no pregnancy' group. (Figure 6.B) Table 6.F shows the outcome of the pregnancies.

FIGURE 6B NUMBERS OF GRADE 1 EMBRYOS TRANSFERRED IN PREGNANCY & NO PREGNANCY GROUPS

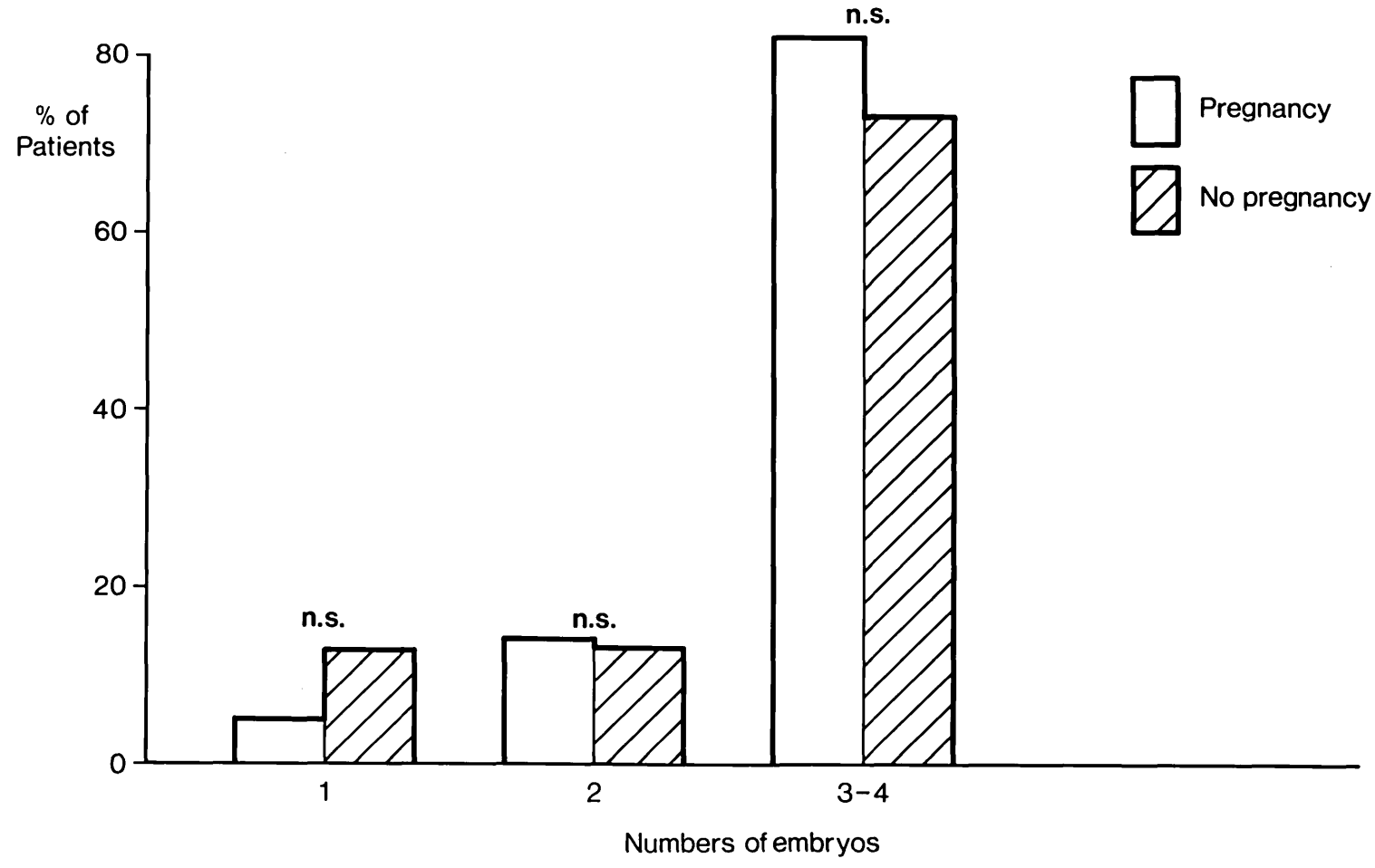


TABLE 6.F

THE OUTCOME OF THE PREGNANCIES

OUTCOME OF PREGNANCIES	
Delivered	16
Aborted < 12/52	1
Aborted > 12/52	2
Ectopic	2
Intra-uterine death	1

2 of the three abortions were twin pregnancies as was the intra-uterine death which occurred at 28 weeks. There were 3 neonatal deaths, one occurring in a twin pregnancy and due to prematurity, and the other two due to fetal abnormality. One of the triplets had oesophageal atresia with a tracheo-oesophageal fistula and one singleton pregnancy had Potter's syndrome.

The 2 ectopics were diagnosed at 7 weeks and 8 weeks. Both had positive fetal heart beats at the time of diagnosis.

6.9 SUMMARY

A purer patient group with only mechanical indications for IVF were selected for Study 2. IVF practices were standardised for the entire group. The oocyte recovery rate for follicles aspirated was around 90%. Only 2% of patients had less than 3 embryos transferred and all pregnancies occurred when 3 or 4 embryos were transferred. There was no significant difference in the implantation rate for 3 or 4 embryos. However a significantly higher number of multiple pregnancies occurred when 4 embryos were transferred. There was a 27% loss rate among the IVF pregnancies.

CHAPTER 7

CYCLE MONITORING OF SERUM OESTRADIOL AND PROGESTERONE LEVELS FOR STUDY 2.

7.1 FOLLICULAR PHASE

Serum E_2 was measured on the day that the maturational dose of hCG was given (day = 0). Results are expressed for all the patients included in the study and then subdivided dependent on whether or not pregnancy resulted (Table 7.A).

TABLE 7.A

SERUM E_2 ON DAY 0

	MEDIAN	RANGE	N
	pmol/L	pmol/L	
All patients	3950	1800-22,500	52
Not Pregnant	4000	1800-22,500	30
Pregnant	3350 (ns)	2050-6,750	22

ns = not significant when compared by Non-parametric analysis (Mann-Whitney).

The range of E_2 for the patients who subsequently established a pregnancy is narrower than for those who did not but this did not reach significance. No patient with an E_2 greater than 6750 pmol/L on day 0 became pregnant.

7.2 LUTEAL PHASE

Blood samples were taken on days 1, 6 and 12 (Day of hCG = Day 0) in the luteal phase and assayed for E_2 and P. Table 7.B shows the median and range for E_2, P and the E_2:P ratio on day 1.

TABLE 7.B

SERUM E_2, P AND E_2:P RATIO ON LUTEAL DAY 1

	MEDIAN	RANGE	N
ALL PATIENTS			52
E_2 pmol/L	3700	850-24,500	
P nmol/L	16	6-60	
E_2:P (x 10^{-3})	255	25-596	
NO PREGNANCY			30
E_2 pmol/L	3900	875-24,500	
P nmol/L	16	6-60	
E_2:P (x 10^{-3})	233	25-596	
PREGNANCY			22
E_2 pmol/L	3624 (ns)	1850-9,250	
P nmol/L	15 (ns)	6-30	
E_2:P (x 10^{-3})	271 (ns)	88-430	

ns = not significant when compared by Non-parametric analysis (Mann-Whitney) to the 'no pregnancy' group.

The day 1 serum levels of both E_2 and P and also the ratio of E_2:P were not significantly different whether or not pregnancy was subsequently established.

However there was a highly significant correlation between the serum E_2 concentration and the serum P concentration on day 1 for all patients ($p<0.001$). (Figure 7.A)

This correlation was also shown when the patients were subdivided in the respective non-pregnant and pregnant groups ($p<0.001$) (Table 7.C).

TABLE 7.C

CORRELATION BETWEEN SERUM E_2 AND P ON LUTEAL DAY 1

	p	r	N
All patients	<0.001	0.80	52
No pregnancy	<0.001	0.78	30
Pregnancy	<0.001	0.89	22

The total E_2 on day 1 is a reflection of the number of follicles that have been stimulated and therefore the number of oocytes recovered.

Table 7.D shows the results of oocyte recoveries and the fertilisation rate.

FIGURE 7A CORRELATION BETWEEN SERUM OESTRADIOL & PROGESTERONE ON LUTEAL DAY 1 – ALL PATIENTS

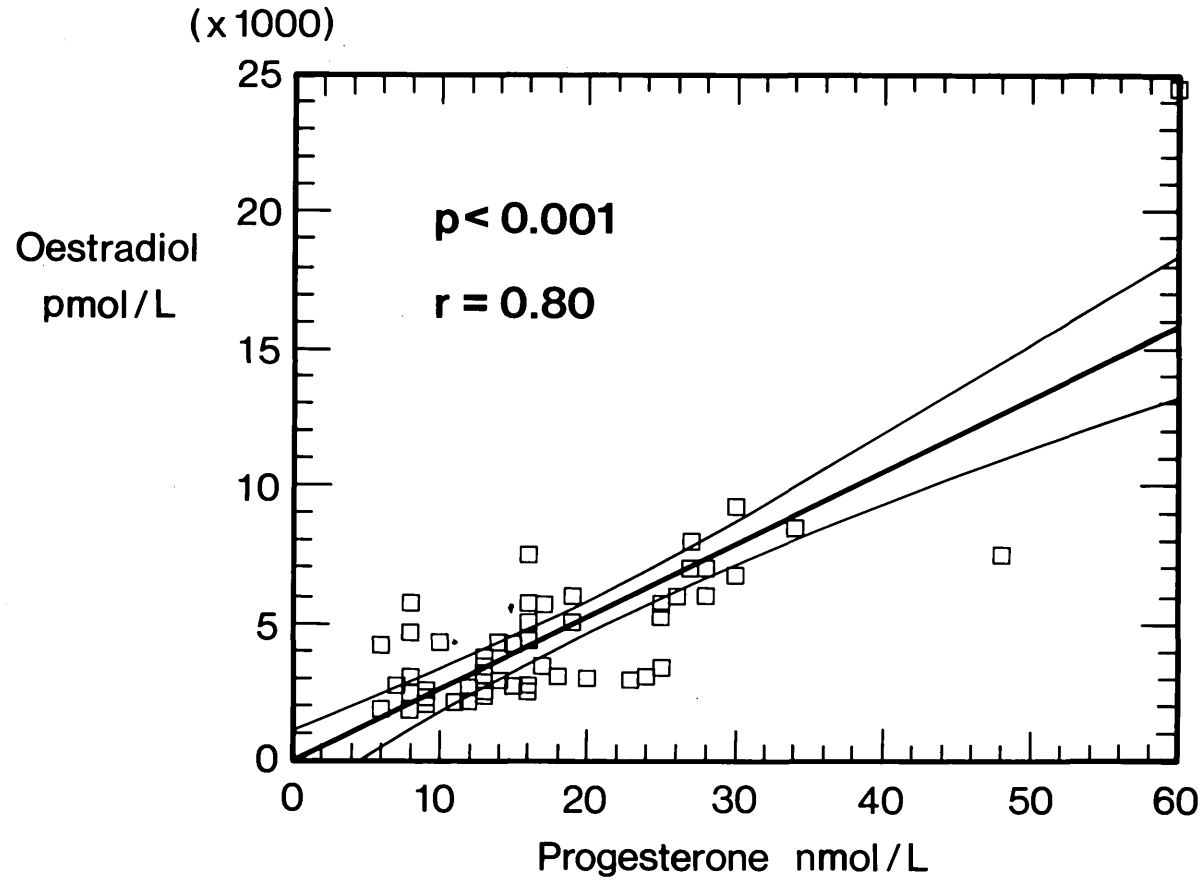


TABLE 7.D

NUMBERS OF FOLLICLES ASPIRATED AND OOCYTES RECOVERED AND THE FERTILISATION RATE.

	MEAN	RANGE	N
ALL PATIENTS			52
No. Follicles aspirated	12.60	4-24	
No. Oocytes recovered	11.04	3-26	
Fertilisation rate	77.7%	36-100%	
NO PREGNANCY			30
No. Follicles aspirated	13.77	4-24	
No. Oocytes recovered	12.03	3-26	
Fertilisation rate	79.7%	46-100%	
PREGNANCY			22
No. Follicles aspirated	11.00 (ns)	5-22	
No. Oocytes recovered	10.36 (ns)	5-21	
Fertilisation rate	76.0%	36-100%	

ns = not significant when compared by Non-parametric analysis (Mann-Whitney)

There were no differences between the no pregnancy group and the pregnancy group in terms of numbers of follicles aspirated, oocytes recovered and the fertilisation rate.

The numbers of follicles aspirated and the numbers of oocytes recovered correlated highly significantly in both groups ($p<0.001$) ($r=0.93$ in the 'no pregnancy' group, $r=0.85$ in the 'pregnancy' group). (Figure 7.B)

Significant correlations were shown between the serum E_2 concentration on day 1 and both the numbers of follicles aspirated and the numbers of oocytes recovered (Table 7.E). The mean day 1 serum E_2 per oocyte recovered was 386 pmol/L with a range of 169 - 1042 pmol/L.

TABLE 7.E

CORRELATION BETWEEN SERUM E_2 ON LUTEAL DAY 1 AND NUMBERS OF FOLLICLES ASPIRATED AND OOCYTES RECOVERED

	p	r	N
ALL PATIENTS			52
Follicles aspirated	<0.001	0.53	
Oocytes recovered	<0.001	0.61	
NO PREGNANCY			30
Follicles aspirated	<0.005	0.52	
Oocytes recovered	<0.001	0.63	
PREGNANCY			22
Follicles aspirated	<0.005	0.62	
Oocytes recovered	<0.05	0.51	

FIGURE 7B CORRELATION BETWEEN FOLLICLES ASPIRATED & OOCYTE YIELD – ALL PATIENTS

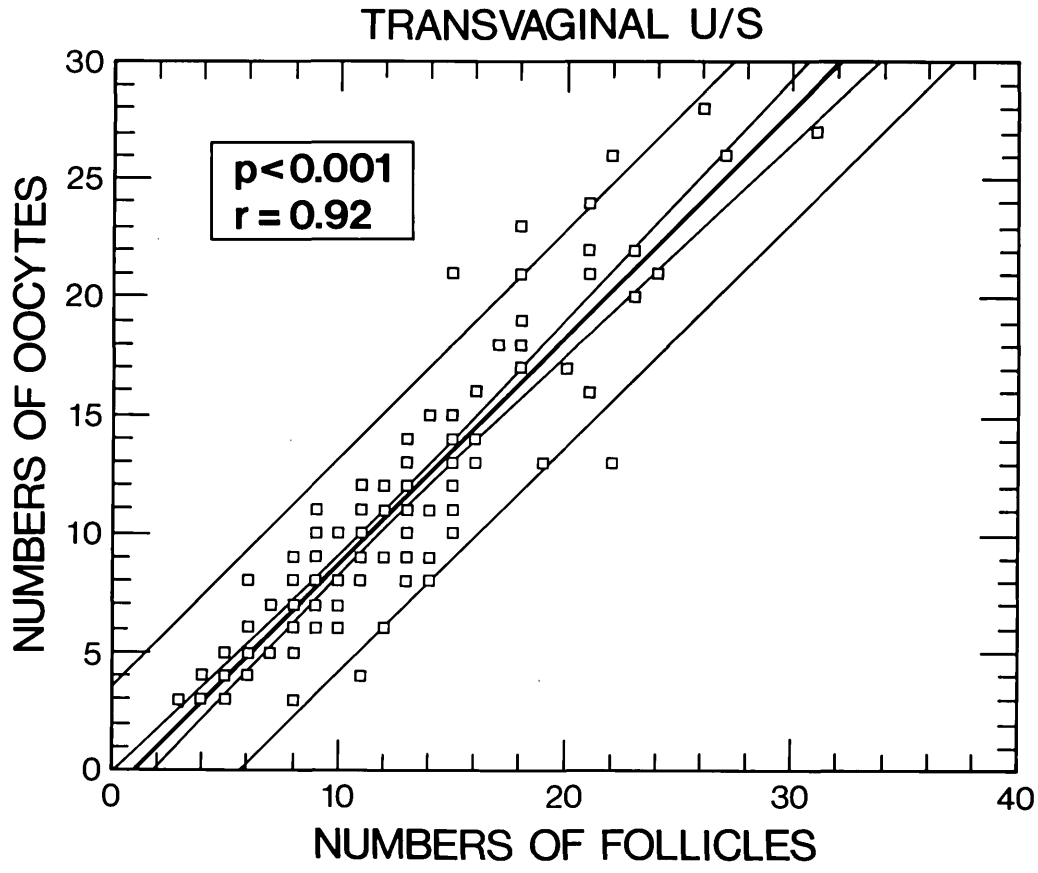


Figure 7.C shows the correlation for all patients taken collectively. The serum P on day 1 also correlated significantly with the numbers of follicles aspirated and oocytes recovered (Table 7.F).

TABLE 7.F

CORRELATION BETWEEN SERUM P ON LUTEAL DAY 1 AND NUMBERS OF FOLLICLES ASPIRATED AND OOCYTES RECOVERED

	p	r	N
ALL PATIENTS			52
Follicles aspirated	<0.001	0.63	
Oocytes recovered	<0.001	0.68	
NO PREGNANCY			30
Follicles aspirated	<0.001	0.64	
Oocytes recovered	<0.001	0.73	
PREGNANCY			22
Follicles aspirated	<0.005	0.64	
Oocytes recovered	<0.05	0.47	

The next two tables (7.G and 7.H) show the median and range for E_2, P and the E_2:P ratio on day 6 and day 12 of the luteal phase.

FIGURE 7C CORRELATION BETWEEN SERUM OESTRADIOL AND OOCYTES RECOVERED

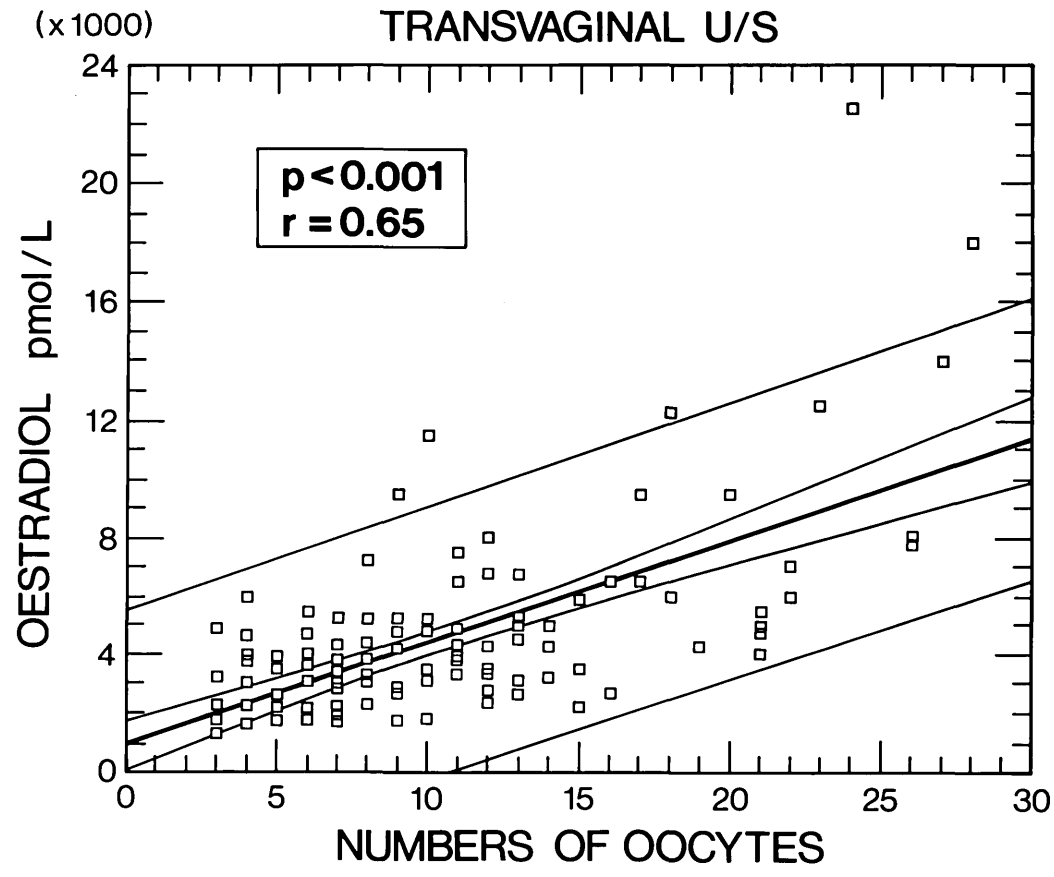


TABLE 7.G

SERUM E_2, P AND E_2:P RATIO ON LUTEAL DAY 6

	MEDIAN	RANGE	N
ALL PATIENTS			52
E_2 pmol/L	3375	1250-16,000	
P nmol/L	180	75-2,360	
E_2:P (x 10^{-3})	19.5	6-56	
NO PREGNANCY			30
E_2 pmol/L	3375	1350-16,000	
P nmol/L	177.5	93-2360	
E_2:P (x 10^{-3})	21.5	8-56	
PREGNANCY			22
E_2 pmol/L	3375 (ns)	1250-7,500	
P nmol/L	180 (ns)	75-440	
E_2:P (x 10^{-3})	18 (ns)	6-50	

ns = not significant when compared by Non-parametric analysis (Mann-Whitney) or analysis of variance.

Extreme results in the 'no-pregnancy' group are caused by isolated cases. For those patients who established a pregnancy the range for both E_2 and P was much narrower although this was not reflected in the E_2:P ratio. Pregnancy did not result if the E_2 exceeded 7500 pmol/L or the P exceeded 440 nmol/L on day 6.

However, there was no significant difference between the 'non-pregnant' group and the 'pregnant group' in either the E_2 or the P levels on luteal day 6.

TABLE 7.H

SERUM E_2, P AND E_2:P RATIO ON LUTEAL DAY 12

	MEDIAN	RANGE	N
ALL PATIENTS			52
E_2 pmol/L	1000	86-15,000	
P nmol/L	26	5-385	
E_2:P (x 10^{-3})	34	15-108	
NO PREGNANCY			30
E_2 pmol/L	1000	86-15,000	
P nmol/L	11	5-385	
E_2:P (x 10^{-3})	39	15-108	
PREGNANCY			22
E_2 pmol/L	2300 (ns)	470-5,000	
P nmol/L	105 ***	12-255	
E_2:P (x 10^{-3})	22 (ns)	16-41	

ns = not significant when compared by Non-parametric analysis (Mann-Whitney) or analysis of variance.

*** $p < 0.001$

Again a much narrower range for E_2 and P for the pregnancy groups is found on day 12. This was also shown for the E_2:P ratio.

No statistical difference was shown for the E_2 levels on luteal day 12 between the 'non-pregnant' and the 'pregnant' group but a highly significant difference was shown for P levels ($p<0.001$). However the E_2:P ratio did not differ significantly.

There was a significant correlation between the E_2 levels on luteal day 6 and the P levels ($p<0.05$) and this correlation was even more significant on day 12 ($p<0.001$) for all patients.

On luteal day 6, this correlation was not shown when the patients were analysed in terms of pregnancy or no pregnancy.

However, on luteal day 12, E_2 levels correlated significantly with P levels in both the 'non-pregnant' group ($p<0.001$) and the 'pregnant' group ($P<0.005$).

E_2 levels on luteal day 6 showed a significant correlation with the E_2:P ratio for all patients ($p<0.005$), the 'non-pregnant' group ($p<0.05$) and the 'pregnant' group ($p<0.05$).

Only in the 'pregnant' group was a significant correlation shown between P levels on luteal day 6 and the E_2:P ratio ($p<0.05$). Neither the E_2 nor the P levels correlated significantly with the E_2:P ratio on luteal day 12.

7.3 SUMMARY

Serum oestradiol and progesterone levels were analysed in this select group of patients and correlations and statistical differences among the various groups were calculated. The importance of so many combinations was to deduce overall trends and important relationships rather than just throw up one statistical result which could have occurred by chance (rather than be of any biological significance).

It was shown that statistical differences were not found before oocyte retrieval even though much narrower ranges for E_2 and P were obtained within the 'pregnancy' group as one would have hoped. The wider ranges of the 'no pregnancy' group were caused by some isolated cases which did not affect overall significances. A lack of difference was also found when the number of oocytes recovered in the 'pregnancy group' was compared with the 'no pregnancy' group. However significant correlations were obtained between E_2 and P concentrations and oocytes recovered.

Differences between the 'no pregnancy' group and the 'pregnancy' group did not appear until late on in the luteal phase. A highly significant elevated progesterone result was found on day 12 in the luteal phase of the 'pregnancy' group.

CHAPTER 8

FOLLICULAR FLUID ANALYSIS OF PROSTAGLANDINS, OESTRADIOL AND PROGESTERONE LEVELS FOR STUDY 2.

INTRODUCTION

Follicular fluids were collected from this select group of patients for study 2 (See Section 6.3). The method of aspiration was as described in Section 3.3. PGE_2 and $PGF_{2\alpha}$ were measured in follicular fluids along with oestradiol and progesterone.

8.1 FOLLICULAR FLUID GROUPING

Follicular fluids were assigned to one of 4 groups dependent upon the outcome of the matched oocyte.

GROUP 1
Follicular fluids from oocytes that did not subsequently fertilise.
GROUP 2
Follicular fluids from oocytes that did fertilise, but were not transferred.
GROUP 3
Follicular fluids from oocytes that did fertilise, were transferred but pregnancy did not result.
GROUP 4
Follicular fluids from oocytes that did fertilise, were transferred and pregnancy did result.

Results were analysed in terms of the outcome of the matched oocyte. Table 8.A shows the number of follicular fluids in each of the 4 groups.

TABLE 8.A

FOLLICULAR FLUID GROUP CATEGORY ACCORDING TO THE OUTCOME OF THE MATCHED OOCYTE.

	NUMBER
No fertilisation	100
Fertilised, not transferred	87
Fertilised, transferred no pregnancy	90
Fertilised, transferred and pregnancy	68
TOTAL	345

Only fertilised oocytes that resulted in cleaved embryos were included in the 3 fertilised groups.

8.2 FOLLICULAR FLUID CONCENTRATIONS OF PGE_2 AND $PGF_{2\alpha}$ ACCORDING TO GROUP CATEGORY

Assays for PGE_2 and $PGF_{2\alpha}$ were performed for all follicular fluids. Table 8.B shows the median and range for PGE_2.

TABLE 8.B

FOLLICULAR FLUID PGE_2 IN THE 4 STUDY GROUPS

	MEDIAN	RANGE	N
	ng/ml	ng/ml	
No fertilisation	2.41	0.2-36.7	100
Fertilised, not transferred	3.42	0.3-27.8	87
Fertilised, transferred no pregnancy	2.80	0.4-17.9	90
Fertilised, transferred, pregnancy	2.65 (ns)	0.5-65.4	68

ns = not significant when compared by Non-parametric analysis (Mann-Whitney)

There was no significant difference in the follicular fluid PGE_2 concentration from oocytes that fertilised and those that did not. When the unfertilised group was compared with each of the three other fertilised groups individually, again no significant difference was shown.

Although the range for PGE_2 was much wider in the pregnancy group, the median was not statistically different.

The results for follicular fluid $PGF_{2\alpha}$ are shown in table 8.C.

TABLE 8.C

FOLLICULAR FLUID $PGF_{2\alpha}$ IN THE 4 STUDY GROUPS

	MEDIAN	RANGE	N
	ng/ml	ng/ml	
No fertilisation	2.05	0.1-21.6	100
Fertilised, not transferred	2.11	0.4-95.0	87
Fertilised, transferred no pregnancy	2.20	0.15-27.6	90
Fertilised, transferred, pregnancy	1.85 (ns)	0.6-33.7	68

ns = not significant when compared by Non-parametric analysis (Mann-Whitney)

The results were not statistically different between the groups when $PGF_{2\alpha}$ levels were compared.

The actual concentrations of both prostaglandins therefore, shows no relationship with the various outcomes of the matched oocyte.

However a highly significant correlation ($p<0.001$) was shown between follicular fluid PGE_2 and $PGF_{2\alpha}$ for all groups with the exception of the fertilised but not transferred group (Table 8.D).

TABLE 8.D

CORRELATION BETWEEN FOLLICULAR FLUID PGE_2 AND $PGF_{2\alpha}$ FOR THE 4 STUDY GROUPS

	p	r	N
No fertilisation	<0.001	0.89	100
Fertilised, not transferred	ns	0.17	87
Fertilised, transferred no pregnancy	<0.001	0.69	90
Fertilised, transferred	<0.001	0.88	68

The ratio of PGE_2 to $PGF_{2\alpha}$ was calculated for all groups (Table 8.E).

TABLE 8.E

FOLLICULAR FLUID PGE_2:$PGF_{2\alpha}$ RATIO FOR THE 4 STUDY GROUPS

	MEDIAN	RANGE	N
No fertilisation	1.50	0.18-5.5	100
Fertilised, not transferred	1.55	0.15-7.66	87
Fertilised, transferred no pregnancy	1.40	0.3-5.91	90
Fertilised, transferred, pregnancy	1.10 ***	0.46-2.00	68

*** $p<0.001$ when compared by Non-parametric analysis (Mann-Whitney) to the other 3 groups.

The PGE_2:$PGF_{2\alpha}$ ratio was not significantly different between the unfertilised group and the combined three fertilised groups. However when the unfertilised group was compared with the three fertilised groups individually, the 'pregnancy' group was the only group to show a significant difference and this difference was highly significant ($p<0.001$). This highly significant difference was also shown when the 'pregnancy' group was compared to the fertilised, not transferred and most importantly to the fertilised, transferred, no pregnancy group.

The range for the ratio in the group that established pregnancy was much narrower, 0.46-2.00 compared to the other three groups (no fertilisation, 0.18-5.5; fertilised not transferred, 0.15-7.66; fertilised transferred, no pregnancy 0.3-5.91). (Figure 8.A).

FIGURE 8A1 NO FERTILISATION

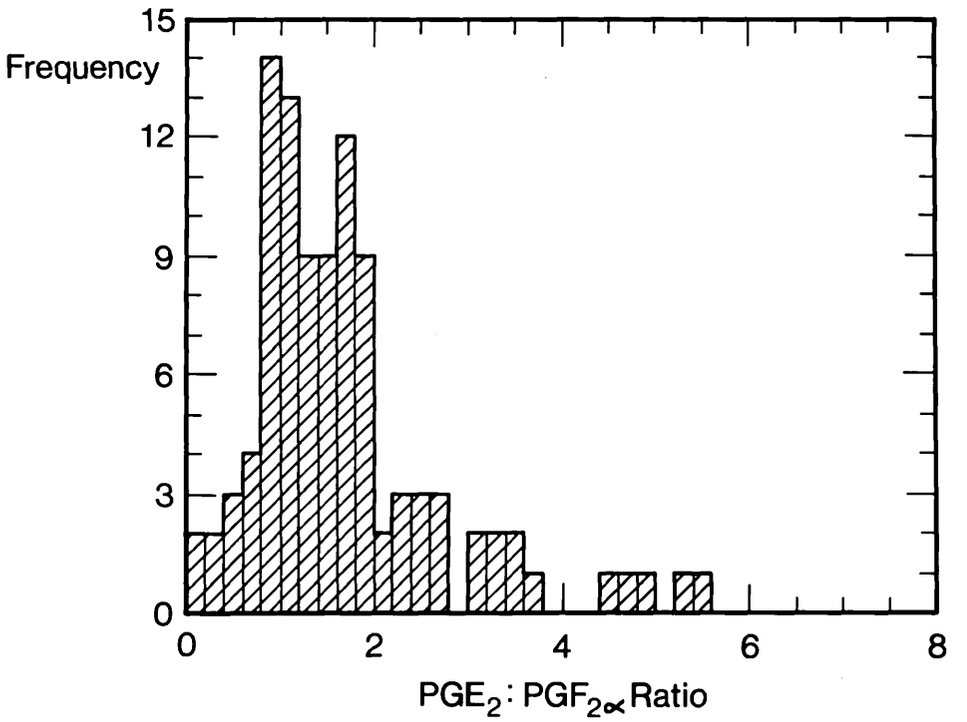


FIGURE 8A2 FERTILISED, NOT TRANSFERRED

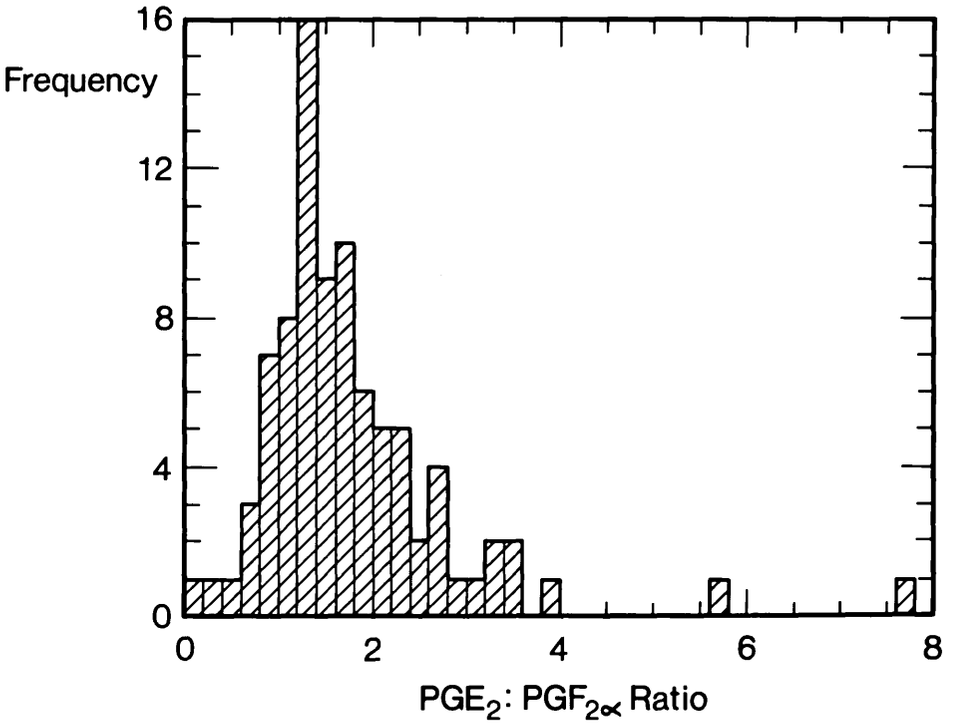


FIGURE 8A3 FERTILISED, TRANSFERRED, NO PREGNANCY

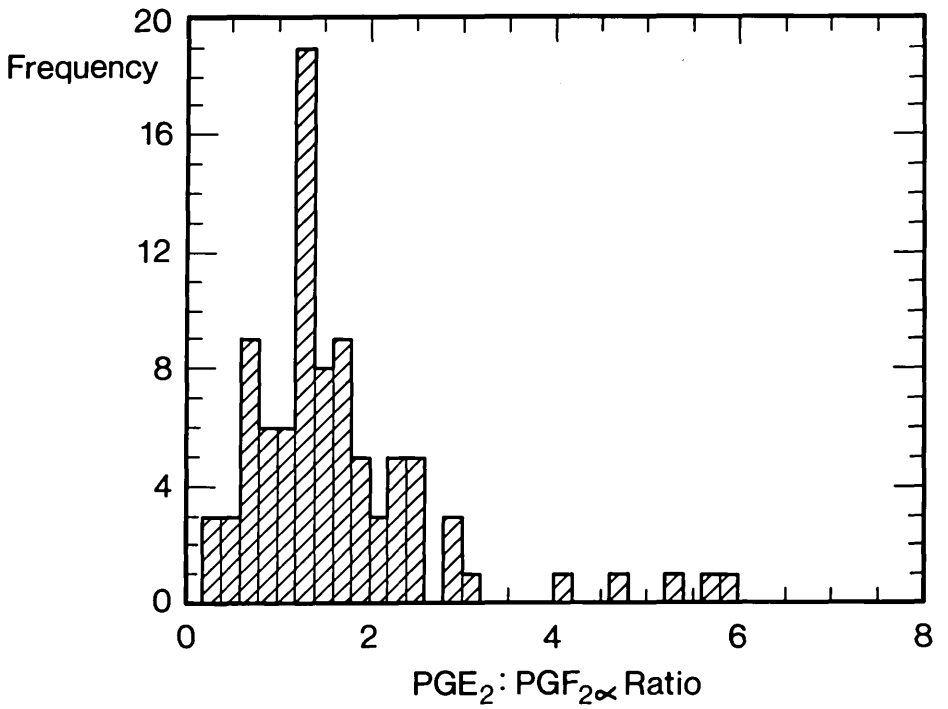
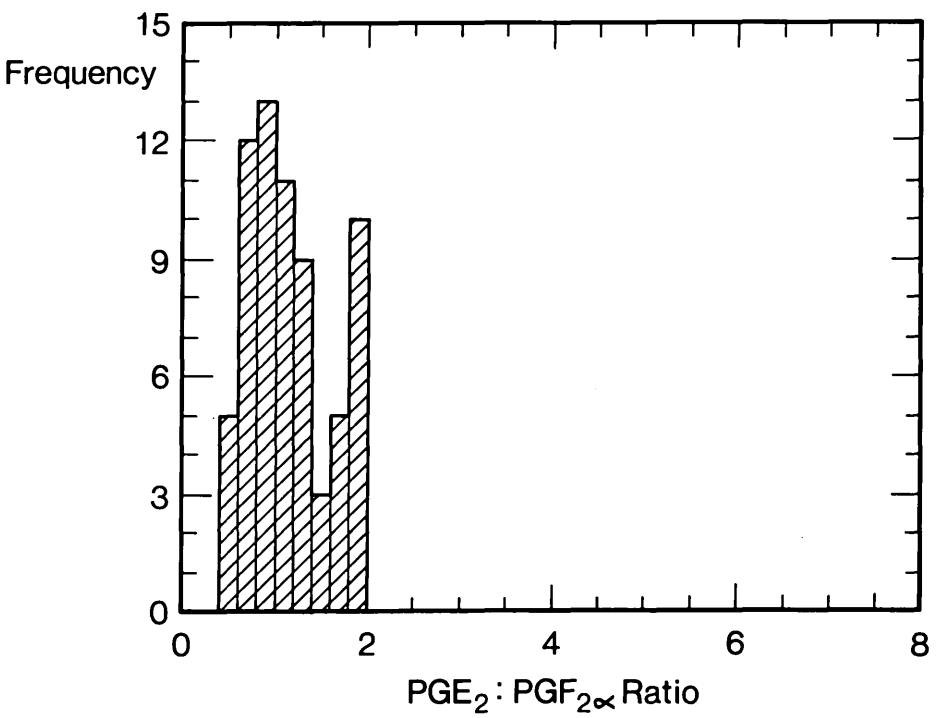


FIGURE 8A4 FERTILISED, TRANSFERRED, PREGNANCY



8.3 FOLLICULAR FLUID STEROID HORMONE CONCENTRATIONS ACCORDING TO GROUP CATEGORY

All follicular fluids were assayed for E_2 and P. The median and range for follicular fluid E_2 and P levels is shown in Table 8.F.

There was no significant difference in follicular fluid E_2 levels shown between the unfertilised group and the fertilised groups either together or individually.

However there was a significant difference in the follicular fluid P levels when no fertilisation was compared with fertilisation ($p<0.005$). When the fertilised groups were compared individually with the unfertilised group this signficance was only seen for the fertilised, tranferred, no pregnancy group. ($p<0.001$)

There was a significant correlation shown between E_2 and P ($p<0.001$) in all groups except the fertilised, transferred, no pregnancy group (Table 8.G).

TABLE 8.F

FOLLICULAR FLUID E_2 AND P LEVELS AND THE E_2:P RATIO FOR THE 4 STUDY GROUPS

	MEDIAN	RANGE	N
NO FERTILISATION			100
E_2 nmol/L	780	50-3500	
P μmol/L	5.95	1-31	
E_2:P (x 10^{-3})	120.5	20-1050	
FERTILISED, NOT TRANSFERRED			87
E_2 nmol/L	765 (ns)	62-3100	
P μmol/L	8.2	1-33	
E_2:P (x 10^{-3})	81.5	16-880	
FERTILISED, TRANSFERRED, NO PREGNANCY			90
E_2 nmol/L	845 (ns)	64-4500	
P μmol/L	12.3 ***	1-50	
E_2:P (x 10^{-3})	78	2-549	
FERTILISED, TRANSFERRED, PREGNANCY			68
E_2 nmol/L	810 (ns)	11-3600	
P μmol/L	8.4 (ns)	1-40	
E_2:P (x 10^{-3})	115	4-355	

ns = not significant when compared by Non-parametric analysis (Mann-Whitney) to the no fertilisation group.

*** $p<0.001$

TABLE 8.G

CORRELATION BETWEEN FOLLICULAR FLUID E_2 AND P

	p	r	N
No fertilisation	<0.001	0.56	100
Fertilised, not transferred	<0.001	0.56	87
Fertilised, transferred no pregnancy	ns	0.18	90
Fertilised, transferred, pregnancy	<0.001	0.52	68

There were no significant differences shown in any of the groups for the E_2:P ratio (Table 8.H).

TABLE 8.H

FOLLICULAR FLUID E_2:P RATIO

	MEDIAN	RANGE	N
No fertilisation	120.5 (ns)	20-1050	100
Fertilised, not transferred	78 (ns)	1.8-549	87
Fertilised, transferred no pregnancy	81.5 (ns)	16-880	90
Fertilised, transferred, pregnancy	115 (ns)	4-355	68

ns = not signficant when compared by Non-parametric analysis (Mann-Whitney).

There was a negative correlation shown between PGE_2 or $PGF_{2\alpha}$ and E_2 and also between PGE_2 and P for the unfertilised group (See Table 8.I). There were no correlations between the prostaglandins and the steroid hormones in the other groups.

TABLE 8.I

CORRELATION BETWEEN FOLLICULAR FLUID PGE_2 AND $PGF_{2\alpha}$ WITH E_2 AND P IN THE 4 STUDY GROUPS

	PGE_2 V E_2	PGE_2 V P	$PGF_{2\alpha}$ V E_2	$PGF_{2\alpha}$ V P
UNFERTILISED				
	$p<0.005$	$p<0.05$	$p<0.01$	p = ns
	r = -0.29	r = -0.21	r = -0.28	r = -0.17
FERTILISED, NOT TRANSFERRED				
	p = ns	p = ns	p = ns	p = ns
	r = 0.006	r = 0.08	r = 0.09	r = 0.06
FERTILISED, TRANSFERRED, NO PREGNANCY				
	p = ns	p = ns	p = ns	p = ns
	r = 0.17	r = 0.13	r = 0.13	r = 0.01
FERTILISED, TRANSFERRED, PREGNANCY				
	p = ns	p = ns	p = ns	p = ns
	r = 0.10	r = 0.14	r = 0.009	r = 0.17

ns = not significant when compared by Non-parametric analysis (Mann-Whitney)

8.4 SUMMARY

The follicular fluid analysis of prostaglandins, PGE_2 and $PGF_{2\alpha}$, oestradiol and progesterone on this more select group was able to substantiate some earlier findings. Results also showed further important statistical and probably biological significances.

The PGE_2:$PGF_{2\alpha}$ ratio values were generally lower and within "tighter" ranges than calculated previously (Chapter 5) as one might expect for this group. Therefore it was possible to show that the fertilised oocytes (not dependent on their embryo grade) associated with a pregnancy outcome had a significantly narrower range of PGE_2:$PGF_{2\alpha}$ ratio than those not associated with a pregnancy.

Interestingly the E_2:P ratio for the 'pregnancy' group was also narrower but the difference from other groups did not reach statistical significance. In addition P levels in follicles associated with oocytes, that were fertilised but no pregnancy resulted, were significantly higher.

CHAPTER 9

TOTAL PROTEIN CONTENT OF FOLLICULAR FLUID AND ANDROGEN CONCENTRATIONS

9.1 PROTEIN CONTENT OF FOLLICULAR FLUID

In order to substantiate the significant correlation between PGE_2 and $PGF_{2\alpha}$ and likewise between E_2 and P, protein concentrations were measured in three sample groups of follicular fluid: unfertilised; fertilised, transferred, no pregnancy; and transferred, fertilised and pregnancy.

Total protein concentration was measured by a standard Coomassie Brilliant Blue (CBB) method. This dye - binding method was originally described for cerebro-spinal fluid (Johnson & Lott, 1978) and subsequently adapted to urine (Lott, Stephen & Pritchard, 1983) CBB binds to protonated amine groups of amino acid residues in the polypeptide chain, and the absorbance maximum for the bound species of the dye decreases at 465nm and increased at 595nm.

Table 9.A shows the range of protein concentrations found in a random selection of follicular fluids from the different groups. The range indicated that a few samples had been diluted with flushing medium.

TABLE 9.A

RANGE OF PROTEIN CONCENTRATIONS

	MODE(mg/ml)	RANGE(mg/ml)	n
Unfertilised	30.8	10.5-46	12
Fertilised, transferred no pregnancy	24.8	3.8 - 50	15
Fertilised, transferred pregnancy	33 (ns)	2.7 - 45.3	12

ns = not significant when compared by Non-parametric analysis (Mann-Whitney).

> 90% were above 20 mg/ml.

These findings are similar to those reported by Stone et al (1988). The median protein concentration in follicular fluid was 39mg/ml and no significant differences were shown between pregnant and non pregnant outcomes.

The PGE_2: protein ratio and the $PGF_{2\alpha}$;protein ratio were calculated. The summary of the prostaglandin:protein ratios is shown in Tables 9.B and 9.C.

TABLE 9.B

FOLLICULAR FLUID PGE_2:PROTEIN RATIO

	MEDIAN	RANGE	N
Unfertilised	0.56	0.13-6.43	12
Fertilised, transferred no pregnancy	0.99	0.1-13.17	15
Fertilised, transferred pregnancy	0.90(ns)	0.22-8.52	12

ns = not significant when compared by Non-parametric analysis (Mann-Whitney).

TABLE 9.C

FOLLICULAR FLUID $PGF_{2\alpha}$:PROTEIN RATIO

	MEDIAN	RANGE	N
Unfertilised	0.60	0.14-3.9	12
Fertilised, transferred no pregnancy	0.66	0.07-9.25	15
Fertilised, transferred pregnancy	0.79(ns)	0.25-8.89	12

ns = not significant when compared by Non-parametric analysis (Mann-Whitney).

The same highly significant correlation found between PGE_2 and $PGF_{2\alpha}$ in follicular fluid was also shown between the PGE_2:protein ratio and the $PGF_{2\alpha}$:protein ratio. ($p<0.001$, $r=0.77$) (Figure 9.A)

A highly significant correlation was also shown between the E_2: protein ratio and the P:protein ratio in follicular fluids ($p<0.001$, $r=0.69$) (Figure 9.B). However, once again there were no significant correlations between the steroids and the prostaglandins.

FIGURE 9A CORRELATION BETWEEN FOLLICULAR FLUID PGE_2 : PROTEIN RATIO AND THE $PGF_{2\alpha}$: PROTEIN RATIO

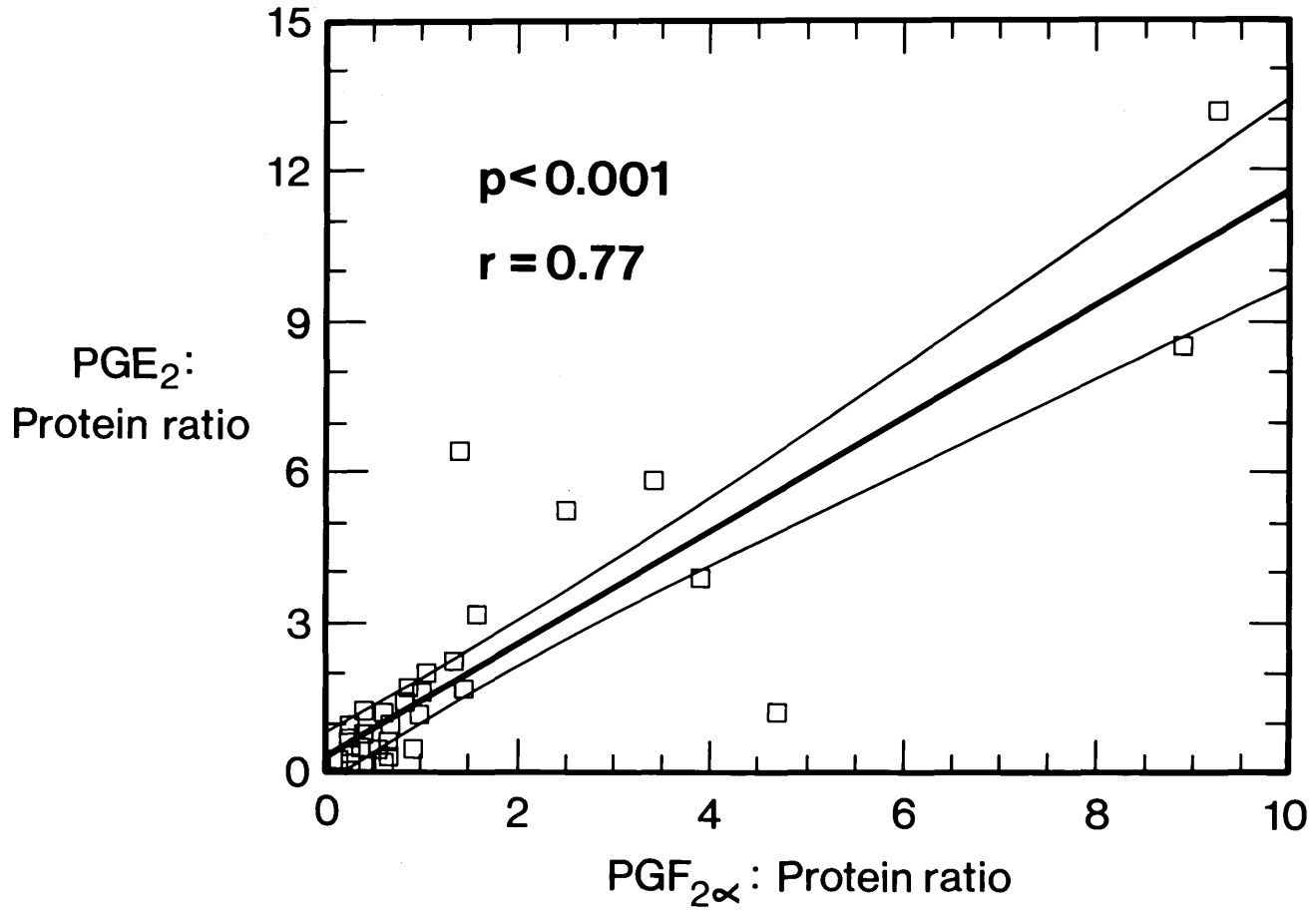
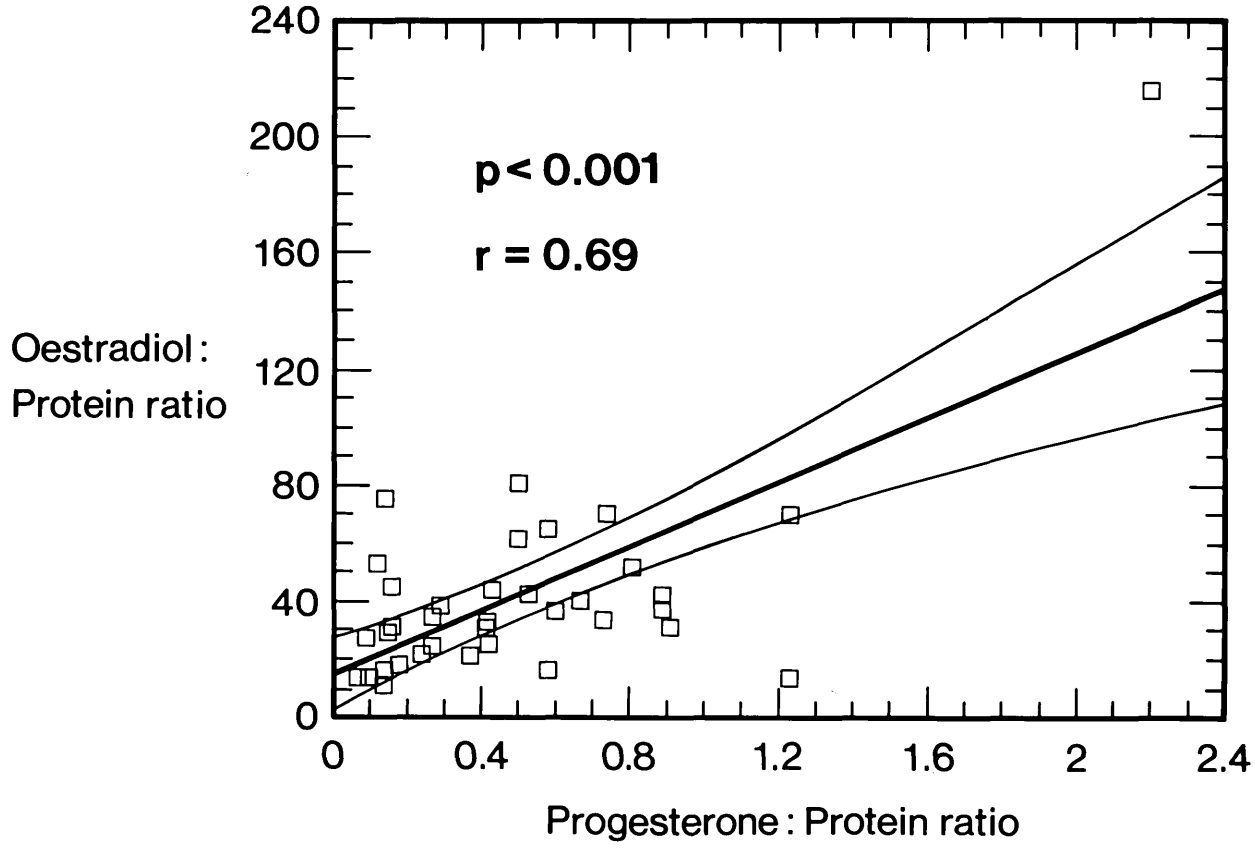


FIGURE 9B CORRELATION BETWEEN E_2 : PROTEIN RATIO AND THE P : PROTEIN RATIO



9.2 ANDROGEN CONCENTRATIONS IN FOLLICULAR FLUID

Many factors are involved in ensuring successful fertilisation, not least of which is the developmental state of the follicle at the time of aspiration. The cumulus-oocyte complex is exposed to the hormonal environment of the follicular fluid, where concentrations can vary quite dramatically in the pre-ovulatory follicle.

E_2 and P content of follicular fluid have been analysed extensively in a preceding chapter. Follicular fluid analysis would not be complete without reference to androgen levels.

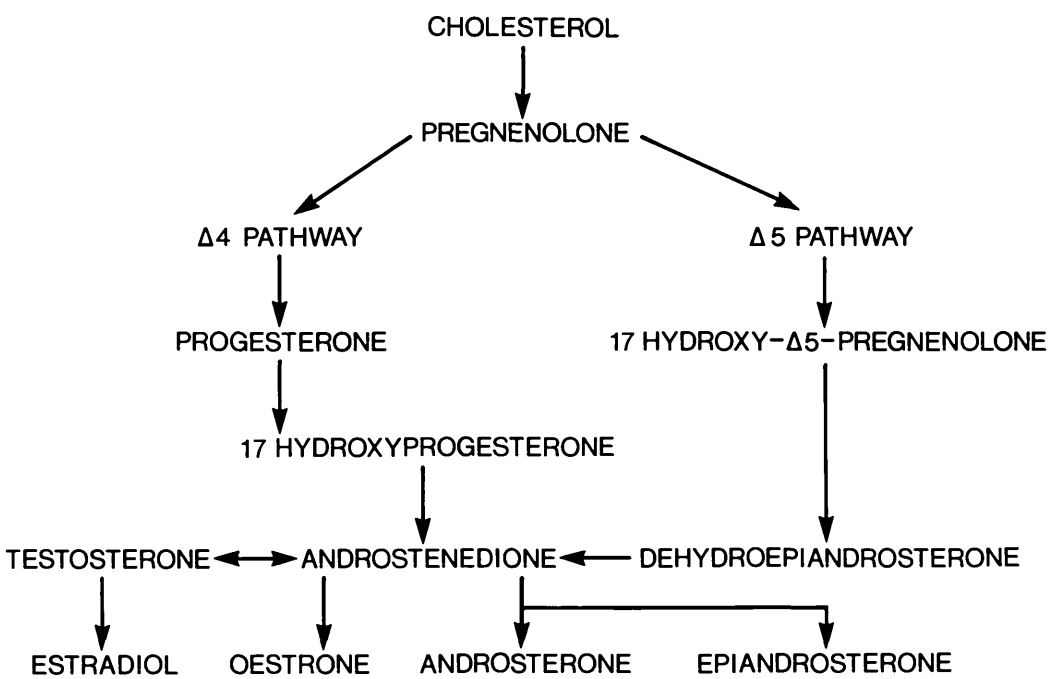
Steroidogenesis in the ovary and in particular the $\Delta 5$ pathway involved in the biosynthesis of androgens and oestrogens is shown in Diagram 9.A.

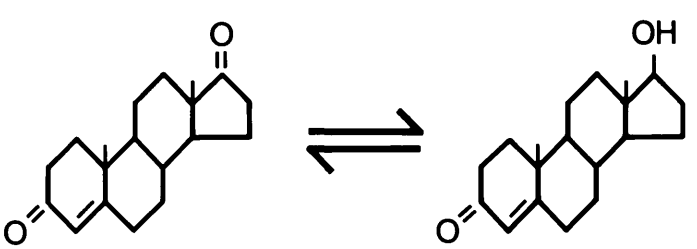
The amount of androstenedione in follicular fluid changes during follicle growth. It is found in higher amounts than E_2 in growing follicles but in smaller quantities in pre-ovulatory follicles (Edwards, 1980).

Fowler et al (1977), using the natural cycle, found higher levels of androstenedione in non-ovulatory follicles from which oocytes did not fertilise, compared to pre-ovulatory follicles. Pre-ovulatory follicles contained androstenedione levels in the range 3-107ng/ml compared to a range of 991-2169 ng/ml in non-ovulatory follicles.

Fowler et al (1978), again demonstrated higher androgen levels in non-ovulatory follicles from hMG stimulated cycles.

DIAGRAM 9A STEROIDOGENESIS IN THE OVARY





Androstenedione Testosterone

Fishel et al (1983), showed no significant differences in androstenedione levels whether or not fertilisation of the oocyte occurred. The majority of fertilised oocytes had a follicular fluid androstenedione:E_2 ratio of around 0.1. However ratios greater than 0.1 were compatible with fertilisation.

Lower androstenedione:E_2 ratios were shown by both Chikhaoui et al (1983) and Chabab et al (1986) to be associated with a favourable outcome.

Chikhaoui et al using CC stimulated cycles showed an androstenedione:E_2 ratio less than or equal to 0.5 to be associated with the highest fertilisation and cleavage rates.

Chabab et al analysed both CC and hMG stimulated cycles and found a ratio of less than 1.0 to be most favourable for fertilisation and pregnancy outcome.

RESULTS

Androstenedione levels were assayed in a sample population from each of three follicular fluid groups: unfertilised; fertilised, transferred, no pregnancy; and fertilised, transferred, pregnancy.

Androstenedione was measured by a commercially available radioimmunoassay (Amersham, UK, Product No. IMB.500).

The main steps of the assay are:

Incubation of reaction mixture	Sample/Standard
30 minutes in 37°C waterbath	+ tracer
	+ antiserum
then	
Addition of precipitating reagent	
15 minutes incubation	
then	
Centrifuge	
then	
Decantation of supernatant	
then	
Retain pellet	
then	
Radioactivity counting in a gamma counter	

The cross-reactivity was 4% for 5α-Androstane-3,17-dione and less than 3% for other steroid hormones and their metabolites.

The intra-assay variation was 5% and the inter-assay variation was 6%. The minimal detection concentration of androstenedione was 0.04ng/ml (μg/l).

Table 9.D shows the median and range for the androstenedione levels in these selected follicular fluids.

Figure 9.C displays the ranges for the three groups in histogram form.

TABLE 9.D

FOLLICULAR FLUID ANDROSTENEDIONE CONCENTRATIONS

	MEDIAN	RANGE	N
	μg/L	μg/L	
Unfertilised	45.5	3.5-503	12
Fertilised, transferred no pregnancy	35.4	13.8-260	15
Fertilised, transferred pregnancy	34.1(ns)	8.2-87	12

ns = not significant when compared by Non-parametric analysis (Mann-Whitney).

Although there were no significant differences shown between the groups a tighter range for androstenedione was seen as the outcome became more favourable.

FIGURE 9C1 RANGE OF ANDROSTENEDIONE IN UNFERTILISED GROUP

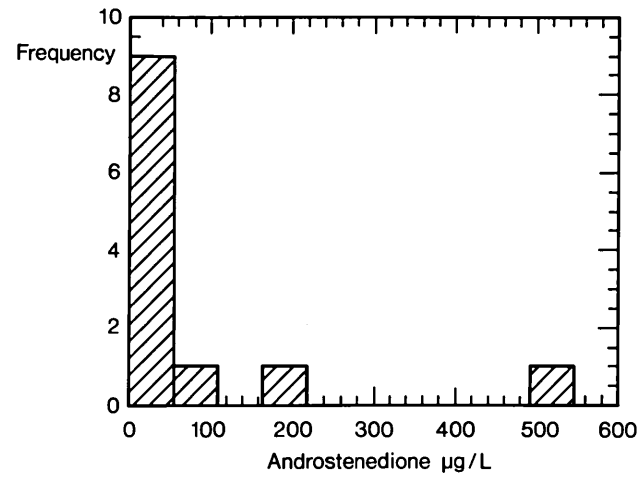


FIGURE 9C2 RANGE OF ANDROSTENEDIONE IN FERTILISED, TRANSFERRED, NO PREGNANCY GROUP

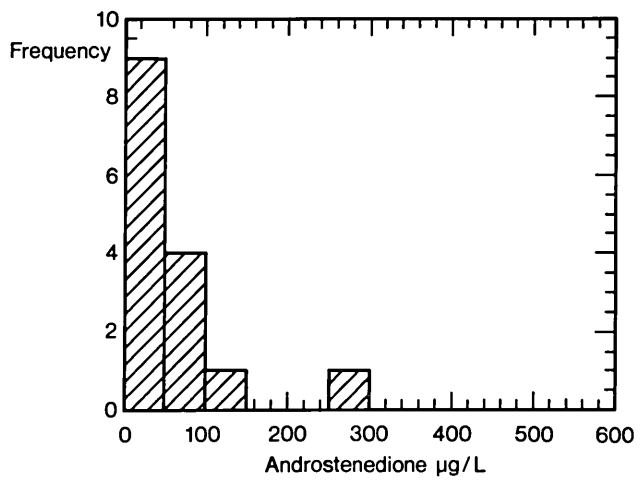


FIGURE 9C3 RANGE OF ANDROSTENEDIONE IN FERTILISED, PREGNANCY GROUP

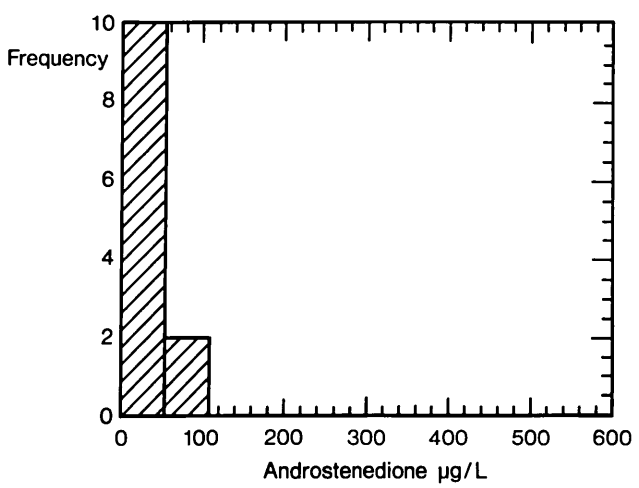


Table 9.E shows the median and range for the androstenedione:protein ratio in each group.

TABLE 9.E

FOLLICULAR FLUID ANDROSTENEDIONE:PROTEIN RATIO

	MEDIAN	RANGE	N
Unfertilised	0.42	0.09-3.86	12
Fertilised, transferred no pregnancy	0.44	0.21-1.24	15
Fertilised, transferred pregnancy	0.34(ns)	0.25-1.57	12

ns = not significant when compared by Non-parametric analysis (Mann-Whitney).

There was no significant difference in androstenedione levels between the groups. Once again results were not affected by taking into account the protein concentration and thus any dilution effect.

The unfertilised group contains isolated high levels and low levels of androstenedione which are not seen in either of the two fertilised groups. However no overall difference was found between any of the groups.

Table 9.F shows the median and range for the androstenedione:E_2 ratio in the three groups. No significant difference was shown.

TABLE 9.F

THE FOLLICULAR FLUID ANDROSTENEDIONE:E_2 RATIO

	MEDIAN	RANGE	N
Unfertilised	0.03	0.1-0.74	12
Fertilised, transferred no pregnancy	0.04	0.02-0.27	15
Fertilised, transferred pregnancy	0.04(ns)	0.01-0.41	12

ns = not significant when compared by Non-parametric analysis (Mann-Whitney).

The fertilised, no pregnancy group was the only group in which a significant correlation was found between androstenedione and E_2. Androstenedione and progesterone were not significantly correlated in any of the groups. However, as previously shown, E_2 correlated significantly with P in all groups. The prostaglandins did not show significant correlations with any of the steroids or ratios. Nevertheless the prostaglandin ratio did once again show to be statistically different in the 'pregnancy' group from the 'no pregnancy' group ($p<0.01$).

9.3 SUMMARY

It was important to show that possible dilution effects with flushing medium were not causing some of the established relationships.

However, if dilution had occurred to a great extent, previous findings should have shown positive correlations between the prostaglandins and the steroids, when not allowing for protein effects. This was not found. The ratios between two variables (i.e. PGE_2:$PGF_{2\alpha}$), of course, would not be affected.

Nevertheless, the significant correlation between PGE_2 and $PGF_{2\alpha}$ levels could be shown if the protein concentration was taken into consideration (Section 9.1) once again prostaglandins did not correlate with steroid levels.

Androstenedione was also measured in follicular fluid samples. The fertilised groups showed narrower ranges than the unfertilised group, but no statistical differences could be found. The fertilised groups also showed a narrower range for the androstenedione:E_2 ratio.

These data showed the presence of extreme biochemical levels in follicular fluids associated with poor outcome, but only with the prostaglandin ratio could important statistically significant results be obtained.

CHAPTER 10

DISCUSSION

In vitro fertilisation is now a well established technique for the treatment of various forms of infertility. Nevertheless despite more than a decade's experience and major advances, success rates remain low.

Implantation of embryos transferred after IVF depends on several factors, many of which remain unclear. Sharma et al, (1988) showed the influence of the number of embryos transferred. The clinical pregnancy rate progressively increased from 8.6% when one embryo was transferred to 29.5% when four embryos were transferred. Jones et al, (1983) emphasized the importance of the endocrine characteristics of the transfer cycle. The highest pregnancy rates were obtained when the serum E_2 continued to rise throughout the follicular phase and on the day following the administration of hCG. The value of E_2 had usually fallen on the day of oocyte collection. Garcia et al, (1984) performed early luteal phase endometrial biopsies in patients whose embryo transfer was not accomplished. Serum levels in pregnancy cyles were compared with the serum P levels in these patients. It was concluded that since the embryo arrives in the endometrial cavity considerably earlier than in the natural cycle (approximately 52 hours compared to 72-96 hours) "advanced" endometrium might be beneficial for implantation.

These three factors, the number of embryos transferred, the endocrine characteristics of the transfer cycle and endometrial morphology at the time of transfer are known to influence the outcome of the IVF procedure. All three factors are inter-related and affected by the changing concentrations of various components of follicular fluid. The studies in this thesis attempted to establish whether differing components of follicular fluid, in particular prostaglandins and steroid hormones were associated with the degree of success and thus possibly "influenced" the outcome of oocytes fertilised in vitro. Success rates of embryo transfer would be improved substantially if only viable embryos could be selected for transfer. The follicular fluid results would be used together with the microscopic evaluation of the embryos to produce a more sensitive assessment of embryo viability.

Reliable assays for the steroid hormones to be measured had been well established, but prostaglandin assays at the start of these studies required careful assessment and refinement. Initially it was established that an extraction process was necessary prior to radioimmunoassay for PGE_2 and $PGF_{2\alpha}$. Previous workers had extracted prostaglandins using various organic solvents or by extensive chromatography on columns of XAD-2 resin (Chapter 1.4, Table 1.B). Extraction on ODS silica mini-columns proved to be a fast, convenient and moderately inexpensive method for the removal of PGE_2 and $PGF_{2\alpha}$ from follicular fluids. Recoveries varied between 80 and 95% with this extraction method whereas without extraction no more than 58% of PGE_2 and $PGF_{2\alpha}$ could be detected. (Chapter 4.6, Figures 4.H and 4.I)

Although prostaglandins are rapidly catabolised within the body, freeze storage of follicular fluid did not influence the results. The variation in results between assays on fresh samples and frozen samples did not exceed 7% for PGE_2 and 8% for $PGF_{2\alpha}$. (Chapter 4.7, Figures 4.J and 4.K) Radioimmunoassays for PGE_2 and $PGF_{2\alpha}$ on extracts were reliable and reproducible. The intra-assay coefficient of variations were 4% and 5% for PGE_2 and $PGF_{2\alpha}$ respectively and the inter-assay coefficient of variations were 7% and 8% for PGE_2 and $PGF_{2\alpha}$ respectively. (Chapter 4.5) The steroid hormones were assayed in unextracted samples using commerical assays designed for direct determination of steroids.

In this thesis, preliminary studies measured E_2 and P in follicular fluid and comparisons were made in regard to the presence or absence of an oocyte, fertilisation, cleavage and embryo grade. Many workers have measured the steroid hormones in human ovarian follicles and attempts have been made to correlate follicular fluid concentrations of these substances with these various outcomes. Literature results, however, have been conflicting. Higher E_2 and P concentrations have been associated with oocyte containing follicles compared to follicles without oocytes (Tsuiki et al, 1988). This study did not confirm these findings. (Table 10.A).

TABLE 10.A

FOLLICULAR FLUID E_2 AND P LEVELS IN OOCYTE CONTAINING AND NON OOCYTE CONTAINING FOLLICLES

	TSUIKI Mean	SOTON Mean
Oocyte		
E_2 nmol/L	1006 (444-2007)	634 (11-3400)
P μmol/L	6.9 (0.42-22.9)	12.72 (1.5-55)
No Oocyte		
E_2 nmol/L	7530 (965-13570)***	526 (68-2300) (ns)
P μmol/L	2.28 (0.028-6.25)***	12.58 (1.5-42) (ns)
Regime	CC/hMG or FSH	LHRH-A/hMG

SOTON = Southampton

*** $p<0.001$

ns = not significant

Fishel et al (1983) and Botero-Ruiz et al (1984) showed that high fertilisation rates were associated with elevated follicular fluid E_2 concentrations whereas Reinthaller et al (1983) associated successful fertilisation with higher follicular fluid P concentrations. Lack of correlation between follicular fluid P or E_2 concentrations and subsequent fertilisation has also been reported. (Berger et al, 1987). No differences between E_2 and P were shown in this study whether or not fertilisation occurred (Table 10.B).

TABLE 10.B FOLLICULAR FLUID E_2 & P LEVELS FOR FERTILISED AND NON-FERTILISED OOCYTES

	FISHEL	REINTHALLER	BERGER	BOTERO-RUIZ	SOTON
	1983	1983	1987	1984	
	MEAN	MEAN	MEAN	MEAN	MEAN
Fertilisation					
E_2 nmol/L	5500	2565	6352	1938	705
P μmol/L	35	27	3.1	19.9	15.2
Non Fertilisation					
E_2 nmol/L	3400*	2202 (ns)	5355 (ns)	1292**	768 (ns)
P μmol/L	12.5**	21*	2.2 (ns)	20.5 (ns)	16.7 (ns)
Regime	CC	hMG	hMG	hMG	LHRH-A/hMG

** $p < 0.01$

* $p < 0.05$

ns = not significant

No significant differences in the steroid hormone levels were found when comparing fast and slow cleaving embryos (Chapter 5.4, Table 5.K) and good quality and poor quality embryos. (Chapter 5.5, Table 5.N). The steroid hormones, therefore did not appear to be potential markers of oocyte quality within the range of follicles assessed as optimal by transvaginal ultrasound in the present studies.

The prostaglandins, PGE_2 and $PGF_{2\alpha}$, were also measured in the preliminary study and similar comparisons made. The involvement of prostaglandins in the process of ovulation has been established in animal studies. Yang et al (1974), Tsang et al (1979), Brown et al (1984) and Espey et al (1986) all showed significantly higher levels of both PGE_2 and $PGF_{2\alpha}$ in follicles destined to ovulate. Ovulation in animals can be prevented by administering a cyclo-oxygenase inhibitor, such as indomethacin. Indomethacin blocked ovulation in rats (Tsafriri et al, 1972, Parr, 1975), monkeys (Wallach et al, 1975) and rabbits (Rhodes et al, 1985). Ovulation can also be prevented by administration of antisera to prostaglandins (Armstrong et al, 1974).

The role of follicular fluid prostaglandins in relation to the subsequent outcome of the associated oocyte and also whether the follicle yielded an oocyte has been studied in humans. The results have been inconsistent.

Both Lumsden et al (1986) and Jeremy et al (1986) showed significantly higher concentrations of both prostaglandins, PGE_2 and $PGF_{2\alpha}$ when the follicle contained an oocyte. My study, in agreement with the findings of Heinonen et al (1986) did not show this difference. (Table 10.C) Comparison between fertilisation and non-fertilisation also showed no significant differences for either PGE_2 or $PGF_{2\alpha}$ concentrations. This is in agreement with other studies (Table 10.D).

Similarly, prostaglandin concentrations did not differ significantly with differing rates of cleavage or embryo grade. The ratio of PGE_2 to $PGF_{2\alpha}$ was not significantly different for any of the parameters analysed. Table 10.E summarises the results of the preliminary findings.

TABLE 10.C FOLLICULAR FLUID PGE_2 AND $PGF_{2\alpha}$ CONCENTRATIONS IN RELATION TO OOCYTE OR NON OOCYTE CONTAINING FOLLICLES

	LUMSDEN 1986 MEAN	JEREMY 1986 MEAN	HEINONEN 1986 MEAN	SOTON MEAN
Oocyte				
PGE_2 ng/ml	3.38	0.43	4.5	4.33
$PGF_{2\alpha}$ ng/ml		0.86	4.1	1.45
No Oocyte				
PGE_2 ng/ml	1.16**	0.07***	5.2 (ns)	3.53 (ns)
$PGF_{2\alpha}$ ng/ml		0.11***	3.1 (ns)	1.45 (ns)
Regime	CC	CC/hMG	CC/hMG	LHRH/A/hMG
Methodology	Methyloxime	XAD-2 Resin	XAD-2 Resin	ODS Silica
Treatment of Samples	Ethanol	Ethylacetate	Diethylether	

*** $p<0.001$

** $p<0.01$

ns = not significant

TABLE 10.D FOLLICULAR FLUID PGE_2 AND $PGF_{2\alpha}$ FOR FERTILISED AND NON FERTILISED OOCYTES

	CHABAB 1986 MEAN	REINTHALLER 1987 MEAN	SOTON MEAN
Unfertilised			
PGE_2 ng/ml	8.03	3.4	4.04
$PGF_{2\alpha}$ ng/ml	3.78	9.3	1.39
Fertilised			
PGE_2 ng/ml	5.66 (ns)	2.6 (ns)	3.05 (ns)
$PGF_{2\alpha}$ ng/ml	3.49 (ns)	9.2 (ns)	1.33 (ns)
Regime	CC/hMG	hMG	LHRH-A/hMG
Methodology	No extraction Bio-Sila Slurry	Ethylacetate ODS Silica	Diethyl Ether

ns = not significant

TABLE 10.E SUMMARY OF PROSTAGLANDIN RESULTS IN PRELIMINARY STUDY

	PGE_2 ng/ml Median	PGE_2 ng/ml Range	$PGF_{2\alpha}$ ng/ml Median	$PGF_{2\alpha}$ ng/ml Range	RATIO Mean	RATIO Range	N
Oocyte	3.4	0.4-86.3	1.35	0.1-22.5	2.77	0.24-16.4	83
No Oocyte	4.21	0.18-80.3	1.45	0.2-16.5	2.27	0.18-15.5	42
Fertilisation	3.25	0.5-327	1.33	0.1-31.5	2.69	0.67-16.4	82
No Fertilisation	4.34	0.4-337	1.4	0.2-52.5	2.45	0.21-10.0	43
2 Cell	3.2	0.5-327	1.5	0.15-31.5	2.9	0.67-16.4	27
4 Cell	3.4	0.5-60.9	1.15	0.1-10.5	2.57	0.67-16.4	40
Grades 1 & 2	4.7	0.7-94.4	1.35	0.1-12.0	3.42	0.93-16.4	41
Grades 3 & 4	2.8	0.5-86.3	1.4	0.1-27.0	2.91	0.67-8.57	22

The wide disparity in previous study findings (Lumsden et al, 1986, Jeremy et al, 1986) and the results of my preliminary study could be explained in terms of lack of overall uniformity. The indications for IVF in their reports were quite diverse and a number of studies analysed small numbers of follicles from an even smaller number of patients. Ovarian hyperstimulation regimes were not standardised and consisted of either clomiphene citrate alone or in combination with hMG, hMG alone or FSH alone. No study was conducted on follicular fluids following a hyperstimulation regime which included an LHRH-A. Oocyte recoveries, in the main, were performed laparoscopically, thus subjecting the follicles to the influence of a prolonged CO_2 pneumoperitoneum and also general anaesthesia. The methodology for the extraction of prostaglandins from follicular fluid differed widely (Chapter 1.4, Table 1.B) as did the methodology for the various radioimmunoassays. Is it surprising therefore that there is disagreement in the literature?

The preliminary study had not shown any obvious significant differences in either prostaglandins or steroid hormone concentrations in follicular fluid when the various outcomes of the associated oocyte were analysed. A non-select group of patients had been studied and IVF practices were not standardised. Therefore, to control for such variability, a purer patient group with only mechanical indications for IVF and standardised IVF procedures was selected for the second, in depth study. The pregnancy rate for this select group was of high enough standard to be introduced as a further parameter for evaluation.

All patients were hyperstimulated with the combination of the LHRH-A, buserelin and hMG. This regime increases the oocyte yield and prevents premature luteinisation (Chapter 2.1). Cycles were monitored by transvaginal ultrasound resulting in more accurate cycle monitoring. All oocyte recoveries were performed using ultrasound guided transvaginal aspiration. Pregnancy rates of 33% per embryo transfer were achieved during the second year of the programme and this compared favourably with results from the best centres.

Firstly, serum concentrations of E_2 and P were measured and comparisons made between the 'pregnancy' and the 'no pregnancy' groups. On luteal day +1, (day of hCG administration = day 0) no significant differences were shown. (Chapter 7.2, Table 7.B) However, narrower ranges for both E_2 and P were found within the 'pregnancy' group. Isolated low and high level cases were within the 'no-pregnancy' group and accounted for the wider range within this group but they were not enough to cause a statistical difference from the 'pregnancy' group. The number of oocytes recovered and embryos transferred were comparable for the two groups. (Chapter 7.2, Table 7.D)

The pregnancy group again demonstrated narrower ranges for both E_2 and P in the mid luteal phase (day +6) but the differences were not statistically significant. (Chapter 7.2, Table 7.G) However, in the late luteal phase, day +12, the 'pregnancy' group displayed significantly higher P levels ($p<0.001$). (Chapter 7.2, Table 7.H)

These findings are consistent with those of Muasher et al (1984) and Dlugi et al (1984) who showed that the mean E_2 and P concentrations in the luteal phase behaved similarly in pregnant and non-pregnant cycles except at the latter part of the luteal phase (after day +11), when placental hCG and possible other factors are exerting their effects on the corpus luteum, ovary and endometrium of pregnant patients.

These steroids were then measured in follicular fluid, the medium in which prostaglandins could also be measured, for indications of their influence on oocytes. Follicular fluids were assigned to one of four groups depending upon the outcome of the matched oocyte.

1. No fertilisation
2. Fertilisation, not transferred
3. Fertilisation, transfer, no pregnancy
4. Fertilisation, transfer, pregnancy

Similar findings to the preliminary study were found when the follicular fluid E_2 concentrations were analysed. There were no differences shown between the no-fertilisation group and the fertilisation group taken either together or individually. In contrast to the findings of the preliminary study the follicular fluid P concentrations were significantly higher ($p<0.005$) when fertilisation was compared to no fertilisation. These findings agree with those of Fishel et al (1983), Reinthaller et al (1987) and Berger et al (1987) (Table 10.B). However, this difference was not consistent when the fertilisation groups were compared individually. (Chapter 8.3, Table 8.F)

Follicular fluid E_2 and P levels and the E_2:P ratio were not significantly different when 'pregnancy' was compared with 'no-pregnancy'. Basuray et al (1988) found significantly higher P levels associated with pregnancy and consequently a significantly lower E_2:P ratio (Table 10.F)

TABLE 10.F

FOLLICULAR FLUID E_2 AND P CONCENTRATION AND THE E_2:P RATIO IN CONCEPTION AND NON CONCEPTION

	BASURAY MEAN	SOTON MEAN
Pregnancy		
E_2 nmol/L	2000	810
P μmol/L	30.9	8.4
E_2:P (x 10^{-3})	65	115
No Pregnancy		
E_2 nmol/L	1809 (ns)	845 (ns)
P μmol/L	17.1 *	12.3 (ns)
E_2:P (x 10^{-3})	106*	78 (ns)
Regime	CC/hMG	LHRH-A/hMG

* $p<0.05$

ns = not significant

Higher androstenedione levels have been demonstrated in follicles from which oocytes did not fertilise, both in the natural cycle (Fowler et al, 1977) and in stimulated cycles (Fowler et al, 1978; Fishel et al, 1983 and Reinthaller et al, 1987). The present studies (Chapter 9.2) whilst showing a narrower range for androstenedione and the androstenedione:E_2 ratio in the fertilisation group, did not show any significant difference when compared with the no-fertilisation group. Similarly no significant differences were found when 'pregnancy' was compared with 'no-pregnancy'.

The study of follicular fluid prostaglandin levels yielded the most interesting findings. The results of the preliminary study were repeated when non-fertilisation was compared with fertilisation. No significant differences were shown for either PGE_2 or $PGF_{2\alpha}$ concentrations. (Chapter 8.2, Tables 8.B and 8.C)

There was a highly significant correlation within three of the four groups (the fertilised, not transferred group being the exception) between PGE_2 and $PGF_{2\alpha}$ levels ($p<0.001$) and a highly significant correlation between E_2 and P levels ($p<0.001$) (with the exception of the fertilised, transferred, no-pregnancy group), but the correlation between prostaglandins and steroids was poor.

No studies to date had established a relationship between the concentrations of PGE_2 and $PGF_{2\alpha}$ and the outcome of the matched oocyte in terms of pregnancy. This study showed that the actual levels of the two prostaglandins did not differ significantly when any two groups were compared. However, the ratio of PGE_2 to $PGF_{2\alpha}$ does have a highly significant relationship with the outcome of the matched oocyte. Chabab et al (1986) found the PGE_2:$PGF_{2\alpha}$ ratio consistently >1 in all stimulated cycles. But Chabab showed no statistical differences for the ratio when no fertilisation; fertilisation, no pregnancy and fertilisation, pregnancy were compared. (Table 10.G)

TABLE 10.G

FOLLICULAR FLUID PGE_2:$PGF_{2\alpha}$ RATIO

	CHABAB MEAN	SOTON MEAN
No Fertilisation	1.77	1.44
Fertilisation no transferred	-	1.57
Fertilisation transferred no pregnancy	1.64	1.42
Fertilisation transferred pregnancy	2.42 (ns)	1.07 ***

*** $p<0.001$

ns = not significant

The studies of Chabab et al (1986), included a large number of variables. Four different hyperstimulation regimes and the natural cycle were analysed and the prostaglandins were assayed in unextracted samples.

This study showed the PGE_2:$PGF_{2\alpha}$ ratios were generally lower and within narrower ranges than calculated previously as one might expect for the select group of patients. The prostaglandin ratio for the 'pregnancy' group was significantly lower ($p<0.001$) than the ratio for all other groups and that included the fertilised, transferred, no pregnancy group. The range for the 'pregnancy' group was narrower (0.45 - 2.00) compared to the fertilised, transferred, no-pregnancy group (0.3 - 5.91), the fertilised, not transferred group (0.15 - 7.66) and the no fertilisation group (0.18 - 5.5). (Chapter 8.2, Figure 8.A)

Therefore for oocytes that have the potential to establish a pregnancy there is a well defined range for the PGE_2:$PGF_{2\alpha}$ ratio. Follicular fluid prostaglandins appear to play a significant role in predicting the outcome of an oocyte, not in terms of specific concentrations of prostaglandins but in the ratio of one to the other, thus possibly providing an indicator for the selection of embryos with the optimal chance of establishing a pregnancy.

CONCLUDING REMARKS

The main aim of this thesis was to establish whether measurement of the components of follicular fluid, in particular the prostaglandins, PGE_2 and $PGF_{2\alpha}$ was of value in the prediction of outcome of the matched oocyte. If that proved to be the case the results would be incorporated with microscopic evaluation of the embryos with the ultimate aim of improving the pregnancy rate of IVF.

The major finding of this thesis, that of the highly significant difference in the follicular fluid PGE_2:$PGF_{2\alpha}$ ratio when pregnancy resulted from the matched oocyte, may represent a biochemical marker of maturity of the associated oocyte. (The $PGF_{2\alpha}$ has to be high enough in relation to the PGE_2 to signify maturity but not too high to suggest over maturity. It is likely that a switch to more $PGF_{2\alpha}$ occurs at optimal maturity, after an initial rise in both PGE_2 and $PGF_{2\alpha}$ after hCG administration).

The action of prostaglandins is still unknown. Edwards (1980) wrote "Prostaglandins have an important although unclear role in pre-ovulatory follicles". He further remarked that it used to be believed that prostaglandins were intermediaries in the luteinisation of granulosa cells, although their role in steroidogenesis was far from clear. Prostaglandins stimulated a resumption of meiosis in follicles cultured in vitro and this effect was independent of changes in steroid biosynthesis. Thus prostaglandins may be involved in oocyte maturation.

Certainly, prostaglandins are synthesised for a reaction at a particular moment in time. They are produced at the site of action and rapidly catabolised, not stored. Thus the range of the prostaglandin ratio found for the 'pregnancy' group reflects the "moment" when conditions are optimal within the follicle for the associated oocyte to go on to establish pregnancy. If the oocyte is recovered beyond this optimal time, because of the fast catabolism of prostaglandins, the ratio is no longer at the optimal level, and regardless of the microscopic evaluation of the resultant embryo, pregnancy will not be established.

The steroid hormones are produced for a considerable length of time, both before and after events. Catabolism is less rapid and therefore the levels and the ratio of the steroid hormones are not as sensitive markers as that of the prostaglandin ratio.

The prostaglandin levels found in the hyperstimulated cycle may not necessarily reflect the physiological situtation. Investigation of the natural cycle is difficult because of small numbers of follicles available for analysis. The success rate for IVF using the natural cycle for one particular group is encouraging, 22.5% reported by Foulot et al (1989), and may provide a means of comparing the natural with the hyperstimulated cycle if this level of success can be maintained.

These studies have established the importance of the optimal PGE_2:$PGF_{2\alpha}$ ratio. The process of extraction and radioimmunoassay for prostaglandins has been improved in this thesis but it may be necessary to improve them further before they are incorporated in the process of embryo evaluation for that current IVF cycle. Preparation of samples and their extraction takes around 2 hours. Overnight incubation of standards and samples with tracer and antiserum is an integral part of the radioimmunoassay. Thus, although it is possible to complete the measurement of prostaglandins within the timescale between oocyte recovery and embryo transfer, development of a faster assay would make this more feasible. It's major value, however, may be realised for the evaluation of the large number of embryos in frozen storage prior to transfer. There again, the technical difficulties associated with the freezing and thawing of embryos will have to be overcome before the usefulness of selecting the very best embryos can be realised.

Finally, it is one matter to show a statistically significant result, it is another to see it as a biologically significant one. It is a further large step to convert it to a clinically useful application. This thesis takes a few important small steps towards finding the best biochemical markers of oocyte quality in an IVF programme.

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