Follicular fluid expression of alpha-defensins and their role in ovulation

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Abstract

Introduction Defensins are cytotoxic peptides and have a well-defined role in host defense. Human alpha defensins 1–3 (HNP1–3) are primarily produced by peripheral neutrophils and constitute about 50% of the azurophilic granule protein. Studies have suggested that peripheral neutrophils and the resident neutrophils in the ovary enhance the release of IL-8 and TNF- α that play a role in ovulation and influence fertilisation rate and IVF outcome. The production of HNP1–3 by follicular fluid and its role in ovulation has never been studied. The aim of this study was to demonstrate the presence of HNP1–3 in follicular fluid and to ascertain its correlation with fertilisation rate and IVF outcome.

Method Women attending the Reproductive Medicine Unit at Liverpool Women's Hospital UK, for IVF treatment were invited to participate in the study. Sixty-three patients were recruited for the study and underwent controlled ovarian

stimulation and oocyte retrieval according to the unit's protocol. Fluid from the first follicle only was collected to minimise blood contamination of the sample and HNP1–3 was estimated using ELISA technique.

Results HNP1–3 was detected in follicular fluid samples. The concentration did not correlate with the fertilisation rate ($r = 0.01$). The concentrations were also not significantly different in the women who did or did not become pregnant following treatment. Subgroup analysis showed that women with endometriosis were not more likely to have higher levels of the HNP1–3 when compared with controls (male factor infertility group).

Conclusion This is the first study to show the expression of HNP1–3 in follicular fluid. HNP1–3 concentrations did not correlate with fertilisation rate or IVF outcome. It did not show an increased expression of HNP1–3 in fluid collected from women with endometriosis suggesting that inflammatory processes associated with endometriosis do not influence HNP1–3 concentration in the follicular fluid. Further studies to evaluate the correlation between HNP1–3 and IL-8 and TNF- α may clarify the role of defensins in ovulation.

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Introduction

Defensins are cytotoxic peptides which have a well-defined role in host defense mechanisms [1]. Alpha- and beta-defensins constitute more than 5% of the total cellular protein in human polymorphonuclear neutrophils [2]. Human alpha defensins 1–3 (HNP1–3) are primarily produced by neutrophils and constitute about 50% of the

azurphil granule protein [1]. Previous studies measuring neutrophil related cytokines IL-8 and G-CSF (granulocyte colony-stimulating factor) have shown significant correlation between the level of defensins and these cytokines in pleural fluid from patients with empyema of bacterial origin suggesting that alpha-defensins production is restricted to neutrophils [3–5].

Like other cationic antimicrobial peptide families, HNP1–3, have other roles in the immune response in addition to antimicrobial activity [6]. Defensins induce production of IL-8 from epithelial cells, act as a chemotactic factor for T cells and enhance cytokine activity in general [1]. Modulation of IL-8 in cell cultures suggest that it may play an important role in ovulation such as aiding follicular rupture and corpus luteum neovascularisation [7]. Ujiko et al. [8] showed that neutralisation of IL-8 inhibited hCG-induced ovulation as well as neutrophil infiltration into rabbit ovaries.

The ovary is not an immunologically exclusive organ and a variety of leucocyte subsets including mast cells, eosinophils, neutrophils, macrophage/monocytes and lymphocytes are known to be present in the ovarian follicle through various stages of its development from immaturity to the formation of the corpus luteum [9–15]. Neutrophils are present in the medulla, theca, follicular fluid and corpus luteum [16]. The resident neutrophils in the ovary are thought to release TNF- α and IL-1 that contribute to the rupture of the follicular envelopes at ovulation and have been shown to increase the ovulation rates in rabbit ovaries [11, 17]. Follicular fluid levels of IL-1 and TNF- α are found to be higher in women undergoing ovulation induction with gonadotrophins as compared with natural unstimulated cycles [18].

Studies on patients undergoing in-vitro fertilisation (IVF) cycles have shown that IL-1 levels in the follicular fluid to be significantly higher in implantation cycles than in nonimplantation cycles [19]. However, in addition to neutrophils, other resident leucocytes—monocytes, macrophages, and even the ovarian stromal and thecal cells could be the source of IL-1.

All these studies provide evidence of the role of the secretory products of neutrophils i.e. IL-1, TNF- α and IL-8 in the process of ovulation. Herriot et al concluded that follicular fluid exerts chemotactic activity toward neutrophils, the concentration of this activity is related to IVF outcome [20]. It is therefore possible that the resident ovarian neutrophils also produce HNP1–3 that may influence IVF outcome.

The production of alpha-defensins by ovarian neutrophils and their role in ovulation whether direct or indirect, has not yet been studied. This study was undertaken to ascertain if the resident neutrophils of the ovary produce HNP1–3 and whether their presence correlates with IVF outcome.

Materials and methods

The Liverpool Research Ethics Committee granted permission for the project. Women attending the Reproductive Medicine Unit at Liverpool Women's Hospital for IVF and ICSI treatment were invited to participate in the study. An information sheet was given to all women, and informed written consent was obtained from those agreeing to take part.

Sample collection

Sixty-three patients were recruited for the study. According to the unit's protocol, oocyte collection was performed using an aspiration needle (Wallace Ltd., Hythe, Kent, UK) under ultrasound guidance with the patient under sedation. The choice of a single- or double-lumen aspiration needle was determined by the total number of follicles of size 16mm or above, present for aspiration. The presence of less than eight to ten follicles of sizes 16mm or over usually meant aspiration using a double-lumen needle to allow flushing of the follicle and ensure retrieval of the oocyte. Follicular fluid from the first follicle only was collected to minimise blood contamination of the sample with peripheral neutrophils that might affect the HNP1–3 levels. Within 20min of collection, the sample was centrifuged at 300rpm for 10min and the supernatant aliquoted and frozen at -20°C until HNP1–3 concentration was measured by enzyme-linked immunosorbent assay (ELISA).

ELISA technique

Human HNP1–3 was measured by the commercial ELISA test kit (HyCult Biotechnology b.v., Uden, The Netherlands). The samples and standards were first incubated in antibody coated wells recognising HNP1–3 for 1h at room temperature. The plate was then washed four times with 200 μl of the wash buffer to remove unbound material and 100 μl of biotinylated second antibody was added to each well. Following further incubation for an hour at room temperature, the wells were washed to remove excess of the second antibody. One hundred microlitres of diluted streptavidin-peroxidase conjugate was then applied to the wells to react specifically with the biotinylated antibody bound onto the detected HNP1–3. Following the standard incubation for an hour at room temperature, the excess streptavidin-peroxidase conjugate was washed away and substrate tetramethylbenzidine (TMB) was added to the wells. The enzyme reaction was stopped by addition of citric acid and absorbance was measured at 450nm in a plate reader.

A standard curve was obtained by using defined standards and the HNP1–3 concentrations were determined

Fig. 1 Expression of HNP1–3 in pregnant and non-pregnant groups

from this curve. StatsDirect statistical software, Stats Direct Limited, version 2.3.3 was used for analysis. Statistical analysis was performed using the Mann–Whitney *U* test for continuous variables and the χ^2 for categorical variables. Mann–Whitney *U* test was performed to investigate the difference in expression of follicular fluid HNP1–3 between the groups of women who did and did not achieve biochemical pregnancy following IVF-ICSI treatment. The Spearman's correlation coefficient was used to determine any correlation between follicular fluid levels of HNP1–3 and fertilisation rates.

Results

A total of 63 women were recruited and follicular fluid samples collected. Of these 42 (67%) samples were visibly

clear at the time of collection. The remaining 20 (32%) samples were faintly bloodstained initially and clear following centrifugation. One sample had haemolysed following centrifugation and was discarded.

The cause of infertility was male factor in 44%, endometriosis in 11%, tubal factor in 18%, unexplained in 19% and anovulation in 8% of couples. The mean age of the women undergoing treatment was 34years. There was no age difference between women who conceived and those who did not. In 60 women (97%) successful fertilisation occurred, with a mean fertilisation rate of 61.7% (SD: 23.6), while two women (3%) experienced failed fertilisation (Table 1).

Measured HNP1–3 levels in the study population showed a non-normal distribution and had a median (IQR) of 0.69 (0.40, 1.54). The HNP1–3 levels in the samples that were visibly clear at the time of collection ($n = 42$) was compared with the samples that were lightly bloodstained prior to centrifugation ($n = 20$). The median and interquartile range of HNP1–3 level in the clear samples was 0.55pg/ml (IQR: 0.38, 1.24) and that of the initially bloodstained samples was 1.07pg/ml (IQR: 0.43, 4.15). The concentrations in the two groups did not reach statistical significance with a *p* value of 0.07 (Table 2).

Data regarding whether the first follicle yielded an oocyte was recorded for 31 (50%) patients. Of these, 18 (58%) contained an oocyte and 13(42%) did not. HNP1–3 concentration in the follicle that yielded an oocyte was similar to that of the follicle that did not yield an oocyte with a *p* value 0.5 (Table 2).

The expression of HNP1–3 in the non-pregnant group had a median and interquartile range (IQR) of 0.73 (IQR: 0.44, 2.13) and that of the pregnant group was 0.57 (IQR:

Fig. 2 Correlation between fertilisation rates and HNP1–3 concentration in follicular fluid

0.38, 1.29). There was no significant difference in the HNP1–3 levels in the pregnant and the non-pregnant groups p value = 0.4 (Fig. 1).

HNP1–3 is known to increase in infections where inflammatory products are released. It was hypothesised that concentration of HNP1–3 would also be increased in women suffering from pelvic and ovarian endometriosis ($n = 7$). Couples undergoing treatment for male factor infertility were used as control group ($n = 31$) for comparison. There was no significant difference in expression of HNP1–3 in these two groups with a p value of 0.5.

No correlation existed between HNP1–3 concentrations in the follicular fluid and fertilisation rates $r = 0.01$ (Fig. 2).

Discussion

This is the first study to show the expression of HNP1–3 in follicular fluid. However, a wide variation was observed in the HNP1–3 concentration in the samples. HNP1–3 concentrations from the follicular fluid samples that were initially bloodstained were similar to that of the visibly clear samples. Although red cell count of the fluid was not performed, our results strongly suggest that the origin of HNP1–3 in the follicular fluid is the resident neutrophils of the ovary.

The HNP1–3 level in the follicular fluid did not correlate with the pregnancy outcome following IVF treatment or with the fertilisation rates. Normally, fertilisation rate is calculated from the cumulative count of all the oocytes collected, whereas our study analysed fluid from the first follicle only. The embryo derived from the follicle in question was not necessarily replaced into the uterus. This may explain the absence of correlation between HNP1–3 concentration and fertilisation rates and pregnancy outcome. Analysis of pooled follicular fluid was avoided to minimise peripheral blood contamination of the sample. Sampling fluid from each follicle separately and tracking of each oocyte and embryo derived from it individually could be more conclusive but this was outside the scope of this study.

Concentrations of HNP1–3 did not also appear to be influenced by oocyte recovery from the follicle. Retrieval of an oocyte from a follicle essentially involves its mechanical dislodging by vacuum suction of the follicular fluid and is not always successful. This could explain similar concentrations of HNP1–3 in all follicles irrespective of oocyte retrieved from them.

This study did not show an increased expression of HNP1–3 in fluid collected from women with endometriosis as compared with women who did not. This suggests that the inflammatory processes associated with endometriosis do not influence HNP1–3 concentrations in the follicular

fluid. It also cannot be attributed to the collection procedure itself as the fluid was removed before any inflammatory reaction could begin. This observation was similar to the results of a previous study done in our unit on IL-8 concentration in women with endometriosis [21].

The presence of HNP1–3 in follicular fluid therefore would suggest a likely role in ovulation. Ovulation has been recognised as an inflammatory process [22]. Ovarian IL-8 has been observed to be chemotactic for neutrophils and is also a potent angiogenic, pro-inflammatory, growth-promoting cytokine [23]. Bukulmez and Arici [16] have shown that local migration of neutrophils is hormonally regulated through local modulation of IL-8. Further studies investigating the correlation between HNP1–3 and other cytokines (IL-8 and TNF- α) known to have a more definitive role in ovulation may provide further information regarding the role of the defensins in folliculogenesis and ovulation.

Appendix

Table 1 Characteristics of the study population

Variables	
Age of study population, Mean (SD)	34 (3.4) years
Duration of infertility, Mean (SD)	4.6 (2.1) years
IVF/ICSI, n (%)	30 (49)
IVF, n (%)	32 (51)
Cause of infertility, n (%)	
Male factor	31 (50)
Endometriosis	7 (11)
Tubal factor	11 (18)
Unexplained	14 (23)
Anovulation	7 (11)

Table 2 HNP1–3 concentration in the study population and relationship between HNP1–3 levels with oocyte presence in the follicle and bloodstaining of fluid during collection

Variables	HNP1–3 levels (pg/ml)	p value
HNP1–3 concentration (pg/ml), $n=62$	0.69 (0.40, 1.54)	
Visibly clear follicular fluid, $n=42$	0.55 (0.38, 1.24)	0.07
Bloodstained follicular fluid, $n=20$	1.07 (0.43, 4.15)	
Oocyte in first follicle (present), $n=18$	0.82 (0.46, 1.54)	0.57
Oocyte in first follicle (absent), $n=13$	0.69 (0.45, 0.95)	

References

1. Ryley HC. Human antimicrobial peptides. *Rev Med Microbiol* 2001;12(3):177–86.
2. Lehrer RI, Ganz T. Antimicrobial peptides in mammalian and insect host defence. *Curr Opin Immunol* 1999;11:23–7.
3. Ashitani J, Mukae H, Nakazato M, Taniguchi H, Ogawa K, Kohno S, Matsukura S. Elevated pleural fluid levels of defensins in patients with empyema. *Chest* 1998;113(3):788–94.
4. Kogan BL, Ganz T, Lehrer RI. Defensins: a family of antimicrobial and cytotoxic peptides. *Toxicology* 1994;87:131–49.
5. Selsted ME, Szklarek D, Ganz T, et al. Activity of rabbit leucocyte peptides against *Candida albicans*. *Infect Immun* 1985;49:202–6.
6. Hancock REW, Diamond G. The role of cationic antimicrobial peptides in innate host defences. *Trends Microbiol* 2000;8(9):402–10.
7. Arici A, Oral E, Bukulmez O, Buradagunta S, Engin O, Olive DL, et al. interleukin-8 expression and modulation in human preovulatory follicles and ovarian cells. *Endocrinol* 1996;137:3762–9.
8. Ujioka T, Matsukawa A, Tanaka N, Matsuura K, Yoshinaga M, Okamura H. Interleukin-8 as an essential factor in human chorionic gonadotropin-induced rabbit ovulatory process: interleukin-8 induces neutrophil accumulation and activation in ovulation. *Biol Reprod* 1998;58:526–30.
9. Hill JA, Welch WR, Faris HM, Anderson DJ. Induction of class II major histocompatibility complex antigen expression in human granulosa cells by interferon gamma: a potential mechanism contributing to autoimmune ovarian failure. *Am J Obstet Gynecol* 1990;162:534–40.
10. Lei ZM, Chegini N, Rao CHV. Quantitative cell composition of human and ovine corpora lutea from various reproductive states. *Biol Reprod* 1991;44:1148–56.
11. Brannstrom M, Mayrhofer G, Robertson SA. Localisation of leucocyte subsets in the rat ovary during periovulatory period. *Biol Reprod* 1993;48:277–86.
12. Brannstrom M, Norman RJ. Involvement of leucocytes and cytokines in the ovulatory process and corpus luteum function. *Hum Reprod* 1993;8(10):1762.
13. Norman RJ, Brannstrom M. Cytokines in the ovary: pathophysiology and potential for pharmacological intervention. *Pharmacol Ther* 1996;69:219–36.
14. Spanel-Borowski K, Rahner P, Ricken AM. Immunolocalisation of CD-18 positive cells in the bovine ovary. *J Reprod Fertil* 1997;111:197–205.
15. Spanel-Borowski K, Ricken AM. Evidence for the maintenance of macrophage-like cells in long-term bovine granulosa cell cultures. *Cell Tissue Res* 1997;288:529–38.
16. Bukulmez O, Arici A. Leucocytes in ovarian function. *Hum Reprod Update* 2000;6(1):1–15.
17. Takehara Y, Dharmarajan A, Kaufman G, Wallach E. Effect of interleukin-1 β on ovulation in the in vitro perfused rabbit ovary. *Endocrinology* 1997;134:1788–93.
18. Loret de Mola JR, Goldfarb JM, Hecht BR, et al. Gonadotropins induce the release of interleukin 1 β , interleukin-6 and tumor necrosis factor- α from the human preovulatory follicle. *Am J Reprod Immunol* 1998;39:387–90.
19. Karagouni EE, Chryssikopoulos A, Mantzavinos T, Kanakas N, Dotsika EN. Interleukin-1 β and interleukin-1 α may affect the implantation rate of patients undergoing in vitro fertilisation-embryo transfer. *Fertil Steril* 1998;70(3):553–9.
20. Herriot DM, Warnes GM, Kerin JF. Pregnancy related chemotactic activity of human follicular fluid. *Fertil Steril* 1986;45:196–201.
21. Gazvani MR, Bates M, Vince G, Christmas S, Lewis-Jones DI, Kingsland C. Follicular fluid concentrations of IL-12 and IL-8 in IVF cycles. *Fertil Steril* 2000;74:953–8.
22. Espey LL. Current status of the hypothesis that mammalian ovulation is comparable to an inflammatory reaction. *Biol Reprod* 1994;49:705–15.
23. Koch AE, Polverini PJ, Kunkel SL, Harlow LA, DiPietro LA, Elner VM, et al. Interleukin-8 as a macrophage derived mediator of angiogenesis. *Science* 1992;258:1798–801.