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Concentrations of placental protein 14 in uterine flushings from infertile women: validation of the collection technique and method of expression of results

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Concentrations of various proteins in uterine flushings have been described as a direct method for assessment of the secretory activity of the endometrium. We investigated levels of the endometrial protein known as placental protein 14 (PP14) in flushings obtained from 271 infertile women. Under transvaginal ultrasonographic control, 2 ml of 0.154 M sodium chloride solution were injected into the uterine cavity and re-aspirated, five times. In contrast to previous studies the recovered volume of each flushing was not consistent (range: 0.05–2.1 ml); the volume varied significantly between serial samples obtained from an individual ($P = 0.02$, one-way ANOVA), different cycle days ($P < 0.0001$, one-way ANOVA) and women with bilaterally blocked versus patent Fallopian tubes ($P < 0.05$, Student's *t*-test). Concentrations of PP14 showed a better correlation with protein content ($r = 0.506$, $P < 0.0001$) than with the recovered volume ($r = 0.087$, $P = 0.095$). We therefore corrected PP14 concentrations for total protein content as an indicator of the efficiency of the flushing process. Corrected PP14 concentrations varied significantly relative to time since the onset of menstruation ($P = 0.001$, Kruskal Wallis ANOVA) with higher levels on days 1–8, as previously observed in plasma samples. No significant difference in PP14 levels was found with different causes of infertility. This study shows that uterine flushing is not a consistent process in women with differing physical characteristics and at varying times throughout the menstrual cycle.

Key words: endometrial proteins/infertility/placental protein 14/proliferative phase/uterine flushings

Introduction

Implantation remains the rate limiting step in female fertility (Edwards, 1994). Even when embryos are carefully selected according to apparent indicators of quality, only 18.8% of cycles actually result in a pregnancy (HFEA, 1997). Attention has, therefore, focused on the role of the endometrium and its secretions in facilitating fertilization and implantation. Adequate endometrial development has traditionally been assessed by histological criteria (Noyes *et al.*, 1950) but more recently the importance of proteins synthesized and secreted by the endometrial glands has been investigated (Fay and Grudzinskas, 1991; Chard and Olajide, 1994). One of the most abundant of these specific secretory proteins is placental protein 14 (PP14). This was originally isolated from placental extracts but is now known to be synthesized by secretory and decidualized endometrium (Bohn *et al.*, 1982; Julkunen *et al.*, 1990). Recently, PP14 has been renamed glycodelin-A after the discovery of a glycoprotein with a well defined amino acid sequence and unique oligosaccharide structure, which reacted with antibodies against human placental protein 14 (Dell *et al.*, 1995). We have, however, continued to use the term 'PP14' in this paper as our assay has not been validated for the detection of the specifically sequenced protein, glycodelin-A.

Mean serum PP14 levels in women with ovulatory cycles rise in the late luteal phase, reach a peak during menstruation and then decline to a peri-ovulatory nadir (Joshi *et al.*, 1982; Julkunen *et al.*, 1986a; Wood *et al.*, 1989; Fay *et al.*, 1990). PP14 levels in uterine flushings increase 6 days after the luteinizing hormone surge to reach a concentration over ×100 that in plasma (Li *et al.*, 1993a). PP14 potently inhibits human spermatozoa–zona pellucida binding *in vitro* (Oehninger *et al.*, 1995) and it has been postulated that the absence of contraceptive PP14 in the peri-ovulatory endometrium may permit successful fertilization (Clark *et al.*, 1996). PP14 has also been shown to possess immunosuppressive properties (Bolton *et al.*, 1987; Pockley *et al.*, 1988; Okamoto *et al.*, 1991). Increased PP14 levels around the time of expected implantation might, therefore, facilitate blastocyst tolerance, permitting successful implantation to occur.

Between 20 and 44% of women with unexplained infertility have retarded endometrial development (Li *et al.*, 1991). This is reflected by immunomorphometric studies which show decreased staining for PP14 (Klentzeris *et al.*, 1994). Luteal phase uterine flushings from women with unexplained infertility have been shown to contain reduced concentrations of PP14 compared to similarly timed flushings from fertile controls. This difference could not, however, be detected in the corresponding plasma samples (Mackenna *et al.*, 1993). The role of ovarian function in the control of PP14 secretion has been extensively studied but the precise nature of any putative ovarian control factor has not yet been identified (Chard and Olajide, 1994). Some authors have suggested that PP14 levels only remain high throughout menstruation if ovulation has occurred in the previous menstrual cycle

(Julkunen *et al.*, 1986a; Joshi *et al.*, 1987; Wood *et al.*, 1989; Fay *et al.*, 1990). Human Fallopian tubal epithelial cells have been shown to produce PP14 (Julkunen *et al.*, 1986b; Saridogan *et al.*, 1997); fluid from hydrosalpinges in women with occluded Fallopian tubes can leak into the endometrial cavity where it may impair uterine receptivity (Meyer *et al.*, 1997). It is not known, however, if levels of PP14 in uterine flushings vary in women with anovulatory or tubal infertility and this is addressed in the current study.

We have, for the first time, examined PP14 concentrations in follicular phase uterine flushings. Fluid was collected from 271 infertile women. We noted great variability in the retrieved volume of the flushings and that some samples were contaminated with menstrual blood. We, therefore, investigated methods to express the results which might correct for the variable efficiency of the flushing process.

Materials and methods

Patients

Flushings were obtained, on one occasion and as an outpatient procedure, from each of 392 women who presented consecutively for infertility investigations. The median age was 31.1 (range: 21–44) years and median duration of infertility was 4.5 (range: 1–18) years. Primary infertility was present in 60.1% (163/271) and 85.2% (231/271) were nulliparous. Flushings were obtained from days 1–16 of the menstrual cycle, where the first day of menstruation was designated as day 1. Unexplained infertility was diagnosed if the couple's Fallopian tubal patency assessment, semen analysis and hormone profile were within the normal range. Women with a mid-luteal phase serum progesterone concentration less than 30 nmol/l were defined as anovulatory. Approval for this study was granted by the East London, City and Hackney Health Authority ethics committee, UK.

Sampling procedure

With the patient in the dorsal lithotomy position, a sterile Cusco's speculum was passed to expose the cervix. This was then scrupulously cleaned with sterile saline solution, particular attention being paid to removing all visible mucus from the external cervical os and as much of the cervical canal as was accessible. A 5-F intrauterine balloon catheter (Schering AG, Berlin) was passed transcervically, without the routine use of a tenaculum; when it had entered the endometrial cavity the catheter balloon was inflated with 1–2 ml of air. The catheter was then gently withdrawn to ensure that the balloon lay at the level of the internal cervical os. Five separate aliquots of 2 ml of 0.154 M sodium chloride solution were then slowly injected and re-aspirated via the catheter side arm, each over approximately 10 s. This was performed under transvaginal ultrasonographic control to ensure that the only fluid retrieved was that which had entered the uterine cavity, not that which had passed along the Fallopian tubes or remained in the cervical canal. The partners of these women all produced a semen sample for analysis on the same day. Couples were asked to refrain from sexual intercourse for 72 h prior to the day of their attendance, eliminating the risk of contamination of the flushings with seminal proteins.

All flushings were flash frozen in liquid nitrogen and stored at –20°C prior to analysis. PP14 levels were estimated in the first 271 sets of flushings which were of sufficient volume for the assay. In the first 60 subjects PP14 was measured in each of the five flushings collected; in a further 211 sets only the first flushing of the sequence was analysed (see results). PP14 concentrations were measured by a radioimmunoassay, using [^{125}I]-PP14 as tracer and rabbit anti-human PP14 serum, which has previously been validated in our laboratories (Howell *et al.*, 1989). The source of the PP14, for both iodination and assay standards, was homogenates of gestational endometrium, purified as has previously been described (Fay *et al.*, 1990). Total protein content was estimated by the bicinchoninic acid method in each sample on the first occasion on which it was thawed (Smith *et al.*, 1985).

Statistical analysis

A non-parametric test, Kruskal Wallis analysis of variance (KW ANOVA), was used to examine PP14 concentrations because a large number of values fell below the detection limit of the assay. Spearman rank correlation was used to examine the relationship between PP14 concentrations and each of protein level and volume of flushing retrieved. The level of statistical significance was chosen at $P < 0.05$.

Results

Variation in retrieved flushing volume

The retrieved volume of each flushing ranged from 0.05–2.1 ml. This volume varied significantly within the five samples obtained for each individual ($P = 0.02$, one-way ANOVA) (Figure 1a). The mean of the five samples also varied according to the menstrual cycle day ($P < 0.0001$, one-way ANOVA) (Figure 1b). The volume of injected saline which could be re-aspirated was consistently higher in women with bilateral Fallopian tubal blockage when compared with those with bilateral tubal patency ($P < 0.0001$, Student's *t*-test with unequal variance) (Figure 1c).

Correction for total protein content

We found a significant correlation between retrieved volume and total protein concentration ($r = 0.59$, $P < 0.0001$). PP14 concentrations showed a stronger correlation with protein content ($r = 0.506$, $P < 0.0001$) than with recovered volume of the same flushings ($r = 0.087$, $P = 0.095$). We thus chose to express all flushing PP14 concentrations per mg of total protein as this appeared to be a more reliable indicator of the efficiency of the flushing process than the re-aspirated volume.

Variation in PP14 concentration with chronological order of re-aspiration

Total protein and PP14 concentrations were measured in each of the five samples collected from the first 60 women investigated. The proportion of the total amount of PP14 recovered varied significantly across this sequence; higher percentages of PP14 being retrieved in earlier specimens ($P < 0.0001$, KW ANOVA) (Figure 2a). Similar variation was seen when the protein content of individual flushings was expressed as a percentage of the total amount of protein ($P < 0.0001$, KW ANOVA) (Figure 2b). Correcting PP14 concentrations for total protein content decreased but did not eliminate this variation between sequential flushings; proportionately more PP14 was still present in the first flushing recovered ($P = 0.002$, KW ANOVA) (Figure 2c).

Menstrual cycle timing

Combining the cross-sectional data obtained from individual women, absolute PP14 concentrations were relatively high on

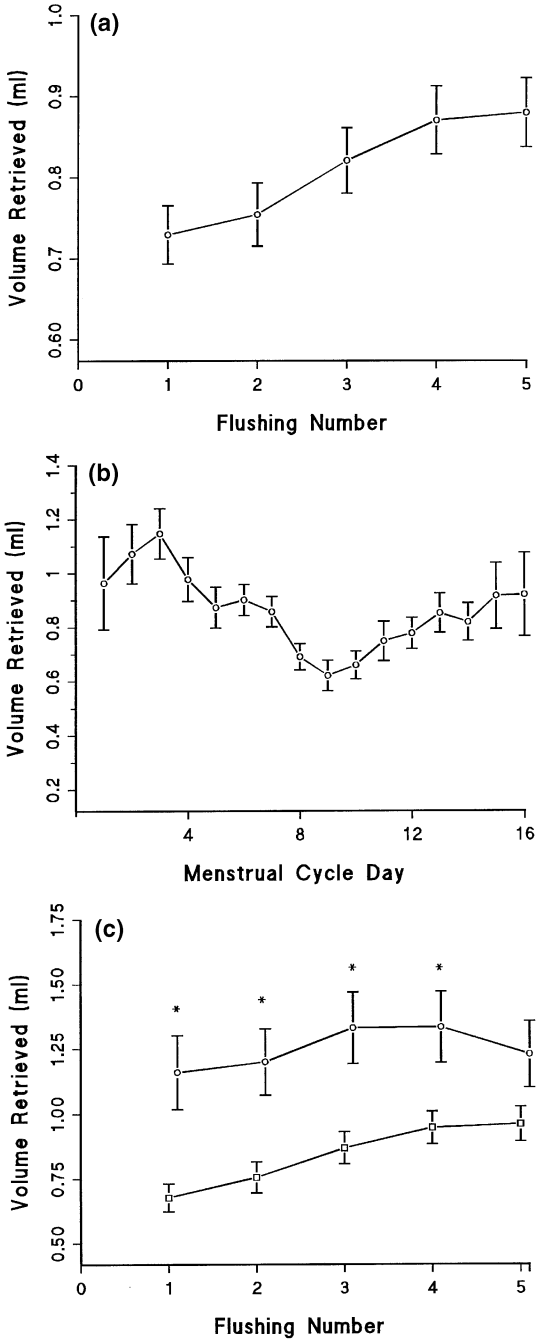

Figure 1. Mean and standard error of the mean volume of each 2 ml flushing retrieved (**a**) from each of the five flushings in a woman, in chronological order of reaspiration ($P = 0.02$, one-way ANOVA), (**b**) relative to day in menstrual cycle (first day of bleeding designated as day 1) ($P < 0.0001$, one-way ANOVA), (**c**) in women with bilaterally blocked (○) and patent (□) Fallopian tubes relative to order of flushing recovery (*$P < 0.05$, for individual flushing numbers, $P < 0.0001$, over all five flushings for patency versus blockage; all: Student's *t*-test).

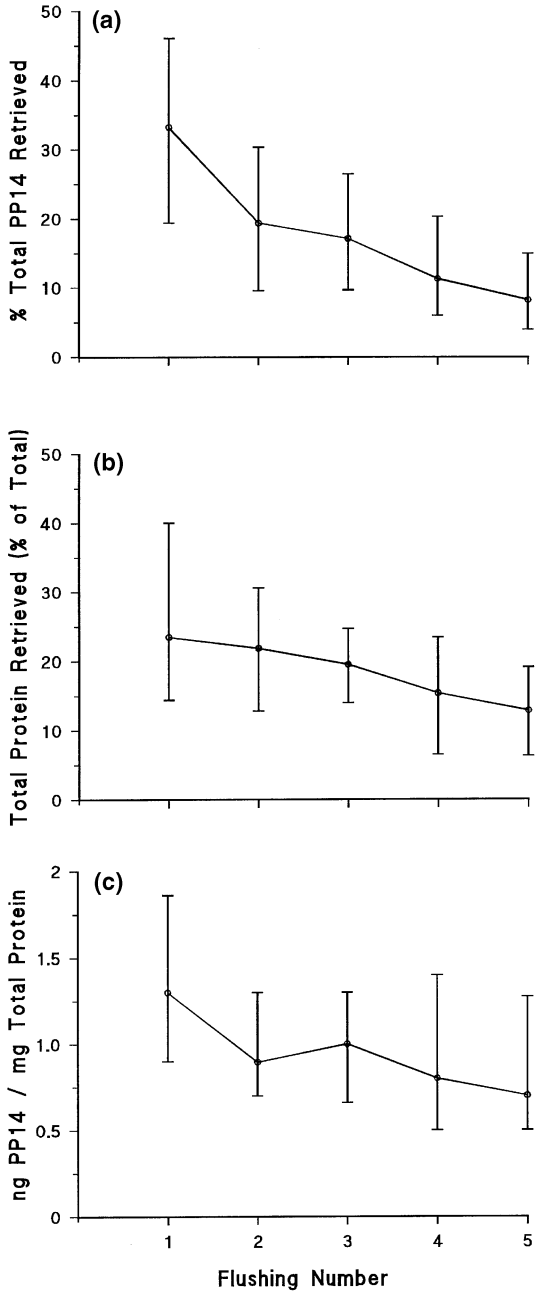

Figure 2. Median and interquartile ranges of (**a**) placental protein 14 (PP14) content of individual flushings expressed as a percentage of the total amount of PP14 retrieved from all five flushings ($P < 0.0001$, KW ANOVA), (**b**) total protein content of individual flushings expressed as a percentage of the total amount of protein retrieved from all five flushings ($P < 0.0001$, KW ANOVA), (**c**) PP14 concentration corrected for total protein content across all five flushings, in chronological order of re-aspiration ($P = 0.002$, KW ANOVA).

days 1–8 and lower on days 9–15, from the onset of the last menstrual period ($P < 0.0001$, KW ANOVA) (Figure 3a). The total protein content varied over the same time period, with higher levels perimenstrually ($P < 0.0001$, KW ANOVA) (Figure 3b). Correcting PP14 levels for protein concentrations did not alter this pattern throughout the proliferative phase ($P = 0.001$, KW ANOVA) (Figure 3c). Median corrected PP14 levels approached undetectable levels from day 10 onwards, being unrecordable on days 11 and 14.

Cause of infertility

There was no difference in the PP14 content of flushings at any stage of the cycle in women with different causes of infertility (male factor: $n = 91$, tubal: $n = 65$, anovulatory:

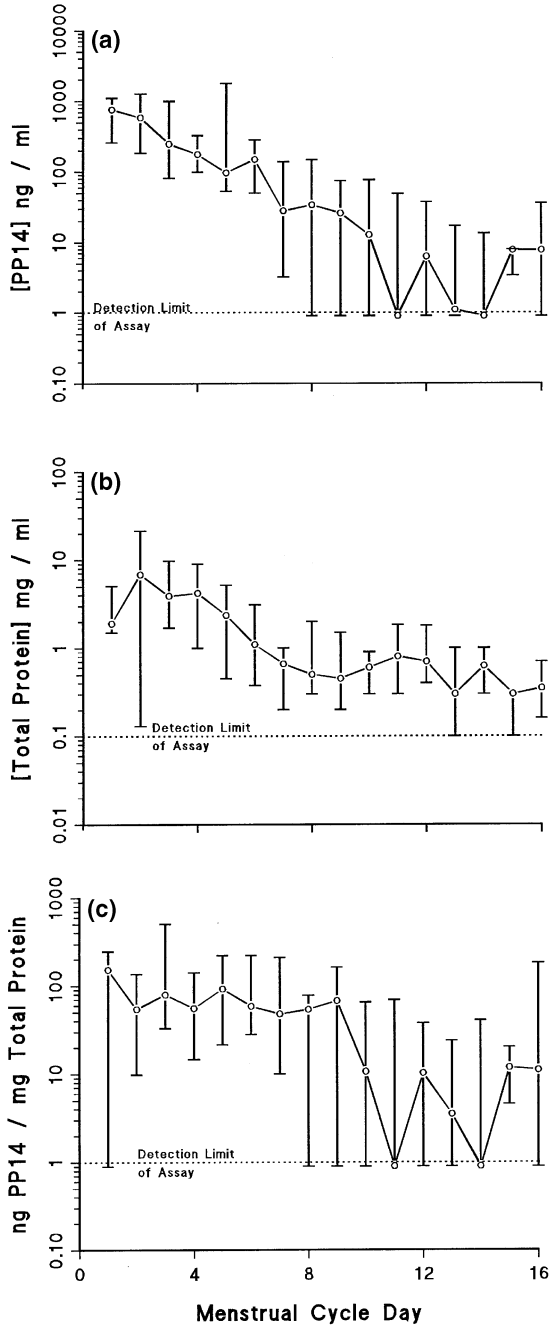

Figure 3. Median and interquartile ranges of (**a**) uncorrected placental protein 14 (PP14) concentration ($P < 0.0001$, KW ANOVA), (**b**) total protein concentration ($P < 0.0001$, KW ANOVA), (**c**) PP14 concentration corrected for total protein content ($P = 0.001$, KW ANOVA), all in flushings and relative to number of days since onset of menstruation. The limit of detection plotted in (**c**) represents an arbitrary value which is the lower limit of detection of the numerator. Note exponential scale on *y*-axis.

$n = 13$, unexplained: $n = 92$) (Figure 4). Only women with a single cause for their infertility were included in this analysis.

Discussion

This study confirms that PP14 can be detected in uterine flushings (Bell and Dore-Green, 1987; Li *et al.*, 1993a) but, for the first time, examines fluid collected during the follicular phase of the menstrual cycle. Blood PP14 levels are elevated in the early part of this phase (Julkunen *et al.*, 1986a; Wood *et al.*, 1989; Fay *et al.*, 1990) and our findings suggest that a similar pattern is observable in uterine fluid. In contrast to previous studies we were unable to find any difference in PP14 concentrations in flushings from women with different causes of infertility (Julkunen *et al.*, 1986a; Fay *et al.*, 1990; Li *et al.*, 1991; Mackenna *et al.*, 1993).

Uterine flushing with saline is necessary because of the difficulty of direct aspiration of the small volume of fluid present in the endometrial cavity (0.2 ml) (Casslen, 1986). It results, however, in uncertainty as to what proportion of fluid retrieved represents diluent and what is actually endometrial secretion/transudation. Previous authors have not considered this to be a problem since more than 90% of the injected saline was retrieved in most cases, using methodology similar to that described in this study but in the luteal phase of the menstrual cycle (Li *et al.*, 1993a, b). The volume of fluid retrieved in the current study, however, showed a high inter-individual variation, ranging from 0.05–2.1 ml for each 2.0 ml of saline injected; this has not previously been described. The recovered volume varied throughout the follicular phase of the menstrual cycle, larger volumes being obtained during menstruation and approaching the expected time of ovulation. Oestrogen increases the permeability of uterine blood vessels, resulting in increased transudation; the pre-ovulatory increase in oestrogen concentration may, thus, lead to an accumulation of fluid in the uterus (Clemetson *et al.*, 1973). Larger volumes were also recovered from the later aliquots in an individual. Not surprisingly, the retrieval process was more efficient in women with bilaterally blocked Fallopian tubes than in those with patent tubes, in agreement with a previous study (Maathuis and Aitken, 1978). The balloon catheter was used to ensure a good cervical seal; backward leakage into the vagina should not account for significant fluid loss, particularly since most women in this study were nulliparous.

The total protein concentration was chosen as the best available estimate of the dilution of true uterine fluid. We found a positive correlation between total protein and volume of individual flushings ($r = 0.59$, $P < 0.0001$). Flushing volume and protein content do not show a negative correlation, perhaps because a high uterine flushing volume does not automatically indicate a dilute sample but may also be caused by the addition of higher volumes of uterine fluid, whose total protein content may be high relative to the volume of saline retrieved. PP14 concentrations were significantly related to the protein content ($r = 0.506$, $P < 0.0001$) but not the recovered flushing volume ($r = 0.087$, $P = 0.095$). The protein concentration also provides an indication of which flushings are most heavily contaminated with haemoglobin and, thus, the highest protein levels were detected perimenstrually. Menstrual or traumatic blood contamination might potentially confound uterine flushing levels of substances which are not specific to the endometrium. We did not further correct for the extent of blood contamination because plasma levels of PP14 are 10–100-fold lower than those in flushings (Li *et al.*, 1993a). Histochemical studies localize PP14 mRNA and its protein, in the early follicular phase to the deep basal glands of the

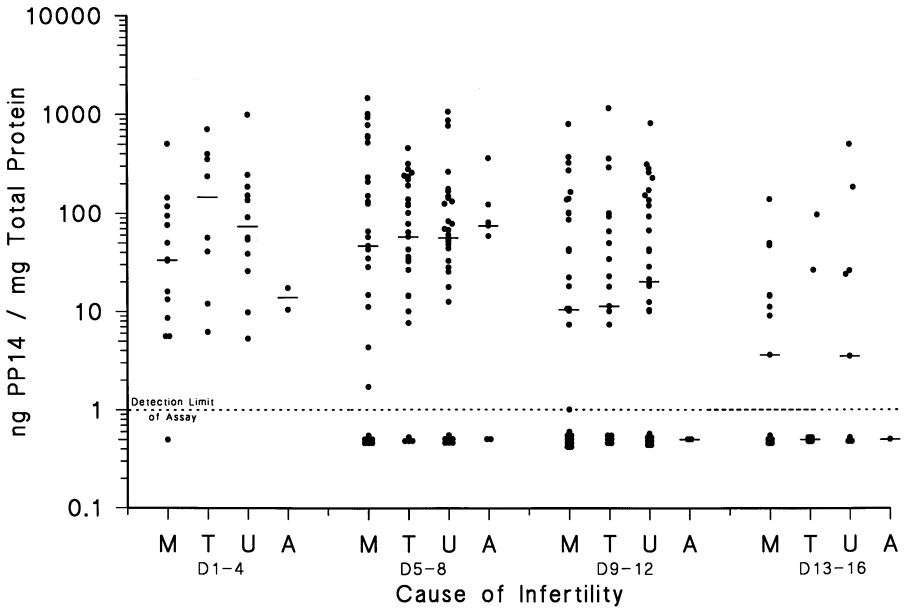

Figure 4. PP14 concentration, corrected for total protein content, for women with male factor (M), tubal (T), unexplained (U) and anovulatory (A) infertility. Dashes indicate median values for each cause. The limit of detection represents an arbitary value which is the lower limit of detection of the numerator. No difference between causes on days 1–4 (D1–4), 5–8 (D5–8), 9–12 (D9–12) or 13–16 (D13–16) of the menstrual cycle (all: $P > 0.05$, KW ANOVA).

endometrium (Seppala *et al.*, 1988; Julkunen *et al.*, 1990). These glands are not shed during menstruation and, thus, uterine flushing PP14 levels, as a potential indicator of endometrial secretory function, should not be confounded by the presence of blood containing endometrial fragments. Correcting for protein content reduced the variation in PP14 concentration in sequential samples from an individual but did not eliminate it. We therefore concluded that different orders of flushings are not comparable between subjects.

Flushing under transvaginal ultrasonographic control allowed visualization of the saline within the endometrial cavity. Problems such as incorrect (intracervical) placement of the catheter, re-aspiration of saline which had not progressed beyond the catheter lumen or retrieving saline from the proximal portion of the Fallopian tubes could be recognized. Ultrasonography can also help to ensure that the recovered fluid is representative of the entire endometrium, in the same way that hysteroscopy is an improvement over 'blind' dilatation and curettage (Stock and Kanbour, 1975). Previous studies have used various means to avoid contamination of uterine flushings (Maathuis and Aitken, 1978). In the present study, abstention from sexual intercourse for 72 h obviated contamination from PP14 in seminal fluid (Julkunen *et al.*, 1984) while the use of ultrasonography identified flushings which might contain PP14 from the Fallopian tubes (Julkunen *et al.*, 1986b; Saridogan *et al.* 1997). Strict attention was also paid to removing cervical mucus, which might distort the total protein content.

Ideally, individual women would be followed sequentially throughout a menstrual cycle. This is not practical but the cross sectional nature of our data is strengthened by the large numbers of women involved ($n = 271$). We examined PP14 concentrations relative to the number of days since the onset of menstruation since this allowed comparison with data previously obtained on peripheral blood PP14 levels over the same period and which used this method of dating samples (Wood *et al.*, 1989; Fay *et al.*, 1990). Our subjects were all actively trying to conceive and, thus, were well motivated to recall the precise date of onset of menses. Our findings showed a similar pattern to that of plasma: high concentrations during menstruation and lower levels thereafter. Despite previous failures to detect PP14 or its mRNA in the endometrium from day 5 of the menstrual cycle until the fifth day after ovulation (Seppala *et al.*, 1988) we found that median PP14 levels in flushings only approached undetectable levels from day 10 onwards, suggesting that there may be a time lag between PP14 production in the endometrial glands and its secretion into uterine fluid.

There was no difference in corrected or uncorrected PP14 concentrations between women with different causes of infertility. Women with male factor infertility were perhaps most likely to have normal endometrial development but their PP14 levels were no different to those in women with unexplained infertility. This might reflect the heterogeneous nature of the latter group, not all of whom have retarded endometrial development. PP14 levels in anovulatory women tended to be very low or unrecordable, as might be expected (Julkunen *et al.*, 1986a; Joshi *et al.*, 1987; Wood *et al.*, 1989; Fay *et al.*, 1990). The difference with other causes of infertility did not, however, reach statistical significance at any stage of the follicular phase, perhaps due to the small number of women with anovulation as the only cause of their infertility. This may reflect the fact that the women had reached a tertiary referral centre by the time of this study; anovulation may have been corrected at earlier stages of the referral chain.

This study has, for the first time, described PP14 levels in

follicular phase uterine flushings. Evidence to support the cyclical variation in serum PP14 levels has been presented although our study had insufficient power to detect a significant difference in flushings taken from women with different causes of infertility. Earlier techniques of fluid recovery and expression of results have been improved and a method of uterine flushing validated for an important endometrial secretory product.

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