Do alterations in follicular fluid proteases contribute to human infertility?

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Received: 31 December 2014 / Accepted: 29 January 2015 / Published online: 15 February 2015
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Abstract

Purpose Cathepsin L and ADAMTS-1 are known to play critical roles in follicular rupture, ovulation, and fertility in mice. Similar studies in humans are limited; however, both are known to increase during the periovulatory period. No studies have examined either protease in the follicular fluid of women with unexplained infertility or infertility related to advanced maternal age (AMA). We sought to determine if alterations in cathepsin L and/or ADAMTS-1 existed in these infertile populations.

Methods Patients undergoing in vitro fertilization (IVF) for unexplained infertility or AMA-related infertility were prospectively recruited for the study; patients with tubal or male factor infertility were recruited as controls. Follicular fluid was collected to determine gene expression (via quantitative polymerase chain reaction), enzyme concentrations (via enzyme-linked immunosorbent assays), and enzymatic activities (via fluorogenic enzyme cleavage assay or Western blot analysis) of cathepsin L and ADAMTS-1.

Results The analysis included a total of 42 patients (14 per group). We found no statistically significant difference in gene

expression, enzyme concentration, or enzymatic activity of cathepsin L or ADAMTS-1 in unexplained infertility or AMA-related infertility as compared to controls. We also found no statistically significant difference in expression or concentration with advancing age.

Conclusions Cathepsin L and ADAMTS-1 are not altered in women with unexplained infertility or AMA-related infertility undergoing IVF, and they do not decline with advancing age. It is possible that differences exist in natural cycles, contributing to infertility; however, our findings do not support a role for protease alterations as a common cause of infertility.

Keywords Ovulation · Infertility · Protease · Cathepsin L · ADAMTS-1

Introduction

Ovulation is a dynamic and intricately orchestrated process that is triggered by a mid-cycle surge of luteinizing hormone (LH); however, the mechanism behind the actual rupture of the follicular wall that allows extrusion of the oocyte is not clearly understood. Longstanding theories on the events leading up to this process have centered on the concept that biochemical and mechanical alterations occur at the level of the follicle or on the follicle wall itself. Early investigations established a role for proteolytic enzyme degradation of the connective tissue matrix of the follicular wall, leading to rupture [1]. More recently, two proteases have been implicated as playing a critical role in the process of follicular rupture [2]. Cathepsin L (CTSL, a lysosomal cysteine protease) and ADAMTS-1 (A disintegrin and metalloproteinase with thrombospondin-like motifs), both selectively induced in granulosa cells by LH during the periovulatory period in wild-type mice, remained at preovulatory levels in anovulatory progesterone receptor (PR) knockout mice [2]. Conclusive

Capsule We performed a prospective, experimental study in IVF patients with unexplained infertility and advanced maternal age to determine if alterations in cathepsin L and/or ADAMTS-1 existed. We found no statistically significant difference in the gene expression, enzyme concentration, or enzymatic activity of either protease when compared to our control. Our findings do not support a role for protease alterations as a common cause of human infertility.

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evidence for the role of ADAMTS-1 in remodeling of the follicular wall to mediate rupture was morphologically and histologically identified in an ADAMTS-1 knockout mouse model, which resulted in a subfertile phenotype with significant reduction in the ovulation rate and the fertilization rate of ovulated oocytes [3]. While the role of cathepsin L in ovulation has not been studied in detail, homozygous female cathepsin L null mice are reportedly infertile [4].

The expression of the progesterone receptor, cathepsin L and ADAMTS-1 in human granulosa cells also increases, under the influence of LH, during the periovulatory period [5–7]. Moreover, a recently published prospective experimental study reported that the expression of ADAMTS-1 was decreased in the granulosa cells of 40 women with polycystic ovarian syndrome (PCOS) undergoing IVF treatment [8].

Up to 30 % of couples with infertility have “unexplained” infertility, meaning no cause for infertility was discovered after evaluation of ovulation, sperm parameters, fallopian tube patency, and uterine anatomy [9]. Commonly used methods to detect ovulation are indirect and are based on measuring LH or progesterone levels; however, in a subset of patients, actual release of the oocyte may not occur despite the hormonal stimulus for ovulation. Although this is controversial, one study evaluating this phenomenon, known as luteinized unruptured follicle (LUF) syndrome, demonstrated an incidence of 25 % in women with unexplained infertility, and an even higher recurrence rate between 78.6 and 90 % in patients undergoing multiple cycles of fertility treatment [10].

While it is well known that fertility declines with advancing age and live birth rates are diminished largely due to aneuploidy, there has been little investigation into the role of ovulatory dysfunction affecting fertility in older patients. It has been well-established that ovulation becomes less regular and more infrequent in the 8–10 years preceding menopause [11]. Patients diagnosed with AMA-related infertility demonstrate a normal evaluation of all other parameters as noted above, and it is plausible that a subset of these patients have defective mechanisms for follicular rupture as well.

Because of the reported involvement of cathepsin L and ADAMTS-1 in female fertility, we sought to perform translational experiments to test the hypothesis that one or both of these enzymes are reduced in human cases of unexplained and AMA-related infertility. In order to evaluate all possible aspects of alterations, we investigated gene expression, enzyme concentration, and enzymatic activity of both proteases.

Materials and methods

Ethical approval

Research approval was obtained from the institutional review board (IRB) at the University of Iowa for all human sample

collections (IRB #201010769). Written informed consent was obtained from all patients included in the study prior to sample collection.

Patient selection

Samples were obtained from patients undergoing in vitro fertilization (IVF) treatment at the Center for Advanced Reproductive Care at the University of Iowa Hospitals and Clinics (Iowa City, Iowa, US). Patients undergoing fresh IVF cycles were prospectively recruited between September 2013 and May 2014.

Enrolled patients were categorized on the basis of diagnosis: control (male factor or tubal infertility), unexplained infertility (all other causes of infertility excluded), and AMA (age ≥ 38 years). We selected our controls on the basis that women with these causes for infertility demonstrate normal ovulatory function clinically and the etiology was not ovarian in nature. Data was prospectively collected on the following clinical characteristics: age (years) at time of cycle start, body mass index (BMI) in kg/m^2 , parity (number of live births), and most recent antral follicle count (AFC) by transvaginal ultrasonography. Additional clinical outcomes were collected for each fresh IVF cycle: total fertilization rate, number of embryos transferred, number of blastocysts cryopreserved, and pregnancy outcome.

All IVF cycles were preceded by one month of oral contraceptive pills. Pituitary desensitization and inhibition of ovulation were achieved by administration of GnRH agonist (leuprolide acetate: Sun Pharmaceutical Industries Ltd., India) or GnRH antagonist (Ganirelix acetate: Merck & Co., Inc., USA). The same gonadotropin formulations were utilized for all patients (follicle-stimulating hormone (FSH) beta, Follistim AQ: Merck & Co., Inc., USA; human menopausal gonadotropin / hMG, Menopur: Ferring Pharmaceuticals Inc., USA). Total gonadotropins utilized were calculated and defined as the total IU of Follistim and Menopur used for the cycle duration. Ovulation was triggered with 10,000 IU of chorionic gonadotropin (APP Pharmaceuticals, LLC, USA) for all patients when at least 2 follicles of ≥ 18 mm in diameter were achieved. Cycle day of trigger was defined as the length of time between the start of stimulation and administration of ovulation trigger. Oocyte retrieval was then performed via transvaginal aspiration 34–36 hours later. Total oocytes retrieved included all oocytes (mature, immature, and atretic) obtained during the follicular aspiration. Mature oocytes were inseminated by either conventional IVF or intracytoplasmic sperm injection (ICSI), and fertilization was confirmed 18 hours following insemination.

Fertilization rate was defined as the number of normally fertilized oocytes (diploid zygotes) divided by the number of oocytes inseminated. The number of embryos transferred was based on institutional policy on either day 3 or day 5 of culture. Supernumerary embryos were all cryopreserved at the blastocyst stage. Pregnancy rate was calculated as ongoing or delivered pregnancy per fresh transfer (excluding first trimester spontaneous abortions).

Sample collection

Fluid was aspirated from the first follicle, selected based on large size and accessible position, into an individual sterile tube without culture medium. Samples were collected without gross contamination of blood. After examination and removal of any oocytes, the first follicle fluid was centrifuged at 3,000 rpm for 15 minutes at 4 °C to eliminate any potential contamination. The supernatant was evaluated microscopically to confirm the absence of blood and other cellular contaminants, then transferred to sterile polypropylene tubes and flash-frozen in liquid nitrogen prior to storage at -80 °C until assays were performed.

The remaining follicular fluid collected during the retrieval was pooled and centrifuged at 3,000 rpm for 15 minutes at 4 °C. After removal of the supernatant, the pellet was resuspended in RNeasy Lysis Solution (Qiagen, USA) in order to preserve any granulosa cell RNA that was present. This suspension was then transferred to a sterile polypropylene tube, flash-frozen in liquid nitrogen, and stored at -80 °C until RNA isolation was performed.

RNA isolation and quantitative real-time PCR (qPCR)

Total RNA was isolated from the pooled follicular fluid pellet obtained following thawing and centrifugation at 5,000 rpm for 15 minutes at 4 °C. The RNA was extracted using QIAshredder spin columns and RNeasy Plus Mini Kits (QIAGEN, USA) according to the manufacturer's instructions. The amount of RNA isolated was quantitated using a NanoDrop 1,000 spectrophotometer (Thermo Scientific, USA). Each sample was then diluted to 10 ng/μL for measurement of gene expression using quantitative real-time PCR (qPCR).

All qPCR assays were run in duplicate using 50 ng of RNA, the iTaq™ Universal SYBR® Green One-Step Kit (Bio-Rad Laboratories, Inc., USA) and a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., USA) with commercially available primers for *CTSL* and *ADAMTS1* (GeneCopoeia™, USA). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, GeneCopoeia™, USA) was utilized as the reference gene. Expression levels of the two genes of interest were normalized to the expression level of the reference gene and quantitated using the delta delta Cq method [12].

Enzyme-linked immunosorbent assays (ELISA)

The concentration of cathepsin L and ADAMTS-1 was determined using ELISA in duplicate 100 μL and 50 μL aliquots, respectively, of thawed fluid from the first follicle supernatant. For cathepsin L, the samples were assayed using the Human Cathepsin L Platinum ELISA kit (eBioscience, Austria)

according to the manufacturer's instructions. The sensitivity of the assay is 1.7 ng/mL and the intra- and inter-assay coefficients of variation are 6.2 and 6.0 %, respectively. For ADAMTS-1, the samples were assayed using the Human A Disintegrin and Metalloproteinase with Thrombospondin 1 ELISA kit (MyBioSource, USA) according to the manufacturer's instructions. The sensitivity of the assay is 0.1 ng/mL and the intra- and inter-assay coefficients of variation are <9 and <10 %, respectively.

Enzymatic activity assay

To quantify the enzymatic activity of cathepsin L, thawed fluid from the first follicle supernatant was used. An assay was developed utilizing the fluorogenic peptide substrate, Z (N-carbobenzoyloxy)-Phe-Arg-AMC (7-Amino-4-methylcoumarin) (R&D Systems, Inc., USA) at a final concentration of 100 μM. Samples were incubated at 37 °C for 275 min and the fluorescent cleaved product was measured on the Synergy 4 Hybrid Microplate Reader (BioTek Instruments, Inc., USA) using 380 nm excitation and 485 nm emission. A blank (fluorescence measurement done at time=0) was subtracted from all the samples and the overall enzymatic activity was expressed as relative fluorescent units (RFU)/mL of follicular fluid. Cathepsin L enzyme from human liver (Sigma-Aldrich, Inc., USA), at a concentration of 1 μg/mL, was utilized as a positive control.

Western blot analysis

Because of the lack of availability of a commercially available fluorogenic or colorimetric assay for ADAMTS-1, we used Western blot analysis to detect the cleavage of endogenous Versican in follicular fluid. Versican is the endogenous substrate for ADAMTS-1 and its cleaved product can be measured with a commercially available antibody [13]. For this analysis, 3 representative samples of first follicle aspirates were selected from each group based on similar ADAMTS-1 concentration obtained with the ELISA assay.

After SDS polyacrylamide gel electrophoresis, proteins were electrotransferred onto a polyvinylidene difluoride membrane over 2 hours. Non-specific binding sites were blocked in 5 % bovine serum albumin (BSA) in Tris-buffered saline (TBS) containing Tween 20 for a total of 2 hours at room temperature. Membranes were then incubated overnight at 4 °C with the primary antibody for cleaved Versican (ab19345: Abcam®, USA) at a dilution of 1:5000. The membranes were thoroughly washed and then incubated for 1 hour at room temperature in goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody (#170-6515: Bio-Rad Laboratories, Inc., USA), at a dilution of 1:3000. After rinsing, the specific proteins were detected with ECL Western Blotting Detection Reagents (Amersham / GE

Healthcare, UK) according to the manufacturer's instructions. Images were developed using an X-Omat film processor (Eastman Kodak Company, USA).

Statistical analysis

From preliminary quantitative enzymatic activity for cathepsin L, we performed a power analysis based on the desired detectable difference in enzyme activity. Using an $\alpha=0.05$, β of 0.2, and an effect size of 25 %, we determined that we would need a total of 12 patients per group.

For analysis of results and creation of figures, we utilized Prism 6 statistical software (GraphPad Software, Inc., USA). We applied ordinary one-way analysis of variance (ANOVA) with Bonferroni correction as indicated. We also utilized linear regression for analysis of the effect of advancing age on gene expression and protein concentration, with significance set at a p -value <0.05 with 95 % confidence intervals. All numerical and graphical data reported are expressed as the mean $\pm$ the standard error of the mean (SEM).

Results

A total of 42 patients aged 28–43 participated in the study and each group had 14 patients. Demographic and clinical characteristics of all patients are listed in Table 1. The mean values are displayed for each variable. We found no statistically significant difference in mean BMI, parity, cycle day of trigger, first follicle volume, fertilization rate, number of supernumerary

embryos cryopreserved, or pregnancy rate among the groups. Mean age ($p<0.0001$), AFC ($p=0.0005$), total gonadotropins administered ($p=0.0001$), and total oocytes retrieved ($p=0.0149$) were all found to vary significantly when comparing the control to the AMA group. The number of embryos transferred in the fresh cycle was also found to be significantly different ($p=0.0009$) when comparing both the control and unexplained infertility groups to the AMA group.

Differential gene expression of CTSL and ADAMTS1

The granulosa cells from the pooled follicular fluid pellets of all samples were used to isolate RNA. In 3 samples from the control and 3 samples from the unexplained infertility groups, the concentration of total RNA extracted was not sufficient for analysis and these samples were thus excluded. The remaining samples (11 control, 11 unexplained infertility, 14 AMA) were analyzed for expression of *CTSL* and *ADAMTS1* using qPCR. Fold-change in gene expression relative to the control group is depicted in Fig. 1a and b for *CTSL* and *ADAMTS1*, respectively. There was no statistically significant difference in expression for *CTSL* or *ADAMTS1* for unexplained infertility or AMA when compared to the control group.

Concentration of cathepsin L and ADAMTS-1

All 14 samples from each group were analyzed for the concentration of cathepsin L (CTSL) and ADAMTS-1

Table 1 Demographic and clinical characteristics of patients according to study group^a

	Control (<i>n</i> =14)	Unexplained infertility (<i>n</i> =14)	Advanced maternal age (<i>n</i> =14)	<i>p</i> -Value ^b
Age (years)	32.07 $\pm$ 0.62	32.50 $\pm$ 0.72	38.43 $\pm$ 0.47	$<0.0001^c$
Body mass index (kg/m ²)	27.35 $\pm$ 1.78	25.14 $\pm$ 1.08	24.03 $\pm$ 0.99	NS
Parity	0.50 $\pm$ 0.25	0.50 $\pm$ 0.14	0.86 $\pm$ 0.31	NS
Antral follicle count	22.29 $\pm$ 1.78	22.07 $\pm$ 1.76	13.07 $\pm$ 1.59	0.0005^c
Total gonadotropins (IU)	2,268 $\pm$ 174.60	2,175 $\pm$ 152.70	3,675 $\pm$ 358.30	0.0001^c
Cycle day of trigger	10.71 $\pm$ 0.35	10.36 $\pm$ 0.29	12.07 $\pm$ 0.59	NS
Total oocytes retrieved	17.64 $\pm$ 1.83	18.07 $\pm$ 2.07	11.14 $\pm$ 1.41	0.0149^c
First follicle volume (mL)	4.18 $\pm$ 0.68	4.06 $\pm$ 0.60	3.18 $\pm$ 0.25	NS
Fertilization rate (%)	69.72 $\pm$ 4.69	67.32 $\pm$ 5.08	73.67 $\pm$ 6.15	NS
Embryos transferred in fresh cycle	1.21 $\pm$ 0.11	1.21 $\pm$ 0.19	2.14 $\pm$ 0.23	0.0009^d
Supernumerary embryos cryopreserved	3.57 $\pm$ 0.80	3.14 $\pm$ 0.90	1.07 $\pm$ 0.40	NS
Pregnancy rate (%)	78.57 $\pm$ 11.38	50.00 $\pm$ 13.87	57.14 $\pm$ 13.73	NS

^a Not all samples used for all assays; statistical significance unchanged with select samples excluded

^b All NS p -Values represent findings across the study groups

^c Significant differences found between the control versus advanced maternal age groups

^d Significant differences found between the control and unexplained infertility versus advanced maternal age groups

Fig. 1 Gene expression of CTSL and ADAMTS1 in human granulosa cells by qPCR analysis. Each symbol represents an individual sample. The mean $\pm$ SEM for each group is noted and is also shown by the bars and brackets. Gene expression of (a) CTSL and (b) ADAMTS1 in the unexplained and AMA-related infertility groups are relative to expression in the control group. There was no statistically significant difference for CTSL or ADAMTS1 when compared to the control group

using ELISA. One sample from the AMA group was below the limit of detection on the CTSL assay, and therefore was excluded from the analysis. The individual sample concentration of CTSL and ADAMTS-1 for each group is depicted in Fig. 2a and b, respectively. There was no statistically significant difference in CTSL or ADAMTS-1 concentration for unexplained infertility or AMA when compared to the control group. We also calculated the concentration of each enzyme per milligram of protein present in the follicular fluid (ng/mg) instead of per ml of follicular fluid (as shown in the Figures); however, this also showed no statistically significant

Fig. 2 Protein concentration (ng/mL) of CTSL and ADAMTS-1 in human follicular fluid by ELISA analysis. Each symbol represents an individual sample. The mean $\pm$ SEM for each group is noted and is also shown by the bars and brackets. Protein concentration of (a) CTSL and (b) ADAMTS-1 is shown for all groups. There was no statistically significant difference for CTSL or ADAMTS-1 when compared to the control group

difference in CTSL or ADAMTS-1 when compared to the control group (figures not shown).

Enzymatic activity of cathepsin L

All 14 samples from each group were analyzed for cathepsin L activity (Fig. 3). No statistically significant difference in cathepsin L activity was demonstrated for unexplained infertility or AMA when compared to the control group. We also calculated the enzymatic activity per milligram of protein present in the follicular fluid (RFUs/mg); however, this also showed no statistically significant difference among the groups (figures not shown).

Fig. 3 Enzymatic activity of cathepsin L in human follicular fluid. Each symbol represents an individual sample. The mean±SEM for each group is noted and is also shown by the bars and brackets. In this assay, an exogenously-added substrate is converted into a fluorescent product and the results are expressed as relative fluorescence units per mL of follicular fluid (RFUs/mL). No statistically significant difference in cathepsin L activity was demonstrated among the groups

Enzymatic activity of ADAMTS-1

As previously described, we utilized 3 representative samples from each group for our analysis, based on similar ADAMTS-1 concentration from the ELISA results. The demographic and clinical characteristics of these select patients are listed in Table 2. The mean values are displayed for each variable.

Within this selected group, we found no statistically significant difference in mean BMI, parity, first follicle volume, AFC, total gonadotropins administered, cycle day of trigger, total oocytes retrieved, fertilization rate,

number of supernumerary embryos cryopreserved, or pregnancy rate among the groups. Mean age ($p=0.0135$) and number of embryos transferred in the fresh cycle ($p=0.0370$) were both found to vary significantly when comparing the control to the AMA group. The Western blot image for cleaved Versican is shown in Fig. 4. The results were not quantitated but simple visual inspection suggests that cleaved Versican is similar in all samples.

Effect of age on gene expression and protein concentration

In order to determine if advancing age had any effect on the expression of *CTSL* or *ADAMTS1*, we analyzed all samples from all groups and plotted the relative expression as a function of age (Fig. 5). While there appeared to be a downward trend in *ADAMTS1* with advancing age, neither *CTSL* nor *ADAMTS1* were found to show a significant change as a function of age.

We performed a similar analysis on the protein concentration of CTSL and ADAMTS-1 (as measured by ELISA, see above) as a function of age. The sample concentrations relative to advancing age for CTSL and ADAMTS-1 are demonstrated in Fig. 6. No trend was observed, and there was no statistically significant difference in the concentration of CTSL or ADAMTS-1 with advancing age. We also analyzed the concentration of CTSL or ADAMTS-1 expressed per milligram of follicular fluid protein (ng/mg) relative to advancing age; however, this also demonstrated no statistically significant difference in the concentration of CTSL or ADAMTS-1 (figures not shown).

Table 2 Demographic and clinical characteristics of select patients for analysis of ADAMTS-1 activity

	Control (n=3)	Unexplained infertility (n=3)	Advanced maternal age (n=3)	p-Value ^a
Age (years)	31.67±1.86	30.33±0.33	37.00±0.58	0.0135 ^b
Body mass index (kg/m ²)	33.78±4.51	24.55±0.86	26.62±3.63	NS
Parity	0±0	0.33±0.33	0.33±0.33	NS
Antral follicle count	22±3.79	15±1.53	15±3.06	NS
Total gonadotropins (IU)	2,625±114.60	2,575±294.70	3,075±766.10	NS
Cycle day of trigger	11±0	11±0.58	11±1.53	NS
Total oocytes retrieved	16.33±3.38	12.67±1.45	10.67±3.18	NS
First follicle volume (mL)	2.93±0.28	4.30±0.95	3.40±0.31	NS
Fertilization rate (%)	64.00±5.97	52.23±11.39	70.60±7.58	NS
Embryos transferred in fresh cycle	1.00±0	1.67±0.33	2.33±0.33	0.037 ^b
Supernumerary embryos cryopreserved	3.67±1.45	1.33±0.88	0±0	NS
Pregnancy rate (%)	100.00±0	66.67±0.33	66.67±0.33	NS

^a All NS p-Values represent findings across the study groups

^b Significant differences found between the control versus advanced maternal age groups

Fig. 4 Detection of cleaved Versican in human follicular fluid by Western blot analysis. Representative samples (3 from each group) were selected for analysis based on similar ADAMTS-1 concentration from ELISA results. ADAMTS-1 cleaves the Versican that is present in follicular fluid and the cleaved Versican is detected with a specific antibody to reveal a 70 kDa band that is present across all samples in all groups

Discussion

There is growing evidence, derived from the study of genetically-modified mouse models that certain

Fig. 5 Gene expression as a function of age for CTSL and ADAMTS1 in human granulosa cells by qPCR analysis. Each symbol represents an individual sample with linear regression analysis used to determine the best-fit line. The expression data are the same as those shown in Fig. 1a and b but they are plotted as a function of the age of the patients for both (a) CTSL and (b) ADAMTS1. There was no statistically significant difference in expression relative to advancing age demonstrated for either CTSL or ADAMTS1

Fig. 6 Protein concentration (ng/mL) as a function of age for CTSL and ADAMTS-1 in human follicular fluid by ELISA analysis. Each symbol represents an individual sample with linear regression analysis used to determine the best-fit line. The protein concentration data are the same as those shown in Fig. 2a and b but they are plotted as a function of the age of the patients for both (a) CTSL and (b) ADAMTS-1. There was no statistically significant difference in the concentration relative to advancing age demonstrated for either CTSL or ADAMTS-1

proteases, specifically cathepsin L and ADAMTS-1, participate in follicular rupture and perhaps other aspects of ovulation and fertilization [2, 3, 6, 13]. This is particularly true for ADAMTS-1 because mice that are null for this enzyme have a substantial defect in ovulation that is secondary to a defect in follicular rupture [3]. In addition, the fertilization rate of ovulated oocytes is reduced in ADAMTS-1 null mice [3]. The involvement of cathepsin L on fertility is not as well defined but, like ADAMTS-1, the expression of cathepsin L is enhanced in the periovulatory period and is dependent on the expression of the granulosa cell progesterone receptor [2, 14]. Mice that are null for the progesterone receptor are anovulatory, and do not express either ADAMTS-1 or cathepsin L when stimulated with LH [2].

These pathways have not been studied as extensively in humans, but it is known that the expression of the

progesterone receptor, cathepsin L and ADAMTS-1 in human granulosa cells also increases, under the influence of LH, during the periovulatory period [5–7]. Moreover, two small independent studies have documented a decrease in the expression of ADAMTS-1 in the ovaries [15] or granulosa cells [8] of women with PCOS, a disease that is associated with decreased ovulation. Interestingly, a similar decrease in ovarian ADAMTS-1 expression was reported in the ovaries of 15 female-to-male transsexuals receiving long-term androgen treatment [15].

Based on these results, we hypothesized that a decrease in the expression of cathepsin L and/or ADAMTS-1 could contribute to the etiology of unexplained infertility and/or the decreased ovulatory response that occurs as a function of age. We show, however, that neither the expression (at the mRNA and protein level) nor the activities of cathepsin L or ADAMTS-1 appear to change in human cases of unexplained or AMA-related infertility. We have also shown that there are no significant differences in the expression (at the mRNA and protein level) or activity of either enzyme as a function of advancing age.

Since our studies were conducted only in women undergoing IVF treatment, an obvious limitation is that the resulting findings cannot be strictly extrapolated to what occurs in a natural ovulatory cycle for women with unexplained or AMA-related infertility. We also recognize that those patients with unexplained infertility may be too heterogeneous a group to truly identify a difference in gene expression, enzyme concentration, or enzymatic activity from the sample size utilized in this study. As in the case of LUF syndrome, alterations may be present only in a subset of patients with unexplained infertility. Finally, it is likely that cathepsin L and ADAMTS-1 are both integral mediators of ovulation and that a coordinated disruption in both is required to prevent ovulation. This conclusion is, in fact, supported by the partial reduction in ovulation of the ADAMTS-1 null mice [3] and the anovulatory phenotype of the progesterone receptor null mice [2] where neither ADAMTS-1 nor cathepsin L are induced.

Conclusions

Further studies are needed to investigate the underlying causes of unexplained and age-related infertility, as these conditions are so common among patients seeking fertility care. We have demonstrated by gene expression, protein concentration, and enzymatic activity the presence of two enzymes integral to ovulation in human follicular fluid; however, our findings do not support the hypothesis that alterations of cathepsin L or ADAMTS-1 are a cause of unexplained or AMA-related infertility.

Acknowledgements We would like to acknowledge the Division of Reproductive Endocrinology and Infertility and the University of Iowa's Center for Advanced Reproductive Care for their support and assistance in sample collection. We would also like to thank Drs. Donna and Mark Santillan for their assistance in sample processing and use of materials from the University of Iowa REI Tissue bank. Finally, we would like to recognize Professor Miriam Bridget Zimmerman for her expertise in statistical analysis.

Conflict of interest The authors declare that they have no conflict of interest.

Study funding This work was supported by funding from the Departments of Pharmacology and Obstetrics and Gynecology at the University of Iowa.

Compliance with ethical standards The authors declare that they have no conflict of interest. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

References

1. Schochet SS. A suggestion as to the process of ovulation and ovarian cyst formation. A preliminary report. *Anat Rec*. 1916;10:447–57.
2. Robker RL, Russell DL, Espey LL, Lydon JP, O'Malley BW, Richards JS. Progesterone-regulated genes in the ovulation process: ADAMTS-1 and cathepsin L proteases. *Proc Natl Acad Sci U S A*. 2000;97:4689–94.
3. Brown HM, Dunning KR, Robker RL, Boerboom D, Pritchard M, Lane M, et al. ADAMTS1 cleavage of Versican mediates essential structural remodeling of the ovarian follicle and cumulus-oocyte matrix during ovulation in mice. *Biol Reprod*. 2010;83:549–57.
4. <http://jaxmice.jax.org/strain/008352.html>. Last accessed November 1, 2014.
5. García V, Kohen P, Maldonado C, Sierralta W, Muñoz A, Villarroel C, et al. Transient expression of progesterone receptor and cathepsin-L in human granulosa cells during the periovulatory period. *Fertil Steril*. 2012;97:707–13.
6. Yung Y, Maman E, Konopnicki S, Cohen B, Brengauz M, Lojkin I, et al. ADAMTS-1: a new human ovulatory gene and a cumulus marker for fertilization capacity. *Mol Cell Endocrinol*. 2010;328:104–8.
7. Freimann S, Ben-Ami I, Dantes A, Armon L, Ben Ya'cov-Klein A, Ron-El R, et al. Differential expression of genes coding for EGF-like factors and ADAMTS1 following gonadotropin stimulation in normal and transformed human granulosa cells. *Biochem Biophys Res Commun*. 2005;333:935–43.
8. Xiao S, Yubin L, Li T, Chen M, Xu Y, Wen Y, et al. Evidence for decreased expression of ADAMTS-1 associated with impaired oocyte quality in PCOS patients. *JCEM*. 2014;99:E1015–21.
9. Practice Committee of the American Society for Reproductive Medicine. Effectiveness and treatment for unexplained infertility. *Fertil Steril*. 2006;86:S111–4.
10. Qublan H, Amarin Z, Nawasreh M, Diab F, Malkawi S, Al-Ahmad N, et al. Luteinized unruptured follicle syndrome: incidence and recurrence rate in infertility women with unexplained infertility undergoing intrauterine insemination. *Hum Reprod*. 2006;21:2110–3.
11. Vollman RF. The menstrual cycle. In: Friedman E, editor. *Major Problems in Obstetrics and Gynecology*. Philadelphia: W.B. Saunders; 1977. p. 1–193.

12. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc.* 2008;3:1101–8.
13. Russell DL, Doyle KM, Ochsner SA, Sandy JD, Richards JS. Processing and localization of ADAMTS-1 and proteolytic cleavage of versican during cumulus matrix expansion and ovulation. *J Biol Chem.* 2003;278:42330–9.
14. Sriraman V, Richards JS. Cathepsin L gene expression and promoter activation in rodent granulosa cells. *Endocrinology.* 2004;145:582–91.
15. Jansen E, Laven JS, Dommerholt HB, Polman J, van Rijt C, van den Hurk C, et al. Abnormal gene expression profiles in human ovaries from polycystic ovary syndrome patients. *Mol Endocrinol.* 2004;18:3050–63.