Ammonia and female sex hormones concentrations in human preovulatory follicular fluid are not directly related

Brak korelacji stężeń amoniaku i żeńskich hormonów płciowych w ludzkim przedowulacyjnym płynie pęcherzykowym

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Abstract

Objective: *The ammonia gradient from the human preovulatory follicular fluid (FF) to blood has been documented. The purpose of the present study was to substantiate whether, following controlled ovarian hyperstimulation, female sex hormones are related to this phenomenon.*

Material and Methods: *Blood was taken from the antecubital veins of 32 randomly selected patients undergoing an in vitro fertilization program prior to oocyte retrieval and FF collection. Ammonia concentrations in blood and FF were determined by the indophenol method, and 17β-estradiol (E2) and progesterone (PGS) concentrations in plasma and FF by radioimmunoassay.*

Results: *The mean ammonia concentration was 39.15 ± 3.25 µM for FF, and 20.15 ± 1.20 µM for blood (p <0.001). In all subjects, the ratios of ammonia concentrations in FF to those in blood were above 1.0, confirming the production of ammonia by the preovulatory follicle. No correlation was found between FF ammonia and E_2 concentrations (Spearman's rank correlation coefficient r = 0.2546; p = 0.160), nor between FF ammonia and the difference in E2 concentration in FF and plasma (r = 0.2416; p = 0.183). Similarly, there was no correlation between FF ammonia and PGS (r = -0.1089; p = 0.553). In support of this finding, no correlation was observed between FF ammonia and the difference in PGS concentration in FF and plasma (r = -0.1133; p = 0.537).*

Conclusions: *Ammonia production is not directly related to intrafollicular female sex hormones concentrations. The accumulation of ammonia is likely to account for the alkaline pH of the human preovulatory FF.*

Key words: **ammonia** / **estradiol** / **follicular fluid** / **human** / **progesterone** /

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Streszczenie

Cel: Udokumentowano istnienie gradientu stężeń amoniaku między ludzkim przedowulacyjnym płynem pęcherzykowym (FF) a krwią. Celem niniejszej pracy było ustalenie czy – po kontrolowanej hiperstymulacji jajników – żeńskie hormony płciowe mają związek z tym zjawiskiem.

Materiał i metoda: Krew żylną ze zgięcia łokciowego pobrano bezpośrednio przed pozyskaniem oocytu i FF od 32 losowo wybranych pacjentek poddanych procedurze zapłodnienia pozaustrojowego. Stężenia amoniaku we krwi pełnej i w FF zmierzono metodą indofenolową, a stężenia 17β-estradiolu (E_2) i progesteronu (PGS) w osoczu i w FF – metodą radioimmunologiczną.

Wyniki: Średnie stężenie amoniaku wyniosło 39,15 ± 3,25 µM w FF, a 20,15 ± 1,20 µM we krwi (p < 0,001). U wszystkich badanych stosunek stężenia amoniaku w FF do stężenia amoniaku we krwi wyniósł powyżej 1,0, co potwierdza produkcję amoniaku przez pęcherzyk przedowulacyjny. Nie stwierdzono istnienia korelacji między stężeniami amoniaku i E2 w FF (współczynnik korelacji rang Spearmana r = 0,2546; p = 0,160), ani między stężeniem amoniaku w FF i różnicą stężeń E2 między FF a osoczem (r = 0,2416; p = 0,183). Podobnie nie wykryto korelacji między stężeniami amoniaku i PGS w FF (r = -0,1089; p = 0,553), co podkreślił brak korelacji między stężeniem amoniaku w FF i różnicą stężeń PGS między FF a osoczem.

Wnioski: Produkcja amoniaku nie jest bezpośrednio powiązana z wewnątrzpęcherzykowymi stężeniami żeńskich hormonów płciowych. Natomiast wewnątrzpęcherzykowa akumulacja amoniaku może być odpowiedzialna za zasadowe pH ludzkiego płynu pęcherzykowego.

Słowa kluczowe: **amoniak** / **człowiek** / **estradiol** / **płyn pęcherzykowy** / **progesteron** /

Introduction

Ammonia (NH_3), in the biological systems tightly coupled with its ionized form of ammonium (NH_4^+), is an intermediate or the end-product of numerous biochemical reactions. This simplest form of a nitrogen-containing compound is highly reactive, volatile and easily crosses cellular and plasmatic membranes. Interestingly, previous work from our laboratory provided evidence of an ammonia gradient from human preovulatory follicular fluid (FF) to blood [1, 2]. The aforementioned studies were strongly suggestive of ammonia production by the granulosa cells, and perhaps by the oocyte, and are in line with animal studies [3, 4].

In the cellular metabolism, one major area with the ammonia involvement is the metabolism of amino acids (AAs). Indeed, there are some observations about the presence of enzymes and coenzymes involved in the metabolism of the amino group in human FF. For instance, the activities of leucine aminopeptidase, glutamate-oxaloacetate transaminase, cystyl aminopeptidase and γ-glutamyl transpeptidase have been reported [see 2].

The comparison of preovulatory intrafollicular and peripheral blood plasma AA concentrations was quite revealing, as it demonstrated significantly lower FF concentrations for all essential and most non-essential AAs [2]. The concentration difference for glutamine (~ 82 µM) was the highest of all AAs. In contrast, glutamate was the only AA that was significantly increased in FF as compared to plasma. An inverse correlation of glutamine concentration in FF with glutamate concentration difference was supportive of the metabolism of glutamine to glutamate and ammonia in follicular cells. Furthermore, the lack of correlation between blood and FF ammonia concentrations, together with the FF urea concentration virtually equal to the plasma value, was suggestive of two important informations. Namely, that the local accumulation of ammonia is purely of intrafollicular origin and that the urea metabolism within the follicle is negligible [2].

Objective

The present study was designed to test the hypothesis of the possible link between FF ammonia concentration and female sex hormones following controlled ovarian hyperstimulation. The FF mirrors the vivid metabolism of both the oocyte and the granulosa cells, which are subject to powerful hormonal stimulation by gonadotropins and in turn release sex hormones. Our hypothesis was prompted by several studies. Firstly, this direction of research was recommended by an authoritative review [5]. Secondly, Bae and Foote documented that, *in vitro*, maturing rabbit oocytes preferentially metabolize glutamine as a substrate, and this metabolism is associated with a vivid ammonia production [6]. Furthermore, Rooke et al. demonstrated that exposure of bovine granulosa cells to ammonium chloride results in their increased production of both 17β-estradiol (E_2) and progesterone (PGS), which is the dominant steroid [7]. Consequently, we anticipated that there could be a link between FF ammonia and PGS. For this purpose, we measured the concentrations of E_2 and PGS in human FF at the final stage of the oocyte's development, before ovulation, and then correlated them with the ammonia concentration. In addition, blood plasma E_2 and PGS concentrations were also measured.

Materials and Methods

Subjects and stimulation protocols

Thirty-two randomly selected patients, aged 32.5 ± 0.8 years (range 24-42 years), attending an in vitro fertilization program in the Department of Gynecology, Medical University of Białystok, Białystok, Poland, were studied. They were white Caucasian non-smoking healthy women who had no history of liver disease. All participants were informed and gave their consent to the study which had been approved by the local Institutional Review Board.

The protocol of ovarian stimulation, as well as the details of medical management were as described [2, 8]. A short protocol of stimulation was applied [9]. Subcutaneous injections of the

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gonadotropin-releasing hormone agonist triptorelin acetate (Decapeptyl; Ferring, Kiel, Germany) 0.1 mg, starting on Day 1 were followed by gonadotropins, follicle stimulating hormone and/or human menopausal gonadotropin, administered in individually adjusted doses for every patient starting on Day 3 of her menstrual cycle. The stimulation was monitored using serum E_2 concentrations, together with ultrasound to measure the follicular size and quantity. The induction of ovulation with human chorionic gonadotropin was performed when the leading follicle was 18-20 mm in diameter, and the serum E_2 concentration per follicle 150-200 ng/L.

Sample collection

Preovulatory ovarian FF was collected from the patients transvaginally using ultrasound as guide for oocyte retrieval. Care was taken to retain only blood-free specimens for later use [10]. Sampling was carried out when follicles ranged from 24 to 26 mm in diameter. Only individual (i.e., unpooled) FFs were collected. Each patient's blood was sampled from an antecubital vein prior to the administration of an anesthetic for the oocyte retrieval procedure.

The FFs were collected into capped, disposable plastic tubes (Life Sciences, Denver, CO, USA). The blood samples were collected into identical preheparinized plastic tubes. A high purity, high-molecular-weight heparin (5000 IU/mL) in the form of a sodium salt was from Polfa (Warsaw, Poland). The tubes were rinsed with heparin and dried.

Both fluids were collected in two portions, the first of which was used for the determination of ammonia, performed within 5 minutes of sampling. The second portion was centrifuged at 10 000 rpm in 4°C for 5 minutes, and the aliquots of plasma and FF were snap frozen in liquid nitrogen and stored at -80°C, until E_2 and PGS analysis was performed within a month. Thus, for routine hormonal monitoring, serum was used, and for the study – in order to obtain parallelism – plasma was the analytical specimen.

Analytical methods

Ammonia concentrations in FF and blood were determined in quadruplicate with the Berthelot-indophenol method using a Wako Chemicals (Neuss, Germany) assay, and read on a spectrophotometer (model DU 640; Beckman Instruments Inc., Fullerton, CA, USA) set at 630 nm. This method has been described and discussed in detail in our previous publication [1]. Briefly, with nitroprusside as a catalyst, one molecule of ammonia reacts with two molecules of phenol to form one molecule of dihydroxydiphenylamine. Subsequently, following oxidation of dihydroxydiphenylamine by sodium hypochloride, the final blue-colored product, indophenol, is formed and quantified by measurement of its absorbance. The results were calculated from a standard curve ranging from 0 to 400 $\mu g \cdot dL^{-1}$ and converted to micromolar values. The intra-assay coefficient of variation calculated on 8 randomly selected samples was 5.6%.

Plasma and FF concentrations of E_2 and PGS were determined by radioimmunoassay using SPECTRIA ESTRADIOL [125I] and SPECTRIA PROGESTERONE [125I] kits from Orion Diagnostica (Espoo, Finland) according to the manufacturer's recommendations. Radioactivities were read in duplicate on Minigamma 1275 Gamma Counter (LKB-Wallac, Turku,

Finland), the results being the mean of both readings. For E_2, the results were calculated from a standard curve in pmol/L and, for comparisons with the literature, converted to values in ng/L using the equation:

$$E_2 \text{ (ng/L)} = E_2 \text{ (pmol/L)} \cdot 0.2724$$

For PGS, the results were calculated using a standard curve in nmol/L. For comparisons with the literature, conversion to ng/mL was accomplished using the equation:

$$PGS \text{ (ng/mL)} = PGS \text{ (nmol/L)} \cdot 0.314$$

Crossreactivity of the E_2 antiserum with sex hormones other than E_2 is <1.0%, and crossreactivity of the PGS antiserum with sex hormones other than PGS is ≤3.9%. The intra-assay coefficient of variation provided by the manufacturer was ≤9.7% for E_2, and ≤ 7.9% for PGS.

Statistical Analysis

The data distribution was first verified for their agreement with normal distribution using the normality plot and Shapiro-Wilk test. Age and ammonia data were in such agreement and they are expressed as means ± standard error of mean (SEM). Their statistical analysis was performed with the Student's *t*-test for paired samples and Pearson's linear correlation. The hormonal data showed distributions different from normal, and consequently were presented as medians and ranges, their differences examined with Wilcoxon test, and correlations with other variables tested using Spearman's rank correlation coefficient. The statistical package was SPSS® 8.0 for Windows PL (SPSS, Chicago, IL, USA). A *p*-value of less than 0.05 was considered statistically significant.

Results

In all subjects, the ratio of ammonia concentration in FF to that in the blood was above 1.0. The mean ammonia concentration was 39.15 ± 3.25 μM for FF, and 20.15 ± 1.20 μM for peripheral blood ($p < 0.001$), values similar to those measured in previously reported sets of samples [1, 2]. Mean concentration difference between the two compartments was 19.00 ± 3.46 μM.

There was no correlation between ammonia concentrations in blood ($r = 0.1160$; $p = 0.527$) or FF ($r = -0.0164$; $p = 0.929$) and the patients' age.

The median E_2 concentration was 371750 pmol/L (range 85200 to 2136000 pmol/L) for FF, and 2913 pmol/L (range 130 to 11990 pmol/L) for plasma ($p = 0.000$), while the median PGS concentration was 7225 nmol/L (range 1620 to 25040 nmol/L) for FF, and 47.1 nmol/L (range 1.4 to 331.6 nmol/L) for plasma ($p = 0.000$). The concentrations of E_2 and PGS in FF from the present study were found to coordinate with those reported by other authors for ovarian preovulatory Graafian follicles following the induction of ovulation [11, 12].

There were correlations of age with: FF PGS ($r = 0.6361$; $p = 0.000$), the difference in PGS concentration in FF and plasma ($r = 0.6418$; $p = 0.000$), and the difference in E_2 concentration in FF and plasma ($r = 0.3659$; $p = 0.039$). Plasma PGS concentration highly correlated with plasma E_2 concentration ($r = 0.7149$; $p = 0.000$), whereas there was no correlation of these hormones' concentrations in FF ($r = 0.1463$; $p = 0.424$).
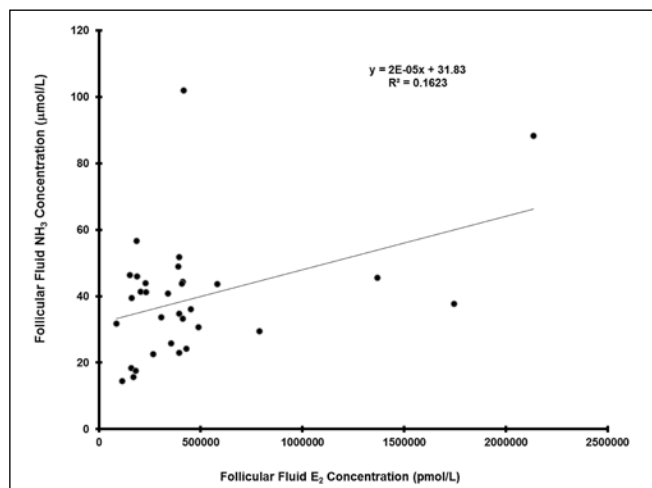
Figure 1. Relationship of 17β-estradiol (E_2) and ammonia (NH_3) concentrations in human preovulatory follicular fluid (p = 0.160; not significant). N = 32.
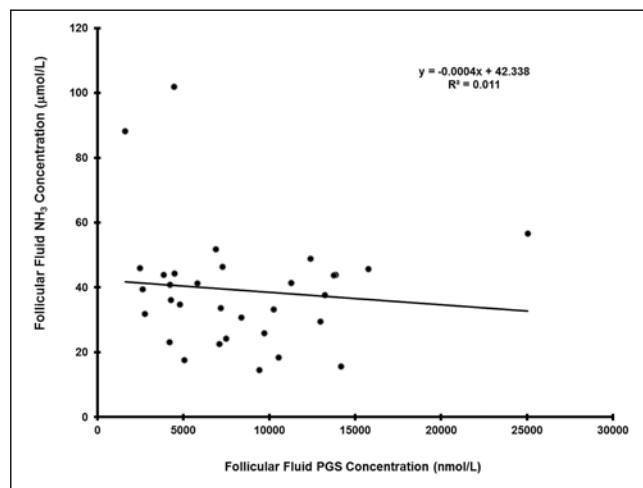

Figure 2. Relationship of progesterone (PGS) and ammonia (NH_3) concentrations in human preovulatory follicular fluid (p = 0.553; not significant). N = 32.

There was no correlation between FF ammonia and E_2 concentrations ($p = 0.160$; $r = 0.2546$) (Figure 1), nor between FF ammonia and the difference in E_2 concentration in FF and plasma ($p = 0.183$; $r = 0.2416$). Similarly, there was no correlation between FF ammonia and PGS ($p = 0.553$; $r = -0.1089$) (Figure 2). In support of this finding, no correlation was observed between FF ammonia and the difference in PGS concentration in FF and plasma ($p = 0.537$; $r = -0.1133$).

Discussion

The present investigation of carefully matched specimens confirms that before ovulation after controlled ovarian hyperstimulation the human oocyte is exposed to ammonia concentrations of 30-40 μM, which is approximately twice the concentration in blood. There is a production of ammonia by preovulatory Graafian follicles which accumulates in FF. The intracellular pH of human oocytes during maturation and fertilization is 7.4, whereas the FF pH is as high as 7.62 [13, 14]. The accumulation of ammonia as certified by this and our previous studies is likely to account for the alkaline pH of the human FF, providing to our knowledge the first chemical explanation of this alkalinity. In other mammals, FF from small ovarian follicles demonstrated higher concentrations of ammonia than FF from more grown follicles, testifying to the tolerance of the oocyte to the increased ammonia content in its environment [3, 7]. In contrast, the acidic environment is deleterious [15].

In mice, the cumulus cells of large antral follicles express a sodium-coupled neutral amino acid transporter SNAT3 (former SN1, Slc38a3) [16]. This is a plasma membrane-bound protein with strong transport activities for histidine, asparagine and glutamine, but not for glutamate [17-19]. The uptake of glutamine by SNAT3 was found to be pH-dependent, i.e. increasing from low to high pH [17, 18, 20]. Thus, the high pH of FF could facilitate the provision of the cumulus cells, and ultimately oocyte, with glutamine. In SNAT3-expressing *Xenopus laevis* oocytes, acidification of the milieu decreased the uptake of glutamine, or even reversed the direction of its transport [20], helping to explain the detrimental role of low extracellular pH.

However, the principal finding of the present investigation is that, contrary to our hypothesis, ammonia production in human preovulatory ovarian FF is not directly related to E_2 or PGS concentrations. The participation of ammonia in the metabolism of estrogens has not been supported by biochemistry books. So far, no study has explored the activities of ammonia-metabolizing enzymes: glutamine synthetase, glutaminase, and glutamate dehydrogenase, within the granulosa cells/oocyte complex. *In vitro*, the activities of both human liver and uterine glutamate dehydrogenases, the enzymes degrading glutamate to 2-oxoglutarate and ammonia, were decreased by the stimulation with 10 μM E_2 [21]. If such a response to high estrogen concentrations is true for glutamate dehydrogenase in the follicular cells, the "glutamate-sparing" effect would be in line with the accumulation of glutamate observed in human preovulatory FF. Glutamate oxidation is necessary for the synthesis of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH). Aromatase, the enzyme complex converting androgens to estrogens, requires NADPH for the action of its NADPH-cytochrome P450 reductase component [22].

In our study of AAs [2], there was a highly significant gradient of serine from blood plasma to FF, while the FF glycine concentration remained unchanged from that of plasma. The data were suggestive of the presence in the preovulatory ovarian follicle of the glycine-cleavage enzyme system operating in the direction from serine to glycine. This readiness for glycine provision needs to be discussed in the light of the cell's constant need for the synthesis of glutathione, or γ-glutamyl-cysteinylglycine. The tripeptide serves as an important intracellular redox buffer. The accumulation of glutamate in FF is clearly the effect of its local cellular release. Among many known glutamate transporters, ones involved in glutamate secretion coupled with cystine uptake are the sodium-independent antiport channel proteins designated as system x_c^- [5, 23-25]. The sodium-dependent X_{AG}^- family of glutamate transporters is also important. These symport channel proteins transport cystine and glutamate inwards, and are thought to tightly cooperate with x_c^- exchangers with the goal of effective cystine provision [23].

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Notably, cumulus cells have been demonstrated to play a critical role in protecting bovine and porcine oocytes against oxidative stress-induced apoptosis through the enhancement of glutathione content in oocytes [26, 27].

Our data warrant interest in the determination of the concentration of ammonia in FF throughout the follicular phase of the cycle in humans. Also, it would be important to study in the follicular cells the presence and function of the system x_c^- antiporters and other transporters involved in the metabolism of glutamine and glutamate, and the activity of ammonia-metabolizing enzymes, together with their hormonal control.

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