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ScienceDirect
Metabolism Clinical and Experimental 59 (2010) 1145 – 1155
Metabolism
Clinical and Experimental
www.metabolismjournal.com

High follicular fluid adenosine levels may be pivotal in the metabolism and recycling of adenosine nucleotides in the human follicle

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Received 26 December 2008; accepted 2 September 2009

Abstract

This study investigated the biochemical relationship between human follicular/oocyte maturity and the levels of follicular fluid purines. Intrafollicular levels of purine metabolites and creatinine are associated with oocyte presence, and the presence of such high levels of adenosine indicates a privileged site with no adenosine deaminase activity. Subgrouping according to oocyte recovery and fertilization revealed differences in correlation between the purine metabolites: Only where an oocyte was recovered and subsequently fertilized did follicular fluid adenosine, adenine, and hypoxanthine levels correlate with each other. Significantly, purines' correlation with levels of the terminal degradation product, uric acid, could only be seen in failed fertilization samples. Given the established metabolic pathways for adenosine triphosphate/adenosine diphosphate/adenosine monophosphate degradation, the results indicate maximization of 2 purine salvage pathways (from adenine and hypoxanthine) that pivot on the presence of high adenosine levels. Such optimized recovery may be necessary to build a store of salvaged adenosine phosphate for oocyte survival.

1. Introduction

Oocyte maturation is arrested during the first meiotic division at the diplotene stage. Only after ovulation does the oocyte proceed to metaphase II. This implies that a follicular factor is responsible for meiotic arrest, and early studies implicated a soluble factor (or factors) present in follicular fluid [1,2].

Follicular fluid is a mixture of serum transudate and granulosa cell secretions. In addition to proteins and steroids, it contains many smaller organic compounds, some of which result from purine and pyrimidine metabolism. The purine metabolites, in particular, have been implicated in inhibition of cyclic adenosine monophosphate (AMP) metabolism and hence regulation of meiosis: hypoxanthine has now been identified in murine [3,4], porcine [5], bovine [6], caprine [7], and human [8] follicular fluid. Certainly in mice, hypoxanthine has been implicated as the follicular fluid soluble agent responsible for oocyte meiosis I arrest [2]. Other purine metabolites—adenosine, adenine, and inosine—have also been identified in human follicular fluid; and adenosine has been implicated as the responsible agent. However, these studies have been inconsistent and have not reached a consensus to date [3,5-8]. Nevertheless, these molecules are present in significant quantities, may correlate with the processes of follicular maturation, and could be reflected in fertility outcome. The failure to arrest meiosis may result in an overdeveloped/premature oocyte that can no longer fertilize or is a blighted ovum (the egg has disintegrated but the follicle remains). However, this underestimates the critical value of purine metabolism to the cell's ability to function: adenosine nucleotide metabolism is more strongly associated with energy and adenosine triphosphate (ATP) availability than with meiotic arrest. In this respect, the greatest physiologic significance to the oocyte is the so-called

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doi:10.1016/j.metabol.2009.09.037

salvage pathways in which hypoxanthine and adenine are recycled to AMP and ultimately to ATP. The oocyte is particularly susceptible to reductions in its energy charge. Its enormous size and membrane integrity are built and maintained by the numerous cumulus "nurse" cells that surround it in the follicle. The oocyte itself is transcriptionally silent; and hence, the cumulus/nurse cells drive the anabolic and metabolic pathways necessary for maintaining the cell. Once ovulation occurs and without the support of the follicular environment, cellular integrity of the oocyte will be largely dependent on the ATP, adenosine nucleotides, and substrates deposited in the oocyte before ovulation. This energy store (which consists of AMP/adenosine diphosphate/ATP precursors as well as glycolytic and Kreb cycle substrates) will be drained to maintain cellular membrane integrity unless fertilization occurs, and de novo synthesis and salvage pathways are coordinated. Hence, the estimated fertilization window postovulation is only 24 hours and will vary according to the total ATP-nucleotide energy charge deposited.

In prior reports on the analysis of follicular fluid metabolites, the follicular fluid samples have been pooled (or grouped by compulsory size cutoff points). The methodology is not accurate because—particularly in small sample number sets—a single sample with extreme levels can bias the data. This is particularly true of follicular fluid analysis, and we have previously shown that the concentrations of different proteins and steroid hormones within follicular fluid can be profoundly influenced by the size of the follicle [9-13]. Thus, when conducting such studies, any quantifiable values generated must be corrected against follicular size; or comparisons of different analytes within the follicular environment must only be drawn from individual and not pooled sample results.

This study analyzed low–molecular-weight (<5 kd) components from *individual* human follicular fluid samples by reversed-phase high-performance liquid chromatography (HPLC). Several purine metabolites—along with other bioorganic molecules—were identified; and their relationship to follicular diameter, oocyte presence, and fertility outcome was examined.

2. Materials and methods

2.1. Subjects and sample preparation

Subjects and follicular fluid collection were as described previously [12]. Briefly, 200 individual follicular fluid samples were taken from 35 patients who participated in a controlled ovarian hyperstimulation program for an assisted reproduction procedure.

Written consent was obtained from each patient, and the study was approved by the East London and The City Health Authority Research Ethics Committee (study no. P/98/222).

A follicular fluid pool from a random selection of 12 follicles (200 μL of each) was created to establish optimized HPLC running conditions. It was mixed thoroughly, and aliquots were frozen at −50°C. The average dilution factor for the follicular fluid pool was calculated. In all quantitative HPLC analyses, follicular fluids were run individually.

2.2. Sample protein removal by filtration centrifugation

Individual follicular fluid samples removed from storage after collection were pretreated by centrifugal ultrafiltration to remove large proteins using 5 kd as cutoff; polyethersulfone membrane Vivaspin 0.5-mL concentrators (Vivascience, Hanover, Germany) were spun for 30 minutes at 400*g* according to the manufacturer's recommendation.

2.3. Reversed-phase HPLC method development

2.3.1. Gradient development

A gradient HPLC system (LKB-Produkter, Bromma, Sweden) was used. Samples were separated on a C18 column (125 × 4.6 mm, 5 μm ODS II Hypersil column) (Thermohypersil, Runcorn, United Kingdom). Samples were injected via a 20-μL Rheodyne (Perkin-Elemer, Monza, Italy) loop injector. Column eluates were monitored at 260 nm and outputted to an HP3396 integrator (GMI, Ramsey, MN). The gradient shape was optimized using the follicular fluid pool. Ammonium acetate (Normapur, Prolabo, France) 50 mmol/L buffered to pH 4.5 was chosen for solvent A, and acetonitrile (ACN; BDH, Yorkshire, United Kingdom) was chosen for solvent B. The flow rate was kept constant at 1 mL/min throughout the linear gradient, with the percentage of ACN varied by the system controller. A 2-minute washout period of 100% ACN and reestablishment of start eluent A were included to prepare the system for subsequent injections.

2.3.2. Standard linearity

Seven standard solutions were made up to standardize the system and also used to help identify some of the peaks resolved in the follicular fluid pool. The known standards were creatinine, uric acid, hypoxanthine, adenine, inosine, guanosine, and adenosine. 7-β-(Hydropropyl theophylline) (7-β-[HT]) was included as the internal calibrant. To assess the linear relationship between the concentration of an analyte and the peak area, a mixture of standards at different known concentrations was injected: the standards were made to a concentration of 25 μmol/L and then diluted to 21.9, 18.8, 15.6, 12.5, 9.4, 6.25, and 3.1 μmol/L before injection. Peak areas were plotted.

2.3.3. Reproducibility

The reproducibility of the system was assessed with 20 μL of the standard mix and the pooled follicular fluid. Means, standard deviations, and coefficient of variation of the retention times (RTs) and areas of the peaks in the standard mix were calculated. The peak areas of the samples and the standard mix were all corrected by dividing each peak area by the area of the internal standard, 7-β-(HT).

2.4. Peak identification in follicular fluids

2.4.1. Retention time

By comparison to the established condition RTs of standards, all samples contained peaks with RTs identical to those of uric acid, creatinine, hypoxanthine, adenosine, and adenine. To identify other peaks in the follicular fluid, further standards with known RTs between 1 and 16 minutes were run; however, none correlated with the unidentified peaks in the follicular fluids.

2.4.2. Spiking

To further confirm that the peaks at 1.36, 1.99, 2.25, 3.62, and 10.28 minutes were uric acid, creatinine, hypoxanthine, adenine, and adenosine, respectively, samples were spiked with 5 μL and 10 mmol/L of each analyte, respectively. Enhanced peaks appeared on rerunning the samples for each corresponding component.

2.4.3. Wavelength ratios

To further confirm the identification of uric acid, column elution was monitored at both 260 and 280 nm. There was an obvious increase in area for the peak eluting at 1.36 minutes when the wavelength was changed from 260 to 280 nm. This further confirms this peak's identity as uric acid, as uric acid has a greater absorbance value at 280 nm.

2.4.4. Diode array spectral characterization

A sample of pooled follicular fluid and of standard mix (both 20 μL) was analyzed by the same HPLC system but equipped with diode array detection at the Purine Research Laboratory, Guy's Hospital (London, United Kingdom). Spectral data were compared between the pool, the standards, and a library of purine-related spectra.

2.4.5. Enzymatic degradation

To further confirm the identity of adenosine eluting at 10.28 minutes, samples were incubated with 1% adenosine deaminase (ADA, wt/vol) in 50 μmol/L potassium dihydrogen orthophosphate, pH 7.4, buffer (Sigma Chemical, Dorset, United Kingdom) at 37°C for 2 hours. This resulted in height reduction of this peak and the appearance of a new metabolite—coeluting with inosine at 3.9 minutes.

2.5. Quantification of analytes for individual follicular fluid samples

Twenty microliters of ultrafiltered individual samples of follicular fluid was injected and separated by gradient reversed-phase HPLC. Reproducibility for HPLC analysis was assessed using the internal standard 7-β-(HT) added to the sample at a concentration of 100 μmol/L. Every 5 samples, the standard calibrant mix was run; RTs and peak area were determined; and mean, standard deviation, and coefficient of variation were calculated. The concentration of each biometabolite in the follicular fluid was calculated from the peak areas of the sample and corresponding standard. Final concentration was calculated by further multiplying by the original sample dilution factor.

2.6. Data plots and statistical analysis

Scatter plots and lines of best fit were calculated using Origin 6.1 software package (OriginLab, Northampton, MA). All descriptive and comparative statistical analyses were performed with the StatsDirect software package (StatsDirect, Cheshire, United Kingdom). In most instances, data were not normally distributed; and nonparametric Mann-Whitney U and Spearman rank correlation tests were applied. $P < .05$ was considered to be statistical significance.

3. Results

3.1. Analytical method validation

There was a linear relationship between the concentrations of analytes between 0 and 25 μmol/L injected and peak area for uric acid, creatinine, hypoxanthine, adenine, inosine, guanosine, and adenosine (data not shown). The reproducibility of the HPLC system was examined using both interspersed rerunning of a mix of standards at 14.28 μmol/L and the internal standard 7-β-(HT). The coefficient of variation for the purine was 0.42% to 13%.

Separation of up to 14 small molecular components in follicular fluid was achieved in this study. Five were identified (adenosine: RT, 10.28 minutes; adenine: RT, 3.62 minutes; hypoxanthine: RT, 2.25 minutes; creatinine: RT, 1.99 minutes; uric acid: RT, 1.36 minutes; and 7-β-[HT]: RT, 15.71 minutes), and the remaining unidentified peaks were labeled *peaks 1 to 8* (Fig. 1).

3.2. Metabolite quantification in follicular fluid

Peak areas for the known biometabolites were corrected against the peak area of the internal standard for individual

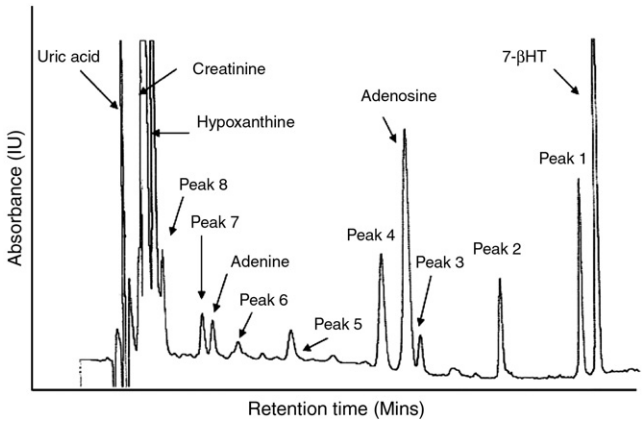

Fig. 1. Typical reversed-phase gradient HPLC chromatograph of follicular fluid with detection at 260 nm. 7-β-(Hydropropyl theophylline) is the internal standard; known peaks are labeled as such; and unidentified peaks were labeled as *peaks 1 to 8*, respectively.

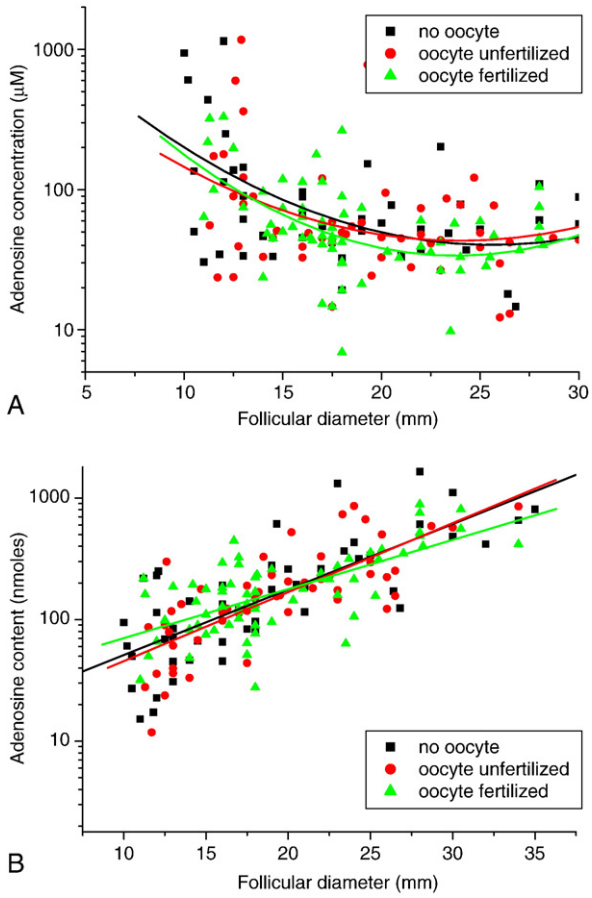

Fig. 2. Scatter plots of adenosine concentration (A) and total content (B) against follicular diameter. A, Polynomial fit of adenosine concentration for 3 groups: no-oocyte-presence group (black line: $r^2 = 0.27$, $P < .0005$), unfertilized-oocyte group (red line: $r^2 = 0.13$, $P = .03$), and fertilized group (green line: $r^2 = 0.29$, $P < .0001$). B, Linear fit of total content of adenosine for 3 groups: no-oocyte-presence group (black line: $r = 0.77$, $P < .0001$), oocyte-unfertilized group (red line: $r = 0.76$, $P < .0001$), and oocyte fertilized group (green line: $r = 0.67$, $P < .0001$).

separations. Adenosine, adenine, hypoxanthine, creatinine, and uric acid concentrations were calculated as previously described; and their concentration and total follicular content were plotted against follicular diameter (Figs. 2-6). In all cases, similar trends were seen when samples were subdivided according to oocyte recovery and fertilization. However, although similar, they were not identical, suggesting that subtle alteration in the total metabolism may occur. In terms of a generalized trend with respect to follicular size, we concentrated on samples in which the recovered oocyte fertilized (the comparison with those samples for which an oocyte was not recovered or failed to fertilize is shown in the legend of Figs. 2-6): both adenosine and uric acid concentration decreased up to approximately 18 to 20 mm and then reached a plateau (Figs. 2A and 6A: $r^2 = 0.29$ and 0.44, both Ps $< .0001$) despite their follicular total content increasing with follicular size (Figs. 2B and 6B: $r = 0.67$ and 0.38, $P < .0001$ and $P = .02$, respectively). Creatinine showed a similar trend to adenosine, although no statistically significant difference was observed between all follicles (Fig. 5). Adenine concentration decreased with follicular diameter (Fig. 3A: $r^2 = 0.23$, $P = .01$), but its total content increased when corrected for the follicular volume (Fig. 3B: $r = 0.44$, $P < .01$). The concentration of hypoxanthine showed a random distribution for all follicles; however, its total content increased with follicular diameter as seen in other follicular components (Fig. 4B: $r = 0.32$, $P < .01$).

Corrected peak areas of unidentified peaks were also plotted against follicular diameter (data not shown). Only peak 7 and peak 8 areas showed a significant decrease with follicular diameter; the others' distribution either varied randomly or remained at a constant level in different follicles.

Concentrations for each identified component were compared with respect to whether oocyte retrieval was successful and for oocyte fertility outcome (fertilized to 2 polar nuclei stage or unfertilized). As the data were not normally distributed, median and interquartile ranges are shown; and comparisons were made by the Mann-Whitney

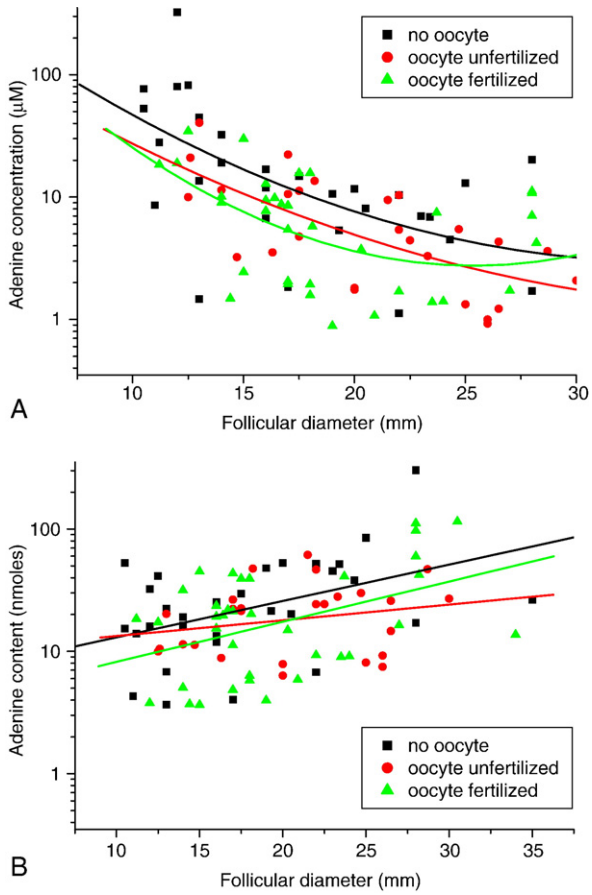

Fig. 3. Scatter plots of adenine concentration (A) and total content (B) against follicular diameter. A, Polynomial fit of adenine concentrations for 3 groups: no-oocyte-presence group (black line: $r^2 = 0.38$, $P = 001$), oocyte-unfertilized group (red line: $r^2 = 0.47$, $P = .0007$), and oocyte-fertilized group (green line: $r^2 = 0.23$, $P = .01$). B, linear fit of total content of adenine for 3 groups: no-oocyte-presence group (black line: $r = 0.44$, $P = .01$), oocyte-unfertilized group (red line: $r = 0.23$, $P = .25$), and oocyte-fertilized group (green line: $r = 0.44$, $P < .01$).

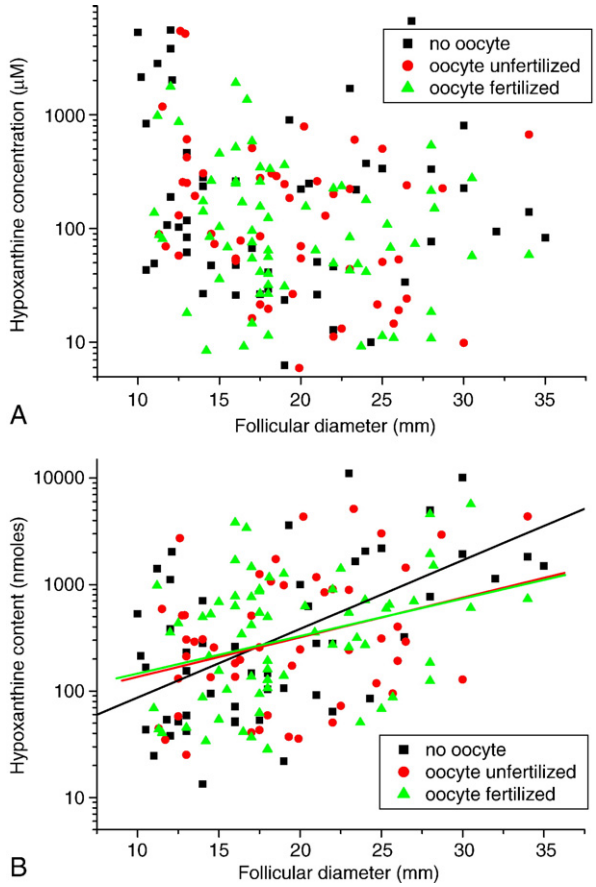

Fig. 4. Scatter plots of hypoxanthine concentration (A) and total content (B) against follicular diameter. A, Median = 95.4 μmol/L, IQR = 41.6 to 277.3 μmol/L. B, Linear fit of total content of hypoxanthine for 3 groups: no-oocyte-presence group (black line: $r = 0.56$, $P < 0001$), oocyte-unfertilized group (red line: $r = 0.33$, $P = .02$), and oocyte-fertilized group (green line: $r = 0.32$, $P < .01$).

U test (Table 1). Adenosine and adenine showed a statistically significant lower median concentration in the group with an oocyte presence ($P = .03$ and .02, respectively), whereas hypoxanthine, creatinine, and uric acid did not. With respect to oocyte fertility outcome, none of the components identified showed a statistically significant difference between fertilized and nonfertilized groups (Table 1).

The correlations between each identified component metabolite and follicular size were examined by Spearman rank correlation test for the following 3 groups: (1) an oocyte was recovered and fertilized, (2) an oocyte was recovered but failed to fertilize, and (3) no oocyte was recovered (Tables 2-4).

In follicular fluids from which an oocyte is recovered and fertilized in vitro, we found that adenosine levels correlated strongly and positively with hypoxanthine and adenine. Adenosine to adenine metabolism is clearly evident from metabolic action of purine nucleotide phosphorylase. However, adenosine conversion to hypoxanthine in the absence of ADA is possible via a longer route pathway of adenosine to AMP to inosine monophosphate (IMP) and subsequent breakdown to hypoxanthine via inosine (Fig. 7). More significantly, adenine strongly correlated with hypoxanthine (Fig. 7); and this can only be achieved in the absence of ADA via the second major purine salvage pathway from adenine to AMP via adenine phosphoribosyltransferase (APRT). Furthermore, the results indicate that the primary salvage pathway (from hypoxanthine to IMP by hypoxanthine guanine phosphoribosyltransferase [HGPRT]) is highly active, such that hypoxanthine levels no longer correlate with uric acid concentrations (Table 2, Fig. 7).

In the group where oocyte was present but failed to fertilize, adenosine levels correlated with hypoxanthine, but not with adenine (Table 3). Adenine levels negatively correlated with uric acid (Table 3). The inverse correlation of uric acid with adenine may suggest a direct metabolic link; however, this cannot be explained via the classic pathways because there was no correlation with adenosine or hypoxanthine as would be expected with or without ADA being present (Table 3, Fig. 8).

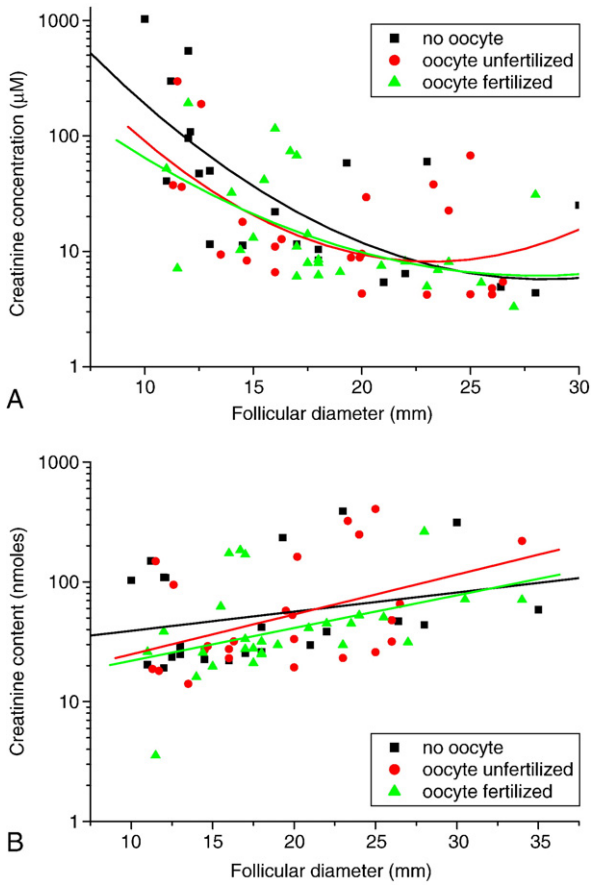

Fig. 5. Scatter plots of creatinine concentration (A) and total content (B) against follicular diameter. A, Polynomial fit of creatinine concentration for 3 groups: no-oocyte-presence group (black line: $r^2 = 0.54$, $P = 0006$), oocyte-unfertilized group (red line: $r^2 = 0.35$, $P = .01$), and oocyte-fertilized group (green line: $r^2 = 0.31$, $P = .01$). B, Linear fit of total content of creatinine for 3 groups: no-oocyte-presence group (black line: $r = 0.27$, $P = .2$), oocyte-unfertilized group (red line: $r = 0.45$, $P = .03$), and oocyte-fertilized group (green line: $r = 0.42$, $P = .02$).

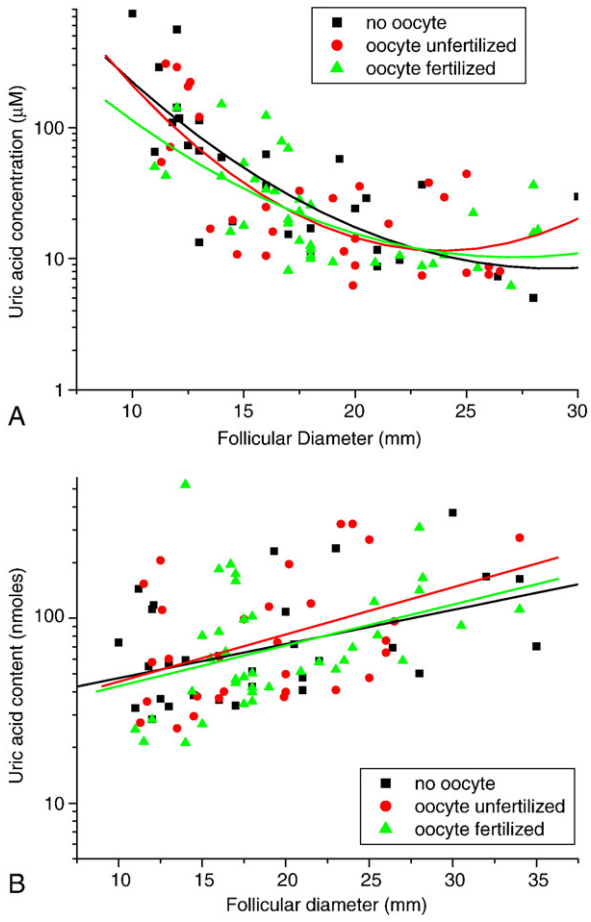

Fig. 6. Scatter plots of uric acid concentration (A) and total content (B) against follicular diameter. A, Polynomial fit of uric acid concentration for 3 groups: no-oocyte-presence group (black line: $r^2 = 0.64$, $P < 0001$), oocyte-unfertilized group (red line: $r^2 = 0.53$, $P < .0001$), and oocyte-fertilized group (green line: $r^2 = 0.44$, $P < .0001$). B, Linear fit of total content of uric acid for 3 groups: no-oocyte-presence group (black line: $r = 0.45$, $P = .01$), oocyte-unfertilized group (red line: $r = 0.43$, $P = .02$), and oocyte-fertilized group (green line: $r = 0.38$, $P = .02$).

In the oocyte-absent group, adenosine levels correlated with adenine and hypoxanthine (Table 4). However, adenine levels did not correlate with hypoxanthine, whereas hypoxanthine levels did correlate with uric acid (Table 4). Given the strength of the correlation, this could indicate that hypoxanthine conversion to uric acid (by hypoxanthine oxidase) is still active and dominant compared with the hypoxanthine HGPRT salvage pathway (Fig. 9).

4. Discussion

In the 1980s, there was considerable interest in the purine metabolites present in follicular fluid and their effect on oocyte development. Downs et al [14] studied their roles as meiotic inhibitors in mice in particular, demonstrated that both hypoxanthine and adenosine are present in follicular fluid, and postulated that hypoxanthine inhibits oocyte meiotic arrest by inhibiting oocyte cyclic AMP phosphodiesterase activity. Later, Lavy et al [8] studied purine metabolite levels in human follicles from both natural and stimulated cycles and proposed that adenosine, not hypoxanthine, was the inhibitor of human oocyte maturation because they found hypoxanthine to be present in subinhibitory concentrations, whereas adenosine levels were within the range effective for the inhibition. In this study, we found that hypoxanthine levels were extremely variable, with a median concentration of approximately 100 μmol/L. However, adenosine levels were supraphysiologic in comparison to serum levels and varied according to follicular size. Indeed, the smaller follicles contained a much greater concentration of adenosine than the larger follicles did. The previous studies were based either on pooled samples in which follicular diameter had not been taken into consideration [2] or on a small number of follicular samples [8]; and thus, their findings only reflected an incomplete picture of the purines' profile in the follicular fluid. In addition, most published data on follicular fluid analysis by HPLC lack details regarding the

Table 1
Descriptive and statistical analysis of the concentration of identified components grouped by oocyte retrieval and fertility outcome

	Adenosine (μmol/L) median (IQR)	Adenine (μmol/L) median (IQR)	Hypoxanthine (μmol/L) median (IQR)	Creatinine (μmol/L) median (IQR)	Uric acid (μmol/L) median (IQR)
No oocyte	55.8 (37.1-110.8)	1.83 (1-13.6)	107.4 (46.5-374.0)	2.0 (2.0-11.6)	10.4 (3.0-57.5)
With an oocyte	46.2 (35.7-73.6)	1.0 (1.0-7.03)	92.6 (40.6-264.7)	2.0 (2.0-8.2)	8.2 (4.0-19.4)
P value	.03*	.02*	.29	.6	.48
Fertilized	45.5 (35.6-64.5)	1.39 (1.0-8.5)	91.4 (40.1-263.3)	2.0 (2.0-7.7)	8.3 (4.0-16.2)
Unfertilized	48.1 (39.2-86.4)	1.0 (1.0-4.4)	109.9 (47.5-283.5)	2.0 (2.0-9.4)	7.6 (4.0-28.7)
P value	.4	.2	.61	.64	.56

IQR indicates interquartile range.
* $P < .05$.

Table 2
The intercorrelations by Spearman rank correlation test were shown between follicular diameter and all components found in follicular fluids in which an oocyte was present and fertilized successfully

Fertilized		Follicular size	Concentration				
			Adenosine	Adenine	Hypoxanthine	Uric acid	Creatinine
Follicular size			***P*<.0001**	*P*=.23	***P*=.006**	*P*=.056	*P*=.24
Concentration	Adenosine	**ρ=–0.43**		***P*<.0001**	***P*=.0001**	*P*=.32	*P*=.32
	Adenine	ρ=–0.13	**ρ=0.41**		***P*<.0001**	*P*=.84	*P*=.1
	Hypoxanthine	**ρ=–0.29**	**ρ=0.46**	**ρ=0.54**		***P*=.71**	*P*=.27
	Uric acid	ρ=–0.2	ρ=–0.11	ρ=–0.02	**ρ=0.04**		***P*<.0001**
	Creatinine	ρ=–0.12	ρ=–0.11	ρ=–0.18	ρ=–0.12	**ρ=0.71**	

Red, green, and blue table entries indicate corresponding pathways shown in Fig. 2.

storage and preparation of the samples. Ischemic changes lead to a rapid fall in adenosine nucleotide levels and a rapid rise in hypoxanthine; and thus, it would be difficult to justify data accuracy.

In this study, low–molecular-weight components (<5 kd) were examined in individual follicles using optimized reversed-phase HPLC conditions. Their possible relationships with follicular diameter were investigated. Two outcome parameters—oocyte retrieval and fertilization—were also examined to see if they are associated with the levels of follicular fluid components.

The results showed that adenosine, adenine, hypoxanthine, creatinine, and uric acid were detected in most individual follicles with median (interquartile range) concentration (in micromoles per liter) of 48.8 (36.9-78.1), 8.1 (2.4-13.0), 97.2 (41.6-277.3), 9.5 (6.6-36.6), and 18.7 (10.5-51.7), respectively. Apart from hypoxanthine and uric acid, other components were all significantly lower in follicles where an oocyte was retrieved. However, further investigation into oocyte fertilization showed that there was no significant association between the absolute levels of any of these individual components and fertility outcome. In terms of biological significance to follicular/oocyte maturation, the similar trend for changing concentration of both adenosine and uric acid (higher in smaller follicles and constant thereafter) could indicate that these metabolites reflect a significant maturation event (at ~18 mm follicular diameter). This could result in adenosine and uric acid being maintained at a constant level (median of 44 and 10.6 μmol/L, respectively) for a healthy follicular environment. Considering the antioxidative capabilities of uric acid and the detrimental effect of reactive oxygen species on the oocyte, this finding could lend support to a beneficial role for these 2 purine metabolites. Hypoxanthine was found to correlate with follicular size only when an oocyte was recovered (Tables 2-3). However, the correlation is strongly enhanced when the recovered oocyte fertilized successfully (Tables 2-3). This could indicate that different purine metabolic pathways are active in the most fertile oocyte follicles.

Nine more peaks resolved by the separation of follicular fluid samples have not yet been positively identified; work needs to be carried out to identify these molecules with reference to spectral libraries or mass spectrometry. Nucleotides can be readily separated by HPLC or, more specifically, anion exchange HPLC or ion-pairing reversed phase liquid chromatography. They are also easily resolved by capillary electrophoresis. When the samples were examined by optimized capillary electrophoresis to resolve nucleotides, no nucleotide peaks were observed [15]. Furthermore, it is well established that nucleotides do not occur (or are not detected) to any significant level in extracellular fluid [16].

Although adenosine has been detected in follicular fluid before, we were surprised at the extremely high levels detected in this study. Adenosine is a potent vasodilator, and

Table 3
The intercorrelations by Spearman rank correlation test were shown between follicular diameter and all components found in follicular fluids in which an oocyte was present but failed to fertilize

Unfertilized		Follicular size	Concentration				
			Adenosine	Adenine	Hypoxanthine	Uric acid	Creatinine
Follicular size			***P*=.05**	*P*=.82	***P*=.03**	*P*=.11	*P*=.93
Concentration	Adenosine	**ρ=–0.28**		*P*=.32	***P*=.0006**	*P*=.98	*P*=.95
	Adenine	ρ=0.03	ρ=0.14		*P*=.71	***P*=.006**	***P*=.04**
	Hypoxanthine	**ρ=–0.3**	**ρ=0.47**	ρ=0.05		*P*=.12	*P*=.5
	Uric acid	ρ=–0.22	ρ=–0.003	**ρ=–0.37**	ρ=0.22		***P*<.0001**
	Creatinine	ρ=–0.01	ρ=–0.008	**ρ=–0.28**	ρ=0.1	**ρ=0.71**	

Red and purple table entries indicate corresponding pathways shown in Fig. 3.

Table 4
The intercorrelations by Spearman rank correlation test were shown between follicular size and all components found in the follicular fluids in which there was no oocyte retrieved

No oocyte		Follicular size	Concentration				
			Adenosine	Adenine	Hypoxanthine	Uric acid	Creatinine
Follicular size			**P=.01**	**P=.04**	P=.2	**P=.01**	P=.07
Concentration	Adenosine	**ρ=-0.36**		**P=0.01**	**P=.0001**	P=.24	P=.09
	Adenine	**ρ=-0.29**	**ρ=0.35**		P=.39	P=.94	P=.44
	Hypoxanthine	ρ=–0.18	**ρ=0.56**	ρ=0.12		**P=.02**	P=.15
	Uric acid	**ρ=–0.34**	ρ=0.17	ρ=–0.01	**ρ=0.34**		**P<.0001**
	Creatinine	ρ=–0.25	ρ=0.24	ρ=–0.11	ρ=0.2	**ρ=0.71**	

Bold and red table entries correspond to proposed pathway shown in Fig. 4.

stressed cardiac myocytes have been shown to release large quantities to induce coronary vessel dilation and overcome acute hypoxia [17]. However, it is normally a short-lived purine metabolite, as the ubiquitous adenosine ADA-1 in tissue (and its larger isoform ADA-2 in serum) rapidly degrades adenosine to inosine that in turn is degraded to hypoxanthine and eventually to uric acid [18]. Indeed, the enzyme is so voracious that the estimated half-life of adenosine in biological fluids/exudates is 1 second. To directly measure adenosine intracellular release by stressed tissues, a microdialysis method has to be used, whereby a dialysis membrane bag is inserted in the tissue (such as muscle) and fluid that excludes protein/ADA enzyme is allowed to diffuse into the dialysis chamber to protect it from ADA degradation [19,20].

Such is the problem that inosine and hypoxanthine levels in biofluids are used as surrogate markers of adenosine, as the metabolism of inosine to hypoxanthine is so much slower and the catabolic enzyme is less ubiquitous in biological fluids than ADA. To confirm the lack of even trace ADA enzymes, we found that the incubation of the samples at 37°C showed no conversion of adenosine to inosine due to endogenous ADA activity in the samples.

In studying adenosine in plasma, researchers normally use ADA inhibitors to prevent its extremely rapid in vitro degradation in blood samples. We did not need to add an

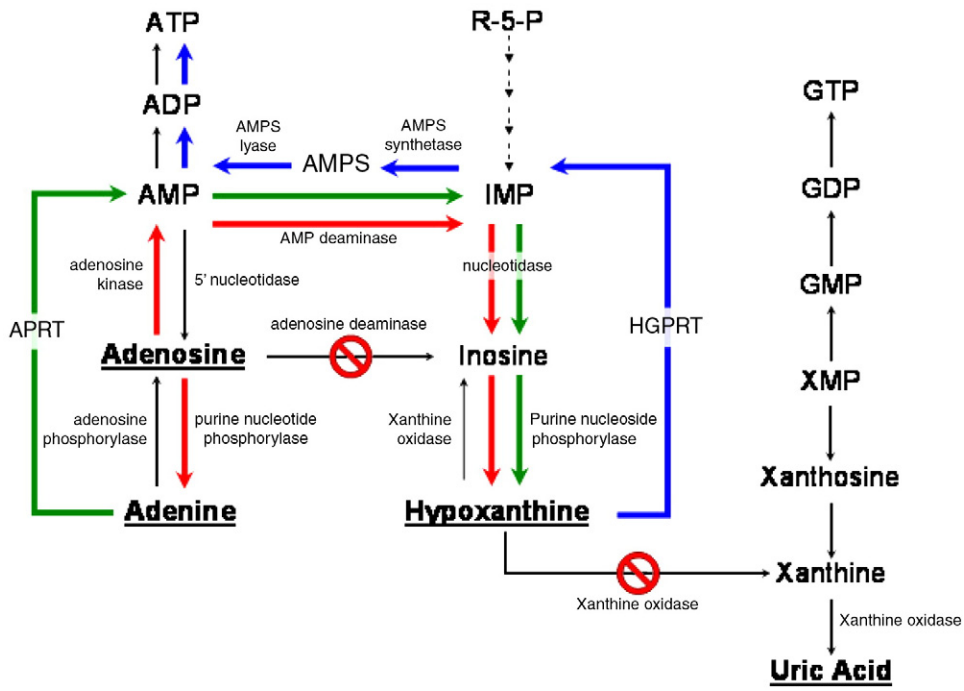

Fig. 7. Purine salvage pathway in follicular fluids that had an oocyte recovered and subsequently fertilized successfully. Thicker lines are consistent with significant correlation (Table 2). Bold and underlined are the detected metabolites. R-5-P indicates ribose-5-phosphate; ⃠ indicates pathway blocked; → and blue text represent a positive correlation for the salvage pathway from hypoxanthine to IMP and presumably AMP/ATP; → and green text represent a positive correlation for the pathway from adenine to hypoxanthine; → and red text represent a positive correlation for the pathways of adenosine's metabolism to hypoxanthine or conversion to adenine.

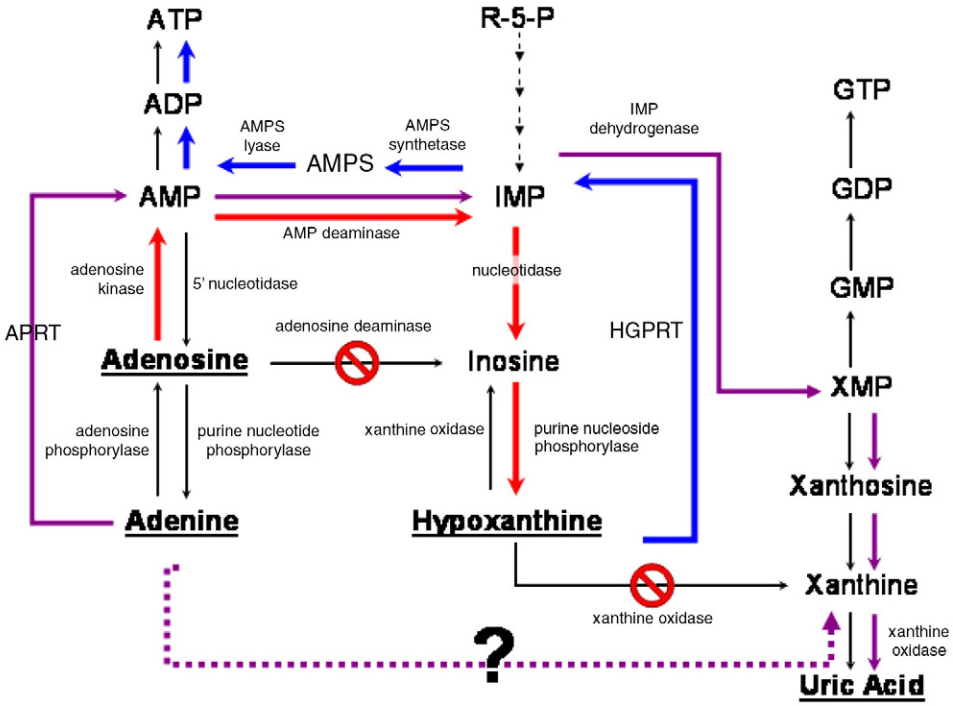

Fig. 8. Purine salvage pathway in follicular fluids that had an oocyte recovered but failed to fertilize. Thicker lines are consistent with significant correlation (Table 3). Bold and underlined are the detected metabolites. R-5-P indicates ribose-5-phosphate; indicates that the pathway is blocked; and blue text represent a positive correlation for the salvage pathway from hypoxanthine to IMP and presumably AMP/ATP; and red text represent a positive correlation for the pathways of adenosine's metabolism to hypoxanthine or conversion to adenine; represents a possible metabolic pathway by which adenine can be converted to uric acid via IMP metabolism to xanthosine monophosphate; indicates that the correlation exists between adenine and uric acid but which cannot easily be fitted to established metabolic pathways.

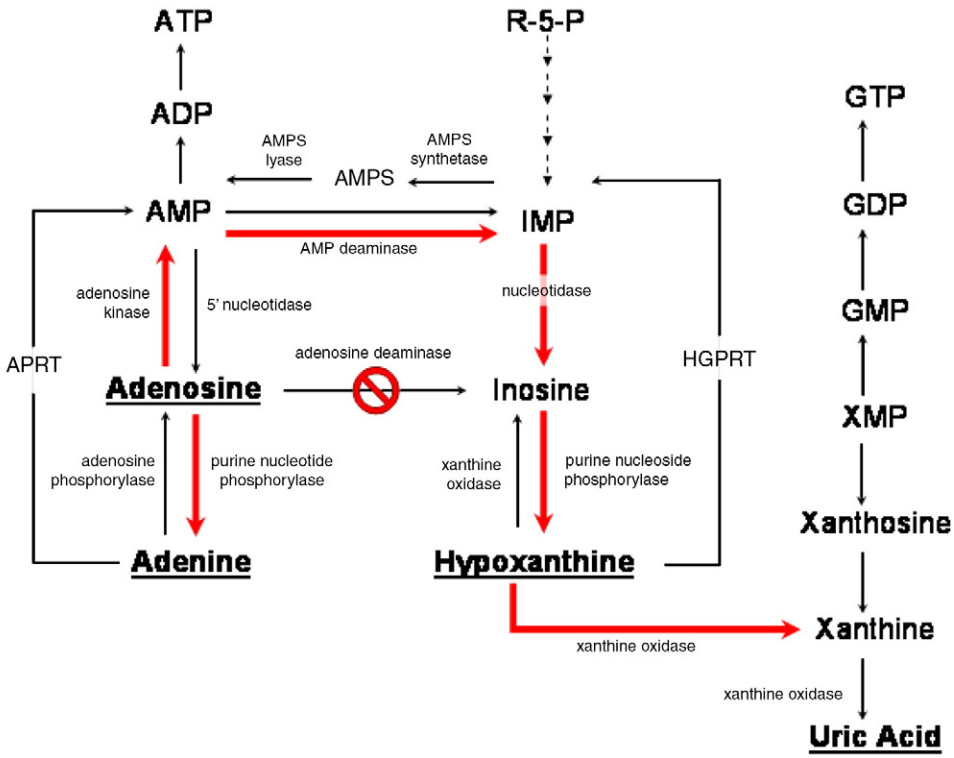

Fig. 9. Purine salvage pathway in follicular fluids that had no oocyte recovered. Thicker lines are consistent with significant correlation (Table 4). Bold and underlined are the detected metabolites. R-5-P indicates ribose-5-phosphate; indicates that the pathway is blocked; and red text represent a positive correlation for the pathways for adenosine's metabolism to hypoxanthine or conversion to adenine, and hypoxanthine's conversion to uric acid.

ADA inhibitor, nor was inosine detected in these follicular fluid samples. To confirm the identity of the adenosine peak in the HPLC chromatographs, ADA was added, shown to reduce peak height; and the appearance of an inosine peak was also demonstrated.

A similar finding with respect to adenosine's presence has been described before by Lavy and his colleagues [8] in 1990. This led them to propose that adenosine may be the major oocyte maturation inhibitor in humans rather than hypoxanthine. However, why the follicular environment should be so protected from ADA expression has not been fully explored before and should be considered more in light of its role in ATP salvage pathways and its established metabolism, rather than as a meiotic inhibitor.

Classic metabolic pathways show that the degradation pathway by which adenosine is converted to adenine can be salvaged directly back to AMP by the enzyme APRT. However, this is often a very rare event, as ubiquitous (and voracious) ADA rapidly coverts any adenosine to inosine. In turn, inosine is converted to hypoxanthine and then to uric acid. A competitive salvage pathway can convert some of this back to AMP via a much more complicated cascade: hypoxanthine is converted to IMP by HGPRT, and IMP to AMP by adenylosuccinate synthetase and adenylosuccinate lyase (Figs. 7-9). Indeed, in red blood cells (which lack a nucleus and hence also gene transcription), these salvage pathways are maximized to maintain the energy charge and metabolic membrane integrity of the erythrocyte for its functional life span.

This study indicates that functional ADA is absent from the follicular environment of the maturing oocyte. The implication of this for the metabolic pathways available for purine salvage and ATP synthesis is significant. Given that ADA rapidly converts any adenosine present to inosine, which in turn is converted to hypoxanthine in most tissues as described above, hypoxanthine is the most abundant and therefore pivotal point for salvage pathways (HGPRT) to recycle this purine to AMP (and thus make it available for ATP synthesis). Hypoxanthine is still abundant in follicular fluid; but it is no longer pivotal, and its presence can only be explained by a secondary conversion pathway: AMP being acted on by AMP deaminase to produce IMP through hypoxanthine via inosine. However, this cannot be a dominant enzymatic cascade, as the buildup of adenosine is testament to the competition for AMP by the adenosine forming enzyme 5′-nucleotidase. Thus, during oocyte maturation, the adenosine purine salvage pathway might become predominant and critical: adenosine levels accumulate; and the route to recycling to AMP (and ATP) is via purine nucleotide phosphorylase to adenine, which is converted to AMP by high-level activity of APRT (Fig. 7).

If we examine closely the statistical relationships between adenosine, adenine, and hypoxanthine levels in the follicular fluid, correlation evidence can be found to support this hypothesis. In all circumstances, adenosine levels correlate with hypoxanthine levels; but no inosine could be detected. In addition, correlation between these metabolites and uric acid changed depending on the 3 circumstances from which the follicular fluids were collected: (1) the oocyte recovered fertilized, (2) the oocyte recovered failed to fertilize, and (3) no oocyte was recovered. In the follicular fluid from which a successfully fertilized oocyte was recovered, we found that all 3 metabolites correlated very strongly with each other but, furthermore, that none correlated with uric acid. This suggests not only that the adenine APRT salvage pathway is fully functional, but also that the hypoxanthine salvage pathway via HGPRT is working efficiently (such that AMP is being salvaged and not terminally degraded to uric acid, Fig. 7). Murine studies have previously shown that the enzyme that converts hypoxanthine to xanthine is absent in the preimplantation conceptus. In the blighted follicles, we see evidence that hypoxanthine salvage is not optimized; and degradation to uric acid appears to dominate in this part of the purine salvage pathways (Fig. 9), as hypoxanthine levels correlate with uric acid. Interestingly, in the follicular fluid from which an oocyte failed to fertilize, the correlation of metabolites would indicate bypass of pathways, as adenine levels correlate directly with uric acid and nothing else (Table 3, Fig. 8). One study has shown that the murine preimplantation conceptus can take up exogenous hypoxanthine and adenine, rapidly converting them to xanthine, presumably as a necessary event to remove the mitotic inhibitory effects of these molecules when fertilization has occurred and rapid cell division is called for in a postfertilized conceptus [18]. One possible pathway for this is the expression and up-regulation of another salvage pathway enzyme—IMP dehydogenase—which converts IMP to xanthosine monophosphate. Subsequent degradation to uric acid down the guanosine phosphate pathway is therefore probable (Fig. 8). However, adenosine also fails to correlate with uric acid levels; and thus, the data also suggest that adenosine can only be converted to adenine and not follow direct salvage pathways back to AMP (Fig. 8). This is inconsistent with data showing that adenosine correlates with hypoxanthine. A possible solution to these findings is that the ova that fail to fertilize not only express IMP dehydrogenase, but also express ADA (albeit at low levels) and thus alter the composition of the follicular fluid environment. Whatever the mechanism, activation of pathways that result in adenine conversion to uric acid would appear to be detrimental to oocyte viability. The metabolic mechanism underlying this may be the reduction of the energy availability charge of the unfertilized ova at ovulation, rather than meiotic arrest.

In terms of systems biology, the role of adenosine is becoming more recognized, not only because of its vasodilatory properties but also because of its direct effects on AMP/ATP salvage and cellular energy charges. Surprisingly, in reproductive biology (and no other tissues), adenosine is simply considered in terms of meiotic inhibition. In studies of heart and brain tissue physiology/pathology, adenosine release into the extracellular environment (under ischemic conditions) is considered not only as an extracellular bioactive cytokine but also a source of

purine/ATP anabolic precursors [21-23]. Indeed, brain tissue cultured in the presence of adenosine survived longer than in culture media lacking this biometabolite. This has been attributed to adenosine salvage pathways and ATP/energy charge dynamics [22]. It is intriguing to speculate on a much broader role for adenosine in follicular fluid oocyte physiology, and further study of this phenomenon (and associated pathways) may yield markers useful in the selection of oocyte for assisted reproduction beyond the current sole criterion of follicular size.

Acknowledgment

The authors thank the staff in Fertility Unit of St Bartholomew's Hospital, London, United Kingdom, for caring for the volunteers during the follicular fluid collection. We thank Dr Stephen Butler for helping us with the graphics. We are also grateful for the financial support for this project from Joint Research Board of Bartholomew's Hospital.

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